BIOSTIMULANTS IN AGRICULTURE

EDITED BY: Youssef Rouphael and Giuseppe Colla

PUBLISHED IN: Frontiers in Plant Science and Frontiers in Microbiology

frontiers Research Topics

ISSN 1664-8714
ISBN 978-2-88963-558-0
DOI 10.3389/978-2-88963-558-0

About Frontiers

Frontiers is more than just an open-access publisher of scholarly articles: it is a pioneering approach to the world of academia, radically improving the way scholarly research is managed. The grand vision of Frontiers is a world where all people have an equal opportunity to seek, share and generate knowledge. Frontiers provides immediate and permanent online open access to all its publications, but this alone is not enough to realize our grand goals.

Frontiers Journal Series

The Frontiers Journal Series is a multi-tier and interdisciplinary set of open-access, online journals, promising a paradigm shift from the current review, selection and dissemination processes in academic publishing. All Frontiers journals are driven by researchers for researchers; therefore, they constitute a service to the scholarly community. At the same time, the Frontiers Journal Series operates on a revolutionary invention, the tiered publishing system, initially addressing specific communities of scholars, and gradually climbing up to broader public understanding, thus serving the interests of the lay society, too.

Dedication to Quality

Each Frontiers article is a landmark of the highest quality, thanks to genuinely collaborative interactions between authors and review editors, who include some of the world's best academicians. Research must be certified by peers before entering a stream of knowledge that may eventually reach the public - and shape society; therefore, Frontiers only applies the most rigorous and unbiased reviews.
Frontiers revolutionizes research publishing by freely delivering the most outstanding research, evaluated with no bias from both the academic and social point of view. By applying the most advanced information technologies, Frontiers is catapulting scholarly publishing into a new generation.

What are Frontiers Research Topics?

Frontiers Research Topics are very popular trademarks of the Frontiers Journals Series: they are collections of at least ten articles, all centered on a particular subject. With their unique mix of varied contributions from Original Research to Review Articles, Frontiers Research Topics unify the most influential researchers, the latest key findings and historical advances in a hot research area! Find out more on how to host your own Frontiers Research Topic or contribute to one as an author by contacting the Frontiers Editorial Office: researchtopics@frontiersin.org

BIOSTIMULANTS IN AGRICULTURE

Topic Editors:
Youssef Rouphael, University of Naples Federico II, Italy
Giuseppe Colla, Università degli Studi della Tuscia, Italy

Citation: Rouphael, Y., Colla, G., eds. (2020). Biostimulants in Agriculture. Lausanne: Frontiers Media SA. doi: 10.3389/978-2-88963-558-0

Table of Contents

frontiers in Plant Science

EDITORIAL
published: 04 February 2020
doi: 10.3389/fpls.2020.00040

Editorial: Biostimulants in Agriculture

Youssef Rouphael[1]* *and Giuseppe Colla*[2]

[1] Department of Agricultural Sciences, University of Naples Federico II, Portici, Italy, [2] Department of Agriculture and Forest Sciences, University of Tuscia, Viterbo, Italy

Keywords: humic acids, mycorrhizal fungi, nutrient use efficiency, physiological and molecular mechanisms, high-throughput phenotyping, plant growth promoting rhizobacteria, protein hydrolysates, seaweed extracts

Editorial on the Research Topic

Biostimulants in Agriculture

PLANT BIOSTIMULANTS: RATIONALE, STATE OF THE ART AND EVOLUTION

Recently, the agricultural sector is facing concomitant challenges of rising the productivity to feed the growing global population and increasing the resources use efficiency, while reducing the environmental impact on the ecosystems and human health. In fact, fertilizers and pesticides play a crucial role in agriculture, representing a powerful tool for growers to increase yield and guarantee continuous productivity throughout the seasons under both optimal and suboptimal conditions. In the last three decades, several technological innovations have been proposed to enhance the sustainability of agricultural production systems, through a significant reduction of synthetic agrochemicals like pesticides and fertilizers. A promising and environmental-friendly innovation would be the use of natural plant biostimulants (PBs) that enhance flowering, plant growth, fruit set, crop productivity, and nutrient use efficiency (NUE), and are able also to improve the tolerance against a wide range of abiotic stressors (Colla and Rouphael, 2015). PBs were initially defined by excluding some functionalities like fertilizers or plant protection products. In 1997, in Grounds Maintenance web-journal, Zhang and Schmidt from the Department of Crop and Soil Environmental Sciences of the Virginia Polytechnic Institute and State University, defined PBs as "materials that, in minute quantities, promote plant growth". By using the statement "minute quantities" for describing PBs, the authors implicitly wanted to discriminate biostimulants from nutrients and soil amendments, which also promote plant growth, but are clearly applied in larger quantities. The PBs mentioned in this web article were two important categories such as humic acids and seaweed extracts, and their action on plants was proposed to be essentially hormonal. In 2012, the European Commission has assigned an *ad hoc* study on plant biostimulants to evaluate the substances and materials involved, which was published by du Jardin (2012) as: "The Science of Plant Biostimulants - A bibliographic Analysis". Based on the scientific literature (250 scientific articles using the term 'biostimulant' in their titles and/or abstracts), the following definition was proposed: "Plant biostimulants are substances and materials, with the exception of nutrients and pesticides, which, when applied to plant, seeds or growing substrates in specific formulations, have the capacity to modify physiological processes of plants in a way that provides potential benefits to growth, development and/or stress responses". du Jardin (2012) concluded that PBs are very heterogeneous materials, and proposed in his study eight categories of substances that acts as biostimulants: humic substances, complex organic materials (obtained from agro-industrial and urban waste products, sewage sludge extracts, composts, and manure), beneficial chemical elements (Al, Co, Na, Se, and Si), inorganic salts including phosphite, seaweed extracts (brown, red, and green

OPEN ACCESS

Edited by:
Hans-Peter Kaul, University of Natural Resources and Life Sciences Vienna, Austria

Reviewed by:
Maria Serrano, Universidad Miguel Hernández de Elche, Spain

***Correspondence:**
Youssef Rouphael
youssef.rouphael@unina.it

Specialty section:
This article was submitted to Crop and Product Physiology, a section of the journal Frontiers in Plant Science

Received: *13 November 2019*
Accepted: *13 January 2020*
Published: *04 February 2020*

Citation:
Rouphael Y and Colla G (2020) Editorial: Biostimulants in Agriculture. Front. Plant Sci. 11:40. doi: 10.3389/fpls.2020.00040

macroalgae), chitin and chitosan derivates, antitranspirants (kaolin and polyacrylamide), and free amino acids and N-containing substances (peptides, polyamines, and betaines); but did not include any microbial biostimulants. Three years later in the frame of a special issue on "Biostimulants in Horticulture" conducted by Colla and Rouphael (2015), a new definition was proposed by du Jardin (2015), which was supported by scientific evidence about the mode of action, nature and types of effects of PBs on agricultural and horticultural crops. PBs were defined by du Jardin (2015) as follows: "A plant biostimulant is any substance or microorganism applied to plants with the aim to enhance nutrition efficiency, abiotic stress tolerance and/or crop quality traits, regardless of its nutrient content". This definition could be completed by "By extension plant biostimulants also designate commercial products containing mixtures of such substances and/or microorganisms". In their special issue Colla and Rouphael (2015) proposed 6 non-microbial and 3 microbial categories of PBs: (i) chitosan (Pichyangkura and Chadchawan, 2015), (ii) humic and fulvic acids (Canellas et al., 2015), (iii) protein hydrolysates (Colla et al., 2015), (iv) phosphites (Gómez-Merino and Trejo-Téllez, 2015), (v) seaweed extracts (Battacharyya et al., 2015), (vi) silicon (Savvas and Ntatsi, 2015), (vii) arbuscular mycorrhizal fungi (AMF; Rouphael et al., 2015), (viii) plant growth-promoting rhizobacteria (PGPR; Ruzzi and Aroca, 2015), and (ix) *Trichoderma* spp. (López-Bucio et al., 2015).

The definition of PBs has been rigorously debated over the last decade, and recently under the new Regulation (EU) 2019/1009, which led to the following: "A plant biostimulant shall be an EU fertilising product the function of which is to stimulate plant nutrition processes independently of the product's nutrient content with the sole aim of improving one or more of the following characteristics of the plant or the plant rhizosphere: i) nutrient use efficiency, ii) tolerance to abiotic stress, iii) quality traits, or iv) availability of confined nutrients in the soil or rhizosphere" (EU, 2019). Based on this definition, PBs are specified on the basis of agricultural functions claims, and include diverse bioactive natural substances: (i) humic and fulvic acids, (ii) animal and vegetal protein hydrolysates, (iii) macroalgae seaweeds extracts, and (iv) silicon, as well as beneficial microorganisms: (i) arbuscular mycorrhizal fungi (AMF) and (ii) N-fixing bacteria of strains belonging to the genera *Rhizobium*, *Azotobacter*, and *Azospirillum*. However, the justification of agricultural claims of a given microbial or non-microbial biostimulant, is considered an important element to allow PBs to be placed on the European Union market; thus members of the European Biostimulant Industry Council (Ricci et al.) proposed general principles and guidelines for trials and assays to follow when justifying PBs claims, that were outlined in details in their policy and practice review article.

More than 700 scientific papers were published in the last 10 years (2009–2019) on "plant biostimulants" (www.scopus.com), where several researchers were able to demonstrate that microbial and non-microbial PBs are capable of inducing an array of morpho-anatomical, biochemical, physiological, and molecular plant responses such as boosting crop productivity, NUE, and increasing tolerance against abiotic stresses (Calvo et al., 2014; Haplern et al., 2015; Nardi et al., 2016; De Pascale et al., 2017; Rouphael et al., 2017a; Rouphael et al., 2017b; Rouphael et al., 2017c; Yakhin et al., 2017; Rouphael et al., 2018a).

This Research Topic collected 50 scientific contributions from high qualified research groups working on PBs and covering the molecular, cellular, and physiological mechanisms underlying plant-biostimulant interactions under different environment and management strategies. Moreover, the present Research Topic compiles many aspects that are helpful to the scientific community, extension specialists, and commercial enterprises to better elucidate the causal/functional mechanism of microbial and non-microbial biostimulants. The elucidation of the agricultural function (i.e., improve nutrient use efficiency, quality, and tolerance to abiotic stresses) and action mechanisms of PBs will permit to develop a second generation of biostimulants where synergies and complementary mechanism can be functionally designed.

NON-MICROBIAL AND MICROBIAL PLANT BIOSTIMULANTS

Protein hydrolysates (PHs) which contain mainly signaling peptides and free amino acids have gained prominence as non-microbial PBs because of their potential to enhance germination, seedling growth, plant growth, fruits, and vegetables quality as well as crop productivity especially under environmental stress conditions (Colla et al.). In their review paper, the authors aimed at uncovering the physiological and molecular mechanisms behind the biostimulant action of animal or vegetal-based PHs on a wide range of agricultural and horticultural crops. Interestingly, the authors also provided for the first-time evidence that plant microbiomes are modified by the application of PHs, and some of the benefits derived from these products might be due in part to changes in the quanti-qualitative composition and activity of these microbial communities.

Seaweed extracts (SWE) represent another important category of organic non-microbial PBs; however red, green, and brown macroalgae are the most common SWE used in agriculture and horticulture with several commercial products present on the market. Macroalgae are typically harvested from seas and oceans, which hampers the chemical composition and quality of its raw material, leading to difficulties in standardization and getting reliable performance of the extracted products. Therefore, searching for controlled production of *in-house algae* is an urgent need for the scientific community and private companies. Chiaiese et al. proposed microalgae as a renewable source of PBs. In their review paper, the authors described the extraction techniques and the bioactive compounds (carbohydrates, proteins, and amino acids) as well as the biostimulatory action of microalgal extracts belonging to the following species: *Chlorella vulgaris*, *Acutodesmus dimorphus*, *S. platensis*, *Scenedesmus quadricauda*, *Dunaliella salina*, *Chlorella ellipsoida*, *Spirulina maxima*, and *Calothrix elenkinii*. On the other hand, developing PBs from

agro-food and industrial by-products could also open new opportunities in a full circular economy approach. Xu and Geelen reviewed examples of PBs derived from agricultural by-products and identified the important criteria to select potential by-products for developing efficient PBs. These criteria included: absence of pesticides and heavy metals, collection and storage at low cost and sufficient availability all year round. Several examples of PBs derived from agricultural and industrial by-products including vermicompost, composted urban waste, sewage sludge, PH, and chitin/chitosan derivatives were discussed in detail.

In addition to non-microbial PBs, the use of microbial PBs such as PGPR and AMF are highly considered as sustainable and efficient tools for securing yield stability under low-input conditions in particular N and P deficiency (i.e., biofertilizer effects), but also as a innovative technology to improve crop tolerance to abiotic stressors in particular extreme temperatures, drought and salinity. In their review papers Backer et al., Granada et al. and Bitterlich et al. described the mechanisms of these beneficial microorganisms regarding nutrient uptake (especially N and P) and tolerance to environmental stress including signals exchange between plant roots and PGPR and AMF. Particularly, Granada et al. reported that the reduction of P-fertilization could be achieved with the use of high efficient P-solubilizing bacterial isolates as crop inoculants. Moreover, based on a long-term study (7 years), Lu et al. reported that no-tillage with straw return had a protective effect on AMF community structures compared to conventional moldboard-plowing or tillage without straw, thereby playing a crucial role in the development of agricultural sustainability in China. In line with Backer et al. and Bitterlich et al. reviews, Turrini et al. elucidated the functional complementarity of AMF and associated microbiota. Particularly, the authors revealed the functional roles of plant growth promoting bacteria (PGPB) and mycorrhizal helper bacteria (MHB), that promote AMF activity and development and thus boost crop productivity under both optimal and sub-optimal conditions. Similarly, Agnolucci et al. demonstrated by using a polyphasic approach (a combination of culture-dependent analyses and metagenomic sequencing.), that AMF inoculum (*Rhizoglomus irregulare* BEG72) is home of a large and diverse community of bacteria with important functional PGP traits (i.e., solubilizing phosphate and producing siderophores and indole acetic acid), and possibly acting in synergy with AMF and providing beneficial effects on crop performance. Finally, Woo and Pepe reported that designing and developing potential *agricultural probiotics* such as *Trichoderma-Azotobacter* consortia is a priority for the PBs sector and should be adopted as a sustainable crop management strategy to improve yield and its qualitative aspect.

IMPLICATIONS OF BIOSTIMULANTS FOR AGRONOMIC AND PHYSIOLOGICAL TRAITS OF CROPS

The stimulation of germination, seedlings and plant growth as well as crop productivity in response to PBs application has been usually associated to the action of signaling bioactive molecules in the primary and secondary metabolisms (Calvo et al., 2014). Different types of hydrolyzed collagen, including granulated gelatin, gelatin hydrolysate and amino acid mixtures simulating gelatin composition, were evaluated in terms of plant growth on cucumber (Wilson et al.). In their study, the authors reported that gelatin hydrolysate treatment increased the expression of genes encoding for amino acid permeases (*AAP3*, *AAP6*) and transporters of amino acids and nitrogen. Therefore, they concluded that gelatin hydrolysate provided a sustained source of N and acted as a biostimulant. Furthermore, Luziatelli et al. conducted a greenhouse experiment on lettuce aiming to assess the effect of three commercial PBs: vegetal-derived PH, vegetal-derived PH enriched with copper and a tropical plant extract on plant growth, and the epiphytic bacterial population. The three commercial PBs boosted the shoot fresh weight with no significant differences between the three organic PBs. The authors were also able to demonstrate that PBs can stimulate the growth of epiphytic bacteria (*Pantoea*, *Pseudomonas*, *Acinetobacter*, and *Bacillus* genus) with PGP and/or biological control activity against pathogens, thus acting synergistically with organic compounds to increase marketable fresh yield of lettuce. Similarly, Mahnert et al. showed the potential of organic biostimulants (containing vermicompost, malt sprouts, stone dust, and organic herbs) to have a positive impact on plant growth and performance by shifting the microbiota on the aboveground parts of the plant as well as in the surrounding. Moreover, Lucini et al. carried out a short term experiment on melon to assess the physiological and metabolomic responses to a biopolymer-based biostimulant containing lateral root promoting peptides and lignosulphonates as well as micronutrients. The vegetal-based biostimulant was applied at four increasing concentrations (0, 0.3, 0.6, 1.2, or 2.4 L ha^{-1}) 2 days after transplanting around the collar level. The substrate drench of a biopolymer-based biostimulant elicits dose-dependent (especially at 0.12 and 0.24 ml $plant^{-1}$) increase of biomass production of melon transplants. The root trait characteristics (total root length and surface area) in biostimulant-treated plants were significantly higher at 0.24 ml $plant^{-1}$ and to a lesser extent at 0.12 and 0.48 ml $plant^{-1}$, in comparison to 0.06 ml $plant^{-1}$ and untreated melon plants. Direct and indirect physiological mechanisms were responsible for better shoot and root biomass production of treated melon transplants. For instance, the signaling molecules in particular bioactive peptides and lignosulfonates may have elicited signal transduction pathway through biosynthesis stimulation of target endogenous phytohormones (Matsumiya and Kubo, 2011). On the other hand, Palumbo et al. reported that humic acids (applied at 0.5 mg and 1 mg C L^{-1} for 2 days) extracted from olive mill water filters and municipal solid waste could be used as valuable biostimulants in maize at both concentrations as demonstrated by their capacity to promote significantly plant growth, activity of marker enzymes, and nutrient accumulation. While on maize, Ertani et al. evaluated the biostimulant effect of 6 seaweed extracts (one extract from *Laminaria* and five extracts from *Ascophyllum nodosum*) supplied for 2 days at 0.5 ml L^{-1}. Thanks to a combination of morphological, chemical, and biochemical approaches, the authors demonstrated

that one of the *A. nodosum* extract was the most efficient in promoting root morphological traits, likely due to its elevated content in indole-3-acetic acid. Such findings illustrate the utility of a robust chemical characterization of commercial seaweed extracts, which predicts the metabolic targets of seaweed extracts-based biostimulants before their commercialization on the market.

Additionally, a significant stimulation of plant growth parameters, yield and yield components of two greenhouse pepper cultivars was observed when seedlings were exposed to *Cladosporium sphaerospermum* (Li et al.). Result of the same study showed that tobacco plants exposed to *C. sphaerospermum* retained higher rates of growth, where it was associated with several putative physiological and molecular mechanisms including cell expansion and cycle, photosynthesis, phytohormone homeostasis, and defense responses.

Concerning flower crops, Cristiano et al. investigated the application effect of an animal-based PH as foliar spray or as substrate drench, applied at three doses (0, 0.1, and 0.2 g L^{-1}) on the agronomical and physiological responses of two snapdragon hybrids. At both PB doses, the application of animal-based PH especially as substrate drench enhanced the performance parameters and ornamental quality traits of snapdragon in a cultivar-dependent manner, compared to untreated control treatment.

In addition to the stimulation action of microbial and non-microbial PBs, the application of these natural substances or microorganisms can have a dual effect including tolerance to both biotic and abiotic stressors. For instance, Sharma et al. study, showed that the exogenous application of jasmonic acid can aid *Brassica juncea* seedlings in recovering from the negative impact of oxidative stress caused by pesticide toxicity, throughout the up-regulation of *RUBISCO*, *NADH*, *CXE*, and *P450* and by triggering the antioxidative defense system of the plants. Similarly, *Trichoderma erinaceum* bio-priming modulated tomato defense transcriptome after the challenged conditions of *Fusarium oxysporum* f. sp. *lycopersici*, where the plants were accompanied by (i) improved accumulation of defense-related WRKY (a class of DNA-binding proteins) transcripts, (ii) increased antioxidative enzyme activities, and (iii) reinforced through a higher number of lignified cell layers, leading to a higher plant growth (Aamir et al.). Finally, Dal Cortivo et al. showed that sedaxane, a succinate dehydrogenase inhibitor with a well know fungicide action, exhibited also a significant hormone-like activity (i.e, auxin-like and gibberellin-like effects) when applied to maize seeds. The authors concluded that sedaxane application can facilitate root establishment and intensify N and phenylpropanoid metabolism in young maize, thus overcoming both biotic and abiotic pressure in early growth stages.

IMPLICATIONS OF BIOSTIMULANTS FOR ABIOTIC STRESSES TOLERANCE

Unfavorable environmental and soil conditions in particular drought, salinity, and extreme temperature are responsible for 70% of yield gap dictated by global climatic changes (Wang et al., 2003). According to the actual climate change scenario, these abiotic stresses are expected to have an increased negative impact, posing serious concerns on crop productivity, and thus food security worldwide (Rouphael et al., 2018b). In order to overcome this situation, the application of non-microbial and microbial PBs has been suggested as one of the most promising and efficient drivers toward further yield stability (Rouphael et al., 2018a).

The application of a legume-based PH (containing amino acids and soluble peptides), as foliar and especially as drench substrate, was found to mitigate the negative effects of drought in tomato grown in controlled environment, by increasing transpiration use efficiency (Paul et al.). The metabolomic approach adopted in this study allowed the identification of the molecular mechanisms of improved drought tolerance following the biostimulant treatment, such as (i) improved tolerance to ROS-mediated (ii) modulation of phytohormones and lipids profiles. The hormonal effects of an animal-based PH (containing L-α amino acids, free amino acids, organic-nitrogen, iron, and potassium) on water-stressed tomato plants were also assessed by a Spanish group (Casadesus et al.). Results of the greenhouse experiment showed that the application of animal-based PH benefited an antioxidant protection and exerted a major hormonal effect in tomato water-stressed leaves by increasing the endogenous content of auxin, cytokinin, and jasmonic acid. Microbial biostimulants based on AMF were also reported to promote tolerance of tomato plants toward drought stress. In the study of Volpe et al., the impact of two AMF strains *Funneliformis mosseae* and *Rhizophagus intraradices* on physiological and molecular responses of tomato were evaluated. The contribution of *F. mosseae* seems more effective on volatile organic compounds production, whereas *R. intraradices* exhibited the best performance traits, leading to a significant higher water use efficiency under severe drought stress. Additionally, *R. intraradices* was demonstrated to be effective against combined abiotic and biotic stress, the latter in terms of attraction toward aphids natural enemies. Moreover, in tomato cultures Bitterlich et al. showed that mycorrhizal plants indeed show higher water extraction rates per unit root length and biomass which was a consequence of AMF-mediated substrate hydraulic properties. The alleviation of substrate water flow resistances in AMF pots allowed for higher root extraction rates and maintenance of transpiration under progressive drought when the potential soil water flow to root systems were limiting transpiration rates (Bitterlich et al.). Because this study indicated that enhanced water extraction capacity in mycorrhizal pots was related to the flow of water from the bulk substrates to the root surface, the same group of authors carried out an additional study in order to see whether AMF substrate colonization under root exclusion is sufficient to alter substrate hydraulic properties (Bitterlich et al.). Indeed, substrate colonization by AMF that engaged in a functional symbiosis stabilized water retention and enhanced unsaturated hydraulic conductivity of the substrate. Theoretically, enhanced hydraulic conductivity in AMF substrates constitutes an effective

enlargement of the water depletion zone around roots. The authors concluded that further studies should investigate how this would quantitatively contribute to water acquisition by plants and the variability of the effect across different soils.

Characterization of several halotolerant PGPR (*Bacillus* spp.) isolated from the rhizosphere of durum wheat cultivated in hypersaline environments, revealed several growth promoting traits (Verma et al.). Several combinations of these PGPR strains were able to boost plant growth traits of mungbean. The authors concluded that specific strains such as *Bacillus* sp. BHUJP-H1 and *Bacillus* sp. BHUJP-H2 can be used as drought tolerant PGPR under open field conditions.

Non-microbial and microbial PBs can be also considered a possible way to enhance tolerance to salinity. Zou et al. reported that the application of crude polysaccharides from brown seaweed *Lessonia nigrescens* or the application of separated and fractionated acidic polysaccharides: LNP-1 at 40.2 kDa and especially LNP-2 at 63.9 kDa, improved the salinity tolerance of wheat seedlings. These beneficial effects were associated to several biochemical and physiological mechanisms such as (i) decreased membrane lipid peroxidation, (ii) increased chlorophyll content, (iii) improved antioxidant activities, and (iv) a better efflux and compartmentation of intracellular ion. The same group of authors, also demonstrated that not only polysaccharides deriving from brown algae but also those deriving from red algae (*Pyropia yezoensis*) can mitigate the negative effects of salinity on wheat seedlings grown under saline conditions (Zou et al.). In their second study, polysaccharides with different molecular weights (3.2, 10.5, 29.0, and 48.8 kDa) were prepared. The authors concluded that the lower-molecular weight samples (3.2 kDa) protected most effectively wheat seedlings against salt stress damage, by coordinating the efflux and compartmentation of NaCl and by enhancing antioxidant activities (Zou et al.). The use of a biostimulant product based on carboxylic acids, containing calcium oxide complexed by ammonium ligninsulfonate was tested on greenhouse lettuce, and it was proven to improve tolerance to nutrient solutions of high electrical conductivity (Bulgari et al.). Lettuce plants treated especially at the higher dose (0.2 ml/L), showed a significant increase in fresh biomass, which was associated to a better biochemical and physiological status (higher chlorophyll content and net photosynthetic rate). Similarly, Wu et al. demonstrated that exogenous 5-aminolevulinic acid application minimized NaCl toxicity on cucumber seedlings through improvement in chlorophyll synthesis, light harvesting capacity, photosynthesis capacity and retarded thylakoid degradation. Moreover, the beneficial role of small bioactive molecules (< 500 Da) such as omeprazole (OMP) a benzimidazole inhibitor of animal proton pumps was reported by Rouphael et al. Salt-stressed tomato plants treated with 10 or 100 μM OMP as substrate drench modulated root system architecture in terms of total length and surface, leading to a higher nutrient uptake and biomass production. Hormonal network was strongly influenced by OMP, eliciting an increase in ABA, a decrease in auxins and cytokinin, as well as a tendency in GA down accumulation. Finally, Albdaiwi et al. selected several potential bacterial isolates possessing plant growth promoting traits including N fixation, auxin and siderophore production and inorganic phosphate solubilization. The authors showed that six halotolerant PGPR strains were able to enhance survival in inoculated plants under high salt stress conditions as reflected by higher agronomic performance (higher germination percentages and seedling root growth) of durum wheat in comparison with non-inoculated plants.

IMPLICATIONS OF BIOSTIMULANTS FOR IMPROVING NUTRIENT USE EFFICIENCY

The use of bioactive natural substances and microbial inoculants can represent a valuable tool to enhance soil nutrient availability, plant nutrient uptake and assimilation (De Pascale et al., 2017). Increasing nutrient use efficiency in particular N and P is fundamental for both economical and environmental reasons. At both optimal and sub-optimal N regimens (112 and 7 mg L^{-1}, respectively) the application of legume-derived PH especially as substrate drench improved leaf number, SPAD (Soil Plant Analysis Development) index, and biomass production of greenhouse tomato (Sestilli et al.). The better agronomic responses of PH-treated tomato was associated to the stimulation of root apparatus that facilitated N uptake and translocation. Moreover, under sub-optimal N concentrations, PH application upregulated the expression of genes encoding for amino acid transporter and ferredoxin-glutamate synthases and glutamine synthetase in roots, which are known to be involved in N assimilation. Furthermore, the biostimulant action of two strains of *Trichoderma* (*T. virens* GV41 or *T. harzianum* T22), under suboptimal, optimal, and supraoptimal levels of N in two leafy vegetables: lettuce and rocket was investigated by Fiorentino et al. The authors reported that *T. virens* GV41 improved Nitrogen Use Efficiency (NUE) of lettuce, and favored the uptake of native N present in the soil of both leafy vegetables. The beneficial effect of microbial-based biostimulants was species-dependent with more pronounced effects recorded on lettuce. The findings also demonstrated that *Trichoderma* inoculation strongly modulated the composition of eukaryotic populations in the rhizosphere, by exerting different effects with suboptimal N regimen compared to N fertilized treatments. In addition to beneficial fungi, bacterial inoculants could also improve the availability of nutrients and their utilization by plants. In Koskey et al. work, 41 rhizobia isolates from root nodules of mild altitudes climbing bean varieties were characterized from a morpho-cultural, biochemical, and genetic point of view in order to select strains with potential biofertilizer properties able to perform under diverse environments. The use of multiple microbial inoculants (bacteria + fungi) containing *Agrobacterium*, *Azotobacter*, *Azospirillum*, *Bacillus*, *Pseudomonas*, *Streptomyces*, *Trichoderma*, and *R. irregularis* was found effective for wheat production compared to the commercial mineral and chemical fertilizers applied at the recommended level for on-farm use in south-western Australia characterized by moderately N and P deficient soil (Assainar et al.). Zinc solubilization by PGPR is relatively a newer approach, thus a research group from Pakistan

screened zinc solubilizing rhizobacteria isolated from wheat and sugarcane and analyzed their effects on wheat (Kamran et al.). The authors reported the potential of *Pantoea*, *Enterobacter cloacae*, and especially *Pseudomonas fragi* to be used as microbial-based biostimulant to overcome zinc deficiency under low input scenario.

IMPLICATIONS OF BIOSTIMULANTS FOR ENHANCING PRODUCE QUALITY

The application of microbial and non-microbial plant biostimulants are able to modify plant primary and secondary metabolism (Colla et al., 2015; Rouphael et al., 2015) leading to the synthesis and accumulation of antioxidant molecules (i.e., secondary metabolites) which are important for human diet. The application of earthworm grazed and *Trichoderma harzianum* biofortified spent mushroom substrate (SMS) induced a significant increase in tomato fruit quality in terms of antioxidant capacity, total soluble sugars, carotenoids (lycopene, lutein, and β-carotene), total polyphenols, and flavonoids contents as well as mineral composition (P, K, Ca, Mg, Fe, Mn, and Zn) (Singh et al.). Moreover, Trejo-Téllez et al. investigated the effect of photosynthetically active radiation (low or high), phosphate (low or high), and phosphite (low, optimal or high), and their interactions on the concentrations of glucosinolates, flavonoids, and nitrate in two *Brassica* species: *Brassica campestris* and *Brassica juncea*. The authors reported that the application of phosphite in the nutrient solution tends to increase phosphate deficiency; therefore, it favors the biosynthesis and accumulation of some target flavonoids and glucosinolates as a possible defense mechanism for coping with nutrient stress.

Concerning fruit trees and grapevines, several authors (Soppelsa et al.; D'Amato et al.; Vergara et al.; Koyama et al.) investigated the application of PBs or exogenous molecules on nutritional and functional quality of fruits. Biostimulant products based on *A. nodosum* seaweed extract, PH, and B-group vitamins had a minor impact on primary apple quality traits (size, flesh firmness, acidity, and total sugars), whereas they induced an improvement of the intensity and extension of red coloration in "Jonathan" apples at harvest in the 2 years trials (Soppelsa et al.). Moreover, the foliar application of Se on olive trees improved nutritional and functional qualities of Extra Virgin Olive Oil (EVOO); since besides the Se biofortification effect, an accumulation of antioxidants molecules was recorded by D'Amato et al. In their study, the biosynthesis and accumulation of key antioxidant molecules such as carotenoids and phenols may have brought advantages to EVOO itself, by improving its oxidative stability and consequently its shelf-life.

In "Redglobe" table grape, treatments with 3 brassinosteroids analogs (24-epibrassinolide, Triol, or Lactone at three concentrations 0.0, 0.4, or 0.8 mg L^{-1}) or a commercial formulation (B-2000R at 0.06 mg L^{-1}) at the onset of véraison, improved total soluble solids, berries color, and anthocyanins without altering yield (Vergara et al.). In line with the previous study, the exogenous application of abscisic acid at different timings (7 or 21 days after véraison; DAV) and concentrations (200 or 400 mg L^{-1}) modulated the biosynthesis of anthocyanins and flavonoids in *Vitis vinifera* × *Vitis labrusca* table grapes (Koyama et al.). The authors showed that two applications (at 7 and 21 DAV) of abscisic acid at 400 mg L^{-1}, resulted in an increase in (i) concentrations of the total and individual anthocyanin, (ii) expression of the key biosynthetic genes *CHI*, *DFR*, *F3H*, and *UFGT*, and (iii) expression of the transcription factors *VvMYBA1* and *VvMYBA2*.

OUTLOOK AND CHALLENGES AHEAD

PBs including natural substances and microbial inoculants appear as a novel and potential category of agricultural inputs, complementing agrochemicals including synthetic fertilizers, and improving tolerance to abiotic stresses, as well as enhancing the quality of agricultural and horticultural commodities. Characterizing the bioactive components of PBs and elucidating the molecular and physiological stimulation mechanisms are still of high interest for the scientific community and commercial enterprises. Due to the complex matrices with different groups of bioactive and signaling molecules, the use of small/medium/large high-throughput phenotyping is the most efficient technology to develop novel biostimulants (Rouphael et al.). Ugena et al. demonstrated that multi-trait high-throughput screening is suitable for identifying new potential biostimulants and characterizing their mode of action under both optimal and sub-optimal (i.e., salinity) conditions. Based on this novel technology, the authors concluded that the mode of action of PBs could be summarized in three groups: (i) plant growth promotors/inhibitors, (ii) stress alleviators, and (iii) combined action. Similarly, Paul et al. reported that the combined use of high-throughput phenotyping and metabolomics could facilitate the screening of new bioactive and signaling substances with biostimulant properties and could provide a biochemical, morpho-physiological, and metabolomic gateway to the mode of actions, underlying PHs action on tomato. Finally, Rouphael and Colla suggested that in the near future the main players of PBs (scientists, private industries, legislators, and stakeholders) should focus on the development of a second generation of these products (biostimulant 2.0) with specific synergistic biostimulatory action through the application of both microbial and non-microbial PBs to render agriculture more sustainable and resilient.

AUTHOR CONTRIBUTIONS

YR and GC have made a substantial, direct, and intellectual contribution to the work, and approved it for publication in Frontiers in Plant Science.

REFERENCES

Battacharyya, D., Babgohari, M. Z., Rathor, P., and Prithiviraj, B. (2015). Seaweed extracts as biostimulants in horticulture. *Sci. Hortic.* 196, 39–48. doi: 10.1016/j.scienta.2015.09.012

Calvo, P., Nelson, L., and Kloepper, J. W. (2014). Agricultural uses of plant biostimulants. *Plant Soil* 383, 3–41. doi: 10.1007/s11104-014-2131-8

Canellas, L. P., Olivares, F. L., Aguiar, N. O., Jones, D. L., Nebbioso, A., Mazzei, P., et al. (2015). Humic and fulvic acids as biostimulants in horticulture. *Sci. Hortic.* 196, 15–27. doi: 10.1016/j.scienta.2015.09.013

Colla, G., and Rouphael, Y. (2015). Biostimulants in horticulture. *Sci. Hortic.* 196, 1–2. doi: 10.1016/j.scienta.2015.10.044

Colla, G., Nardi, S., Cardarelli, M., Ertani, A., Lucini, L., Canaguier, R., et al. (2015). Protein hydrolysates as biostimulants in horticulture. *Sci. Hortic.* 196, 28–38. doi: 10.1016/j.scienta.2015.08.037

De Pascale, S., Rouphael, Y., and Colla, G. (2017). Plant biostimulants: innovative tool for enhancing plant nutrition in organic farming. *Eur. J. Hortic. Sci.* 82, 277–285. doi: 10.17660/eJHS.2017/82.6.2

du Jardin, P. (2012). The science of plant biostimulants-a bibliographic analysis. Contract 30-CE0455515/00-96, *ad hoc* study on bio-stimulants products. http://ec.europa.eu/enterprise/sectors/chemicals/files/fertilizers/final_report_bio_2012_en.pdf.

du Jardin, P. (2015). Plant biostimulants: Definition, concept, main categories and regulation. *Sci. Hortic.* 196, 3–14. doi: 10.1016/j.scienta.2015.09.021

EU. (2019). Regulation of the european parliament and of the council laying down rules on the making available on the market of EU fertilising products and amending Regulations (EC) No 1069/2009 and (EC) No 1107/2009 and repealing Regulation (EC) No 2003/2003. https://eur-lex.europa.eu/legal-content/EN/TXT/?uri=OJ:L:2019:170:TOC.

Gómez-Merino, F. C., and Trejo-Téllez, L. I. (2015). Biostimulant activity of phosphate in horticulture. *Science Hortic*. 196, 82–90. doi: 10.1016/j.scienta.2015.09.035

Haplern, M., Bar-Tal, A., Ofek, M., Minz, D., Muller, T., and Yermiyahu, U. (2015). The use of biostimulants for enhancing nutrient uptake. *Adv. Agron.* 130, 141–174. doi: 10.1016/bs.agron.2014.10.001

López-Bucio, J., Pelagio-Flores, R., and Herrera-Estrella, A. (2015). *Trichoderma* as biostimulant: exploiting the multi level properties of a plant beneficial fungus. *Sci. Hortic.* 196, 109–123. doi: 10.1016/j.scienta.2015.08.043

Matsumiya, Y., and Kubo, M. (2011). "Soybean peptide: novel plant growth promoting peptide from soybean," in *Soybean and Nutrition*. Ed. H. El-Shemy (Rijeka: In Tech Europe Publisher), 215–230. doi: 10.5772/19132

Nardi, S., Pizzeghello, D., Schiavon, M., and Ertani, A. (2016). Plant biostimulants: physiological responses induced by protein hydrolyzed-based products and humic substances in plant metabolism. *Sci. Agric.* 73, 18–23. doi: 10.1590/0103-9016-2015-0006

Pichyangkura, R., and Chadchawan, S. (2015). Biostimulant activity of chitosan in horticulture. *Sci. Hortic.* 196, 49–65. doi: 10.1016/j.scienta.2015.09.031

Rouphael, Y., Franken, P., Schneider, C., Schwarz, D., Giovannetti, M., Agnolucci, M., et al. (2015). Arbuscular mycorrhizal fungi act as biostimulants in horticultural crops. *Sci. Hortic.* 196, 91–108. doi: 10.1016/j.scienta.2015.09.002

Rouphael, Y., Cardarelli, M., Bonini, P., and Colla, G. (2017a). Synergistic action of a microbial-based biostimulant and a plant derived-protein hydrolysate enhances lettuce tolerance to alkalinity and salinity. *Front. Plant Sci.* 8, 131. doi: 10.3389/fpls.2017.00131

Rouphael, Y., De Micco, V., Arena, C., Raimondi, G., Colla, G., and De Pascale, S. (2017b). Effect of *Ecklonia maxima* seaweed extract on yield, mineral composition, gas exchange and leaf anatomy of zucchini squash grown under saline conditions. *J. Appl. Phycol.* 29, 459–470. doi: 10.1007/s10811-016-0937-x

Rouphael, Y., Colla, G., Graziani, G., Ritieni, A., Cardarelli, M., and De Pascale, S. (2017c). Phenolic composition, antioxidant activity and mineral profile in two seed-propagated artichoke cultivars as affected by microbial inoculants and planting time. *Food Chem.* 234, 10–19. doi: 10.1016/j.foodchem.2017.04.175

Rouphael, Y., Kyriacou, M. C., Petropoulos, S. A., De Pascale, S., and Colla, G. (2018a). Improving vegetable quality in controlled environments. *Sci. Hortic.* 234, 275–289. doi: 10.1016/j.scienta.2018.02.033

Rouphael, Y., Kyriacou, M. C., and Colla, G. (2018b). Vegetable grafting: a toolbox for securing yield stability under multiple stress conditions. *Front. Plant Sci.* 8, 2255. doi: 10.3389/fpls.2017.02255

Ruzzi, M., and Aroca, R. (2015). Plant growth-promoting rhizobacteria act as biostimulants in horticulture. *Sci. Hortic.* 196, 124–134. doi: 10.1016/j.scienta.2015.08.042

Savvas, D., and Ntatsi, G. (2015). Biostimulant activity of silicon in horticulture. *Sci. Hortic.* 196, 66–81. doi: 10.1016/j.scienta.2015.09.010

Wang, W., Vinocur, B., and Altman, A. (2003). Plant responses to drought, salinity and extreme temperatures: towards genetic engineering for stress tolerance. *Planta* 218, 1–14. doi: 10.1007/s00425-003-1105-5

Yakhin, O. I., Lubyanov, A. A., Yakhin, I. A., and Brown, P. H. (2017). Biostimulants in plant science: a global perspective. *Front. Plant Sci.* 7, 2049. doi: 10.3389/fpls.2016.02049

Zhang, X., and Schmidt, R. E. (1997). The impact of growth regulators on alpha-tocopherol status of water-stressed *Poa pratensis* L. *Int. Turfgrass Soc. Res. J.* 8, 1364– 2137.

Conflict of Interest: The authors declare that the research was conducted in the absence of any commercial or financial relationships that could be construed as a potential conflict of interest.

frontiers in Plant Science

REVIEW
published: 13 October 2017
doi: 10.3389/fpls.2017.01762

Plant Hormesis Management with Biostimulants of Biotic Origin in Agriculture

*Marcela Vargas-Hernandez[1], Israel Macias-Bobadilla[1], Ramon G. Guevara-Gonzalez[1], Sergio de J. Romero-Gomez[2], Enrique Rico-Garcia[1], Rosalia V. Ocampo-Velazquez[1], Luz de L. Alvarez-Arquieta[1] and Irineo Torres-Pacheco[1]**

[1] Laboratory of Biosystems Engineering, Autonomous University of Queretaro, Faculty of Engineering, Campus Amazcala, Queretaro, Mexico, [2] Laboratory of Microbiology, Autonomous University of Queretaro, Faculty of Chemistry, C.U. Cerro de las Campanas, Queretaro, Mexico

Over time plants developed complex mechanisms in order to adapt themselves to the environment. Plant innate immunity is one of the most important mechanisms for the environmental adaptation. A myriad of secondary metabolites with nutraceutical features are produced by the plant immune system in order to get adaptation to new environments that provoke stress (stressors). Hormesis is a phenomenon by which a stressor (i.e., toxins, herbicides, etc.) stimulates the cellular stress response, including secondary metabolites production, in order to help organisms to establish adaptive responses. Hormetins of biotic origin (i.e., biostimulants or biological control compounds), in certain doses might enhance plant performance, however, in excessive doses they are commonly deleterious. Biostimulants or biological control compounds of biotic origin are called "elicitors" that have widely been studied as inducers of plant tolerance to biotic and abiotic stresses. The plant response toward elicitors is reminiscent of hormetic responses toward toxins in several organisms. Thus, controlled management of hormetic responses in plants using these types of compounds is expected to be an important tool to increase nutraceutical quality of plant food and trying to minimize negative effects on yields. The aim of this review is to analyze the potential for agriculture that the use of biostimulants and biological control compounds of biotic origin could have in the management of the plant hormesis. The use of homolog DNA as biostimulant or biological control compound in crop production is also discussed.

Keywords: hormesis, agriculture, nutraceutic, elicitor, homolog DNA

OPEN ACCESS

Edited by:
Giuseppe Colla,
Università degli Studi della Tuscia, Italy

Reviewed by:
Georgia Ntatsi,
Agricultural University of Athens, Greece
Antonio Ferrante,
Università degli Studi di Milano, Italy

****Correspondence:***
Irineo Torres-Pacheco
torresirineo@gmail.com

Specialty section:
This article was submitted to Crop Science and Horticulture, a section of the journal Frontiers in Plant Science

Received: *07 August 2017*
Accepted: *26 September 2017*
Published: *13 October 2017*

Citation:
Vargas-Hernandez M, Macias-Bobadilla I, Guevara-Gonzalez RG, Romero-Gomez SdJ, Rico-Garcia E, Ocampo-Velazquez RV, Alvarez-Arquieta LdL and Torres-Pacheco I (2017) Plant Hormesis Management with Biostimulants of Biotic Origin in Agriculture. Front. Plant Sci. 8:1762. doi: 10.3389/fpls.2017.01762

INTRODUCTION

Currently, there are generalizable processes from which different terminologies have been constructed, including those from which they may be described; hormesis is a process of this type, present in all organisms (Calabrese et al., 2007). In toxicology, hormesis is defined as a biphasic response to a toxic compound (stressor), which at low doses induces a beneficial effect and at high doses produces a toxic effect. However, at the physiological level, this can be translated as an adaptive response of an organism to a low level of stress factor, accompanied by overcompensation, when the homeostasis readjustment has been interrupted (Calabrese et al., 2007; Mattson, 2008; Calabrese, 2009). This allows the organism to acclimate to its new environment, a key factor in

the evolutionary process. The factors responsible for inducing hormesis are known as hormetins or stressors. In this sense, it has been established that in plants the challenge with different levels of stress constitutes an adaptive process, having reminiscence with the phenomenon of hormesis abovementioned. This stress can be established as "eustress" (beneficial stress) if the effect is similar to the hormetic effect in low doses of a toxin, or "distress" (harmful stress) if the level of this generates an irreversible or negative damage in the plant (Hideg et al., 2013). The level of eustress or distress toward the same factor (e.g., a biostimulant) is not always the same due to the process of adaptation of the plants, thus it is important to take into account these terms when talking about hormesis to establish a strict difference between low dose and high dose of a hormetic factor. It is considered potentially toxic an agent that disrupts homeostasis, and the hormetic effect can be observed as a reparative process that slightly or modestly overshoots the original homeostatic level (Calabrese et al., 2007). In this sense, several plant stressor of biotic origin disrupt homeostasis at molecular level by inducing adaptive responses in organisms that cause increased growth and induce defense processes against biotic and abiotic stresses in several crops, although at cellular level the effects might not clearly been observed by some elicitors. Great efforts have been made to define the concept of biostimulant. According to du Jardin (2015), those compounds or microorganisms that have the function of improving nutrition, efficiency, and tolerance to abiotic stress and/or quality traits of crops are called biostimulants. A more recent definition is that proposed by Yakhin et al. (2017): "A formulated product of biological origin that improves plant productivity as a consequence of the novel, or emergent properties of the complex of constituents, and not as a sole consequence of the presence of known essential plant nutrients, plant growth regulators, or plant protective compounds." Some of the key elements of its definition are the composition of biotic origin, its ability to modify physiological processes to increase the productivity of plants, and protect them from abiotic stress (Yakhin et al., 2017). However, compounds protecting plants from biotic stresses are called biological control compounds. It should be clarified that many of the biotic origin compounds not only have the ability to protect plants from either abiotic or biotic stress, but against both, it is to say they have a function of both biostimulant and biological control compounds (Lucas et al., 2014). If the biostimulant or biological control compound is of biotic origin it is called elicitor (du Jardin, 2015). Elicitors are factors that trigger plant immunity in a dose–response manner. Low dose of elicitors normally induces a eustress condition and at high dose a distress in plants (Mandal et al., 2013; Garcia-Mier et al., 2015; Zunun-Pérez et al., 2017). This behavior is similar to the hormetic effect of physical and chemical factors (Hooper et al., 2010; Tierranegra-García et al., 2011; Mejía-Teniente et al., 2013; Baenas et al., 2014; Calabrese, 2014a; Liu et al., 2016). In order to unify concepts in this sense, it would be necessary to carry out experimentation evaluating elicitor's dose–response curves to determine the hormetic effects of these compounds. Thus, it is clear that mild-stimuli activates plant defense provoking a eustress situation using an elicitor. The stresses coped by plants may have a biotic or abiotic origin provoking an increase in metabolites to cope the stress (Tierranegra-García et al., 2011; Mejía-Teniente et al., 2013; Baenas et al., 2014; Liu et al., 2016); however, when an individual feeds on a "stressed plant" is good for health, a concept called "xenohormesis" (Hooper et al., 2010). Xenohormetic potential of crops can be increased by the hormesis management due to plant possess receptors for molecular patterns (MPs) of different origin as microbial-associated molecular patterns (MAMPs), pathogen-associated molecular patterns (PAMPs), damage-associated molecular patterns (DAMPs), and HAMPs that potentially triggers secondary metabolites pathways. The aim of this review is to analyze possibilities where eustress management using biostimulants or biological control compounds of biotic origin in plants create a hormetic condition to induce equilibrium between xenohormetic potential and yields in crops. The adequate management of this phenomenon it is considered that will be of great importance because of the climate change scenario for agriculture. The use of homolog DNA as biostimulant or biological control compound in crop production is also discussed.

HORMETIC DOSE–RESPONSE IN PLANTS

Hormesis can be defined as a biphasic response in which high doses of a toxic agent could cause inhibition while low dose of the same toxic can cause stimulation (Calabrese, 2009). This process is described by a U or J shape in which there is an initial disruption of homeostasis (i.e., toxicity) followed by a modest overcompensation response that eventually leads to a re-establishment of homeostasis. The changes suffered in cells or organisms at low doses of a stress condition reflect in environmentally induced altered phenotype, the above can be translated in a quantitatively plasticity potential (Calabrese and Mattson, 2011; Calabrese, 2014a). It is this modest overcompensation response, which is seen as the hormetic low-dose stimulation. A study carried out in *Arabidopsis thaliana* at low doses of a synthetic elicitor 2-(5-bromo-2-hydroxy-phenyl)-thiazolidine-4-carboxylic acid (BHTC) enhanced root growth, while high doses of this compound inhibited root growth, besides inducing defense (Rodriguez-Salus et al., 2016). At low levels BHTC triggers a coordinated intercompartmental transcriptional response manifested in the suppression of photosynthesis- and respiration-related genes in the nucleus, chloroplasts, and mitochondria as well as the induction of development-related nuclear genes, while at high doses induce typical defense-related transcriptional changes (Rodriguez-Salus et al., 2016). Hormesis has been widely characterized in the toxicology field, however, considerable efforts have focused on studying this process on the plant biology and agricultural areas in order to enhance crop production (Calabrese, 2014a). Also a major research need is the extension of hormesis beyond chemical stressors to abiotic (e.g., habitat) and biotic stressors (e.g., species introductions, organism interactions) (Chapman, 2001). Despite the fact that hormesis is a generalizable process that can occur

in all organisms, hormesis has been primarily associated with only chemical and physical factors and a limited number of studies on plants (Calabrese and Blain, 2005; Balasubramaniyam, 2015).

The spectrum of endpoints displaying hormetic dose responses is also broad being inclusive of growth, longevity, numerous metabolic parameters, disease incidences (including cancer), and various performance endpoints such as cognitive functions, immune responses, among others (Calabrese and Blain, 2005). Reports of ca. 8000 dose responses within a hormesis database concluded that hormesis has specific characteristics, which are highly generalizable, being independent of the biological model, endpoint measurement, and chemical/physical stress inducing agent; and also the response observed typically falls within a range between approximately 30 and 60% higher than control values (Calabrese and Blain, 2005; Calabrese et al., 2007; Calabrese, 2013, 2014b). Studies in plants have been conducted mainly measuring the endpoint of growth, metabolism, mutagenic, survival, reproduction meanwhile the immune responses are less known (Calabrese and Blain, 2005). The plant hormesis have been often carried out using ion metals, herbicides, or phytotoxins (Poschenrieder et al., 2013; Belz and Duke, 2014). However, as well as the use of environment stress factors the same basic problems have been observed with the use of herbicides, the potential harmful effect in crop plants caused by the toxicity is observed (Belz and Duke, 2014). The hormesis management can be a powerful strategy to satisfy the demand of the prevailing agriculture to maintain desirable yields in crops and increase the xenohormesis potential. But it is indispensable to consider that for the assessment and characterization of the hormesis process the experimental designs require more doses, greater simple population, and a heightened need for replication (Calabrese, 2014b). The hormesis management with biostimulants or biological control compounds is founded in the recognition of plant receptors through which the induction of secondary metabolism modification can be achieved.

Hormesis Mechanism in Plants

Generally, cellular and molecular mechanisms under the effect of hormesis include the activation of the growth factor signaling pathways, ion channels, kinases and deacetylases, and transcription factors responsible for the production of cytoprotective proteins such as chaperones [i.e., heat-shock proteins (HSPs)], antioxidant enzymes (i.e., superoxide dismutases and glutathione peroxidase), and growth factors (i.e., insulin-like growth factors and brain-derived neurotrophic factor), as well as cell survival genes (Mattson, 2008). In plants, the mechanism of hormesis is still unknown, however, some mechanisms have been proposed, (1) a stress factor may have a mode of action as a growth stimulator, (2) the mechanism of hormesis is dependent on the mechanism of the herbicide as a phytotoxin at low concentrations, and (3) a more indirect mechanism for hormesis, overcompensation, related in part with induction of plant defenses (Belz and Duke, 2014). Other authors proposed that low concentrations of toxic metals induce hormetic effects through activating plant stress defense mechanisms (Poschenrieder et al., 2013). The induction of reactive oxygen species (ROS) by mild stress (eustress) leading to the activation of antioxidant defenses, stress-signaling hormones, or adaptive growth responses is the most probable pathways for hormetic responses (Poschenrieder et al., 2013).

In plants exposure to abiotic stressors brings to oxidative stress by affecting antioxidative defense machinery, electron transport system, or induction of lipid peroxidation, however strict redox level, regulated by enzymatic and non-enzymatic antioxidants, maintains cellular redox homeostasis and control of signaling pathways (Singh et al., 2016). Biotic stressors also induce ROS production. For example, redox homeostasis is necessary for symbiosis between legumes and rhizobia, inoculation of *M. truncatula* seedlings with pathogenic or symbiotic bacteria induces the oxidative burst in the host, with a major difference in the levels of ROS production (Peleg-Grossman et al., 2012). Strawberry plant cells treated with AsES elicitor obtained from *Acremonium strictum* exhibited a triphasic production of H_2O_2 and a rapid intracellular accumulation of NO (Martos et al., 2015).

Some of the redox sensitive pathways, observed in organisms, which induce adaptations includes NF-κB, the MAPK family, the phosphoinositide-3-kinase (PI3K)/Akt pathway, p53 activation, and the HSPs, adaptations are also mediated by H_2O_2, a ROS byproduct, which upregulate gene expression (Ji, 2014). Environmental stresses like drought, heavy metals, and UV radiations enhance ROS, provoking damage in biomolecules including proteins. Production of HSPs is essential for folding and repair of damaged proteins and serves to promote cell survival conditions (Calabrese et al., 2016). HSPs respond also to biotic stresses such as pathogen infection and insect attacks (Park and Seo, 2015). Heat stress (37°C) and *Xanthomonas campestris* pv. vesicatoria infection distinctly induce CaHSP70a in pepper leaves, mediating the hypersensitive cell death response (HR) by *X. campestris* pv. vesicatoria (avrBsT) infection. Strong induction of defense- and cell death-related genes in transient CaHSP70a overexpression was also observed (Kim and Hwang, 2015). However, there is a complex and integrative array of signal transduction pathways that mediate hormetic stimulatory responses (Calabrese, 2013).

Biostimulants and Biological Control Compounds Affecting the Hormesis Management

Hormetins according to Stebbing (1987, 1998) depends to a greater extent by organisms rather than the chemical, and then any agent that can disrupt homeostasis would be expected to induce a hormetic response to the induced damage (Calabrese, 2008). It must be also taken into account that organisms respond in a hormetic manner to signals that indicate stress, toxicity, or disruptions in homeostasis (Calabrese, 2008). Plants perceive MAMPs, DAMPs, HAMPs, or PAMPs as signals of danger and induce defense mechanisms, thus disturbing homeostasis trying to cope the potential problem.

Biostimulants are a proposed concept describing any substance or microorganism applied to plants with the aim to enhance nutrition efficiency, abiotic stress tolerance, and/or crop quality traits, regardless of its nutrients content (du Jardin, 2015). According to biostimulants definition among organisms either beneficial or pathogens such as bacteria, fungus, virus, nematodes, and plants may be included. It should be clarified that if we are talking about compounds that protect plants from biotic stresses they are called biological control compounds. Plants can recognize molecular of biotic origin (elicitors) because they are the signal of damage done to plants by other living organisms or environmental conditions. Little is known about the evaluation of hormesis in plants by compounds derived of organisms (Calabrese and Blain, 2005; Belz and Duke, 2014). Studies of herbicide hormesis propose that the mechanism of action is related to the target site of the herbicide, or are produced by overcompensation to moderate stress induced by the herbicides or a response to disturbed homeostasis (Belz and Duke, 2014). The hormetic dose response model in plants can lead to the determination of the biostimulant concentration in which the highest adaptive response studied is observed. The responses, which can be evaluated in hormesis, are disease resistance, production of some secondary metabolites, yields, and growth among others (Calabrese and Blain, 2005; Mattson, 2008; Calabrese and Mattson, 2011). The mechanism increasing plant fitness by biotic elicitors is inducing a defense response in plant translated in increased ROS levels leading to oxidative stress inducing disruption of the redox homeostasis and negative effects on macromolecules as proteins, DNA, RNA, and lipids, which are necessary for cell functioning (Sewelam et al., 2016). Adaptive response causes the recovery of homeostasis, by activation or repression of several defense genes and metabolic pathways (Bhattacharyya et al., 2014). Currently, there are many studies of the effect of biotic elicitors in plants. However, in these studies more doses are necessary to be evaluated in order to observe a hormetic dose response as in toxicology studies (**Figure 1**). The developments of analysis of the hormetic dose responses in plants need rigorous criteria in homogeneous selection of the individuals, several doses of the biostimulant or biological control compound, exposure time, and greater sample sizes (Calabrese and Blain, 2005; Mattson, 2008). Other phenomena such as pre- and post-conditioning must be taken into account when evaluating the hormesis response.

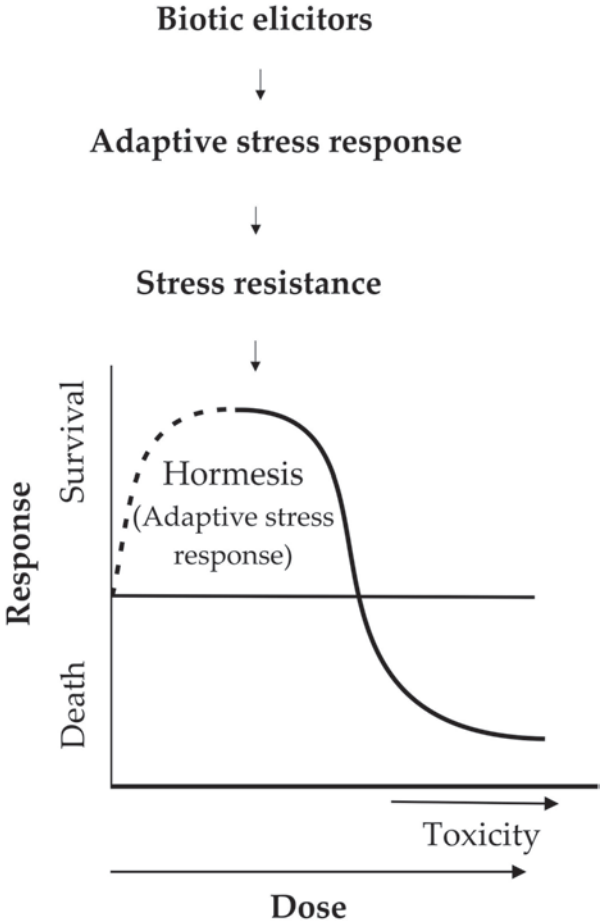


FIGURE 1 | The hormesis induction through elicitors of biotic origin in plants.

PLANT RECOGNITION AND DEFENSE MECHANISMS

Plants, due to the lack of mobility, are exposed to adverse environmental factors that cannot be avoided, and having a direct influence on their development. Adverse factors include not only physical conditions such as temperature salinity, drought, radiation, but also plant–organism interaction. Although it is unknown whether the physiological benefits of pathogen challenges fulfill the characteristic pattern of hormesis, the finding that life-history traits can be improved by a single dose of pathogen challenge suggests that immunization as a type of hormesis, can be induced by host responses to pathogen challenge, and even when the source of stress response is not a pathogen, appears to be driven by the expression of genes associated with immunity (McClure et al., 2014). An interesting study in *Drosophila melanogaster* with a single topical dose of dead spores of the entomopathogenic fungus, *Metarhizium robertsii* displayed a close relationship between the defense system and hormesis, suggesting that hormetic responses to stress might be greater in animals lacking functional immune responses and that hormesis should increase susceptibility to infection (McClure et al., 2014).

It is likely that there is a close relation between physical and chemical induced hormesis in plants and plant defense pathways. It has been proposed that low concentrations of toxic metals induce hormetic effects through activating plant stress defense mechanisms (Poschenrieder et al., 2013). Organisms possess genetic information to produce changes in phenotype, which lead the process of moderate adaptation. These plant changes include a range of more effective plant defenses. On the other hand, there are factors that also limit their development, including coexistence with other living organisms, both beneficial and non-beneficial. An important process in

the plant–organism interaction is the recognition between self and non-self (Coers, 2013). It is therefore important, after the recognition of the microorganism, to give a response with a high degree of specificity, according to determinate microorganism, depending on one or few genes presented by both plant and pathogen (Brodeur, 2012). Specificity also occurs in the organs, some organisms develop on a single tissue or, in aerial or parts under the ground (Jones and Dangl, 2006; Strugala et al., 2015). The onset of this process is given by physical and chemical signals followed by recognition of MAMPS or PAMPs, and/or DAMPs by transmembrane pattern recognition receptors (PRRs), resulting in PAMP-triggered immunity (PTI) (Sanabria et al., 2010). After this, pathogens release effectors, which are recognized by host causing an effector-triggered susceptibility (ETS), causing disease resistance and HR (Wiesel et al., 2014). Pathogens try to avoid effector-triggered immunity (ETI) through a constant struggle to evade the defense system of the plant by the synthesis of compounds named effectors. This recognition process prevents the spread of disease throughout the plant by restricting the invasion through systemic induced resistance (SIR) (Stotz et al., 2014). The time of inducing the stress responses is important for the plant surveillance, the faster the plant responds to the pathogen attack, the easier is to cope the infection (Newman et al., 2013). After a plant pathogen encounter with its host, host susceptibility decreases to subsequent pathogen attacks. In addition to the foregoing, one of the SIR is systemic acquired resistance (SAR) characterized by giving a perdurable resistance for a long time, characterized by localized necrosis, expression of pathogenesis-related (PR) genes, and accumulation of salicylic acid (SA; Dempsey and Klessig, 2012). The event that results in the encounter between the host and microorganism can provoke symbiosis, disease, or disease resistance; however, non-pathogenic microorganisms can also induce a systemic resistance in plants although to a lesser degree (Pieterse et al., 2014). Products resulting of defense mechanism are cell wall reinforcement, production of ROS, and the synthesis of phytoalexins, and PR protein.

Plant resistance to a pathogenic microorganism depends on their specificity. Differences in the degree in which plants are being infected and failure in infection depend on changes of genotypes caused by evolution in both plants and pathogens (Antonovics et al., 2013). There are two kinds of resistance in plants, host and non-host resistance (NHR), in which responses during infection are very similar (Thakur and Sohal, 2013). NHR is present in entire plant species to a non-adapted pathogen. Two kinds of these have been proposed Type I and II NHR, the first does not produce visible symptoms whereas Type II NHR a rapid hypersensitive response is observed followed by cell death (Cheng et al., 2012). Host resistance, on the other hand, is given by the specificity of the pathogen race or plant cultivar, and is mediated by the interactions of resistance genes I and avirulence genes (Avr), this process is explained by gene for gene model (Cheng et al., 2012). Resistance of the plant and avirulence of the pathogen are present when recognition of the R genes and corresponding Avr genes occurs (Thakur and Sohal, 2013).

PRIMING OF PLANT DEFENSES

Although plants do not spend in the implementation of defense when there is absence of enemies, when these are presented, plants can suffer irreparable damages during the time required to mount defenses once attack occurs (Frost et al., 2008). As part of evolution, plants have developed a priming process to ward off these dangerous situations. Stress factors have the ability to induce priming including some elicitors derived of organisms such as plants or microorganisms. For example, primed tomato plants with elicitors such as chitosan (CHT), SA, and jasmonic acid (JA) have the ability to promote resistance in plants against a higher stress provoked by *Ralstonia solanacearum*, reducing vascular browning and wilting symptoms of tomato (Mandal et al., 2013).

The process of priming exhibits certain characteristics, it establishes in the exposed tissue to the elicitor and the distal parts, and the response due to the priming defeats a wide spectrum of microorganisms, in posterior attacks the activation is faster, stronger, and last longer or has attenuated repression (Conrath et al., 2015; Martinez-Medina et al., 2016). Pathogen attack on AMF-inoculated in tomatoes provoked strong defense responses by induction of PR proteins, PR1, PR2, and PR3, as well as defense-related genes LOX, AOC, and PAL, in addition, the induction defense responses in AMF pre-inoculated plants was much higher and more rapid than in un-inoculated plants (Song et al., 2015). The main advantage offered by priming is the reduction of the metabolic cost for plants, for example, to attract natural enemies of the herbivore can produce a minor cost than induction of direct defenses and on the other hand the ability to maintain fitness in complex environments (Conrath et al., 2015; Martinez-Medina et al., 2016).

After experiencing for the first time the stress plants have the ability to respond differently to the following stress exposures (Avramova, 2015). When plants are primed by different stress factors various types of systemic plant immunity can be induced, including SAR and ISR (Aranega-Bou et al., 2014; Pieterse et al., 2014), the above is presented in **Figure 2**. The responsible mechanism for priming in plants has not been completely deciphered. Some of the components playing a central role in SA-mediating priming in *A. thaliana* are the mitogen-activated protein kinases 3 and 6 (Beckers et al., 2009), transcription factors MYC2 (Pozo et al., 2008), elevated levels of PRRs such as FLS2, CERK1, and epigenetic modifications (Jaskiewicz et al., 2011; Tateda et al., 2014). Another advantage for agricultural application is that the priming characteristic in plants can pass down generations, that means an epigenetic component of transgenerational defense priming exist, showing progeny enhanced defense responses (Avramova, 2015). Changes in chromatin structure in responses to environmental stresses are inherited through mitotic and meiotic divisions (Avramova, 2015). Some chemicals have the ability of boosting defense responses and therefore also priming processes, however, the use of chemicals presents negative impacts to environment and also a determined degree of toxicity for plants (Belz and Duke, 2014). Process of priming in plants by the use of elicitors is related to conditioning a term proposed by Calabrese et al.

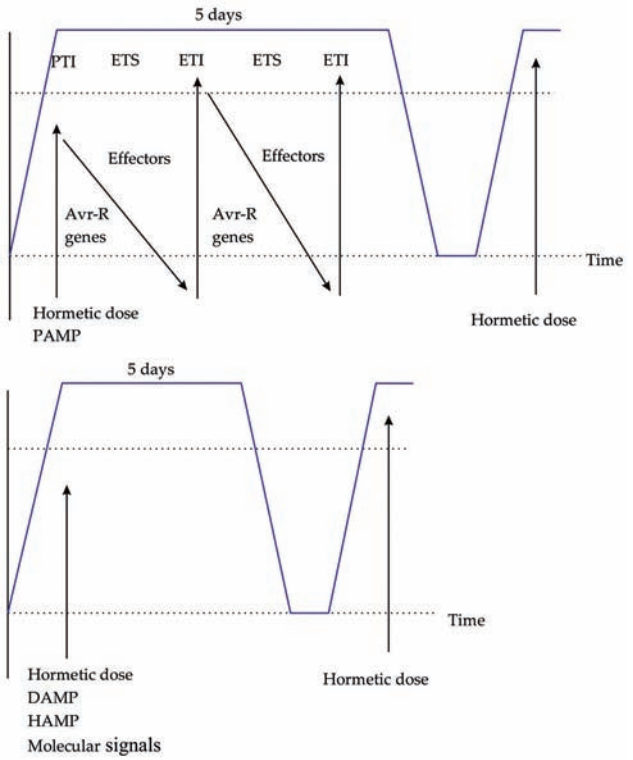


FIGURE 2 | The plant immune system triggered by PAMPS, DAMPS, HAMPS, and molecular signals. **(A)** Zig-zag model describing how plants detect PAMPs dose and induce PAMP-triggered immunity. **(B)** Hormetic dose of DAMP, HAMP, and molecular signals induces a defense response, subsequent responses are greater. Blue line indicates the defense response duration in days.

(2007), defined as the process describing when an organism is first exposed to low doses of a stress factor, it has the ability to activate or up-regulate existing cellular and molecular pathways that allows it to withstand subsequent stresses that are more severe.

BIOSTIMULANTS AND BIOLOGICAL CONTROL COMPOUNDS AS FACTORS INDUCING HORMESIS RESPONSES

Natural compounds called elicitors induce similar defense responses in plants as induced by the pathogen infection. The chemical structure of biostimulants and biological control compounds varies and includes organic molecules for example carbohydrate polymers, lipids, glycopeptides and glycoproteins, or chemical such as SA, CHT, and hydrogen peroxide, among others (Thakur and Sohal, 2013). Several reports have documented the effect of elicitors of biotic origin in plant defense, increasing the levels of H_2O_2 in plants (Sharma et al., 2012), turning on the expression of *pal* and *cat1*, indicators of oxidative stress-specific signaling and *pr1* as indicative of biotic stress (Mejía-Teniente et al., 2013), inducing structural barriers, toxic chemicals, and attraction of natural enemies. Evaluation of the effect of elicitors has been limited to the use of phytotoxins. However, some elicitors may have the ability to induce a similar hormetic response in plants, **Table 1**.

Proposal of Homolog DNA As Elicitor in Plants

DNA is an essential molecule for organisms, which possess the information for survival. As a consequence of evolution, cells are able to detect several pathogen-derived or host, derived substances released when there is damage, including DNA (Hornung and Latz, 2010; Pisetsky, 2012). DNA can act as DAMP, alerting the presence of serf-damage or as MAMP or PAMP if there is the presence of a foreign organism (Gallucci and Maffei, 2017). Recently, studies have demonstrated the inhibition effect of extracellular random fragmented homolog DNA in plants in a dose-dependent manner in comparison with the heterologous DNA (Mazzoleni et al., 2014, 2015). This effect can be biologically general because it occurs in various organisms (Cartenì et al., 2016). DNA recognition by the organism is necessary for the aforementioned process to exist. Recognition of DNA both own and foreign is the task of the PRRs (Gallucci and Maffei, 2017). In mammals, it is known that the recognition is given by Toll-like receptor (TLR9) and cyclic GMP–AMP synthase (cGAS) and absent in melanoma 2 (AIM2), depending on the localization in either the endosomal compartment or in the cytoplasm (Gallucci

TABLE 1 | Reports of hormetic and hormetic-like curves under the effect of elicitors of biotic origin..

Treatment/doses	Species/endpoint	Maximum effect	Reference
H_2O_2 (0, 0.1,0.5, 1.0, or 1.5 mM)	*Vigna unguiculata* (leaf area, shoot length, root length, shoot fresh weight, shoot dry weight, root fresh weight, and root dry weight)	0.5 mM for all parameters	Hasan et al., 2016
SA (0, 50, 100, 150, 200 mg l^{-1})	Rice seed yield	100 mg l^{-1}	Tavares et al., 2014
SA (0, 1.38, 13.8, 69.09, and 138.12 mg l^{-1})	Young barley seedling (length and fresh weight)	SA 1.38 mg l^{-1}	Salitxay et al., 2016
JA (0, 50, 100, and 150 mM)	*Calendula officinalis* (cell weight)	50 mM	Wiktorowska et al., 2010
Chitosan (0, 25, 50, 75, and 100 ppm)	*Vigna radiata* (L.) Wilzek dry mass per plant, harvest index, and photosynthesis	75 ppm	Mondal et al., 2013
Chitosaccharides (1, 10, 50, 100, 500, and 1000 mg l^{-1})	Symbiotic interaction between *Bradyrhizobium* and soybean (number and dry mass of nodules of roots)	100 mg l^{-1}	Costales et al., 2015
Fungal elicitor *Verticillium dahilae* Kleb. (0.1, 0.2, 0.3, 0.4, 0.5, 0.6, and 0.7 mg l^{-1})	*Artemisia annua* (cell growth)	0.4 mg l^{-1}	Hong et al., 2000
Extract of the polysaccharide fraction of *T. atroviride* D16 (30, 60, and 180 mg l^{-1})	*Salvia miltiorrhiza* (growth of hairy roots at 6, 12, and 18 days)	30 mg l^{-1} (6, 12 days) 69 mg l^{-1} (18 days)	Ming et al., 2013
Microbial metabolic products from microorganisms as *Streptomyces* and *Bacillus* (0, 1, 2, and 3 ml l^{-1})	Young barley seedlings (length and fresh weight)	1 ml l^{-1}	Salitxay et al., 2016
MeJA (0, 30, 100, 300, and 1000 ml l^{-1})	Two lily genotypes *L. longiflorum*, and *L. speciosum* regenerated *in vitro* (bulblet FW, regenerated bulblets)	30 ml l^{-1} (bulblet FW) 300 ml l^{-1} (bubbles per explant in *L. longiflorum*)	Jasik and de Klerk, 2006
MeJA (1, 5, 10, and 20 ml l^{-1})	Young barley seedlings length and fresh weight	1 ml l^{-1}	Salitxay et al., 2016
Pectin (2, 4, and 6 mg l^{-1})	*C. officinalis* suspension cultures (cell growth)	2 mg l^{-1} (12 years 24 h) 4 mg l^{-1} (72 years 96 h)	Wiktorowska et al., 2010
Parthenin (12 concentrations in the range of 0.03–6 mmol)	*L. sativa* var. capitata cv. Maikönig or cv. Hilro (root length under different parameters)	0.23–0.65 mmol	Belz and Cedergreen, 2010

and Maffei, 2017). It is not clear the mechanism of DNA recognizement by plant cells or the function of extracellular self-DNA in the organisms (Mazzoleni et al., 2014, 2015). Although plants have putative plant PRRs, no extracellular DNA receptor has been identified, but PR proteins are proposed being good candidates as receptors (Gallucci and Maffei, 2017). Some authors consider that the recognition of DNA in plants is similar to that presented by animals through sensors, including TLR9 (Paludan and Bowie, 2013; Pradhan et al., 2015).

Currently there is no information about beneficial effects at low doses in plants. Studies, just reported that low doses of the homologous DNA of an organism has greater damage than the DNA of other organisms (Paludan and Bowie, 2013; Pradhan et al., 2015). It was proposed that extracellular DNA plays a function as DAMP, to certain low doses of the compound. Probably, DNA excreted by plants and further metabolized to sequences of 50–2000 bp have a very specific signature for each species to be recognized as proper by the plant. Bacteria through DNA restriction–modification (R–M) distinguish the same from the strange through DNA methylation. The same effect is observed by TLR9 that specifically recognizes unmethylated CpGs (Krieg et al., 1995; Pohar et al., 2015; Gallucci and Maffei, 2017); while in plants specific responses depend on DNA fragmentation (Gallucci and Maffei, 2017). Thus, DNA methylation patterns could be one possible mechanism for self-DNA recognition in plants, although more research should be addressed in this sense.

This discovery opens new opportunities by exploiting the best characteristics of self-DNA in both agricultural and pharmacological industries, as highly species-specific inhibitory products, limiting the effect for other species (Mazzoleni et al., 2014, 2015). As the next step it is necessary to prove if homolog-DNA possesses a stimulation effect in at low doses. On the other hand, it is also important to elucidate the mode of action of the self-DNA to classify it as a biostimulant, biological control compound, or both. One of the main advantages is the use of self-DNA as elicitors in plants, by inducing machinery of defense and, as a result, all the plant by-products.

CONTROLLED ELICITATION IN PLANTS AND XENOHORMESIS

Nowadays conventional farming practices are aimed to increase yields and decrease losses provoked by pests, diseases, weeds, and workability (Pradhan et al., 2015). To achieve this, the strategy has been reducing stress in crops that in consequence increase the production of primary metabolites thus obtaining

yield gain. However, the secondary metabolites in those crops tend to decrease in the edible part (García-Mier et al., 2013). Xenohormesis hypothesizes explain how organisms have evolved to respond to stress signaling molecules produced by other species in their environment (Lamming et al., 2004). Many of the polyphenols are synthesized by plants during times of stress and induce survival and stress resistance of heterotrophs, to this interspecies communication of stress signals is called "xenohormesis" (Howitz et al., 2003). Some of the secondary metabolites as quercetin or resveratrol possess low degree of toxicity, suggesting that the health benefits are not related to mild cellular damage but from the evolution adaptative modulation of enzymes and receptors of stress-response pathways (Howitz and Sinclair, 2008). Resveratrol found in diverse species but it is mainly found in grapes (*Vitis vinifera*), a polyphenol possessing several biological activities, prevent early mortality and help in general health in mammalians (Smoliga et al., 2011).

In contrast, in organic agriculture, as it is done under conditions of constant stress during the development stage, the crops produce a greater amount of nutraceuticals. Several recent works involving the use of biotechnological techniques to establish the efficient production of nutraceutical compounds have been reported, one promising strategy is the use of stress conditions that turn on the defense responses and produce the synthesis and accumulation of bioactive compounds (Naznin et al., 2014; Shrivastava et al., 2015). Resveratrol can be induced in grapes by both biotic such as UV–C radiation and $AlCl_3$, or abiotic factors, including fungi, JA, SA, and H_2O_2 (Hasan and Bae, 2017). There are various components throughout the process of plant defense, including space-temporal level, post-transcriptional and post-translational modifications, compartmentalization, metabolite stability, substrate availability, among others (Sewelam et al., 2016). On the other hand, diverse aspects also must be taken into account related to the biostimulants and biological control compounds use, such as dose, period of application, specification of its duration, plant age, and developmental stage. It seems that the production of different metabolomics profiles will vary depending on stress circumstances. In this context, the determination of the limits of hormesis is a strategy that can be managed for the generation of agricultural practices that allow to take advantage of the crops, managing an adequate balance of yield and nutraceutical production. Thus, there is a possibility of obtaining health-related products that may be of interest by using different type of biotic elicitors, and then considerable efforts may be focused on the search for new ways to turn on the plant defense and within this context. A search for new sources of biostimulants and biological control compounds will provide the basis to develop a strategy based on inducing plant defense in a controlled manner by understanding the interaction of signal transduction pathways induced by a specific biotic elicitor. This may lead to the synthesis and accumulation of a desired nutraceutical compound and optimize its yield production. In order to increase cultivars production some strategies are proposed:

(1) Avoiding the activation of the defense metabolism until harvest, in order to avoid the defensive reaction of the plant during its development.
(2) Focusing the defensive metabolism effort only in the edible part of the plant and thereby reducing the production of secondary metabolites in non-edible parts.
(3) Differential induction of specific metabolic pathways for the synthesis of nutraceuticals testing with different doses and types of elicitors.
(4) Management of increased elicitors' doses throughout the phenological stages during plant cultivation. Low doses during vegetative stage, and high doses during fruit development.

Although there has been progress in the characterization of hormetic curves with the application of abiotic stressors, there is still an area of opportunity related to study of biotic elicitors. The above would confer advantages to the strategy presented here because it should influence the absence of negative effects on the environment and, consequently, on health. Some authors have previously recognized the use of biotic elicitors such as a tool to implement sustainable agriculture (Stenberg et al., 2015). The use of biostimulants and biological control compounds for priming activation in agriculture produces a greater increase of yields when they are applied in combination with chemicals than when applied alone (Conrath et al., 2015).

CONCLUSION

Based on the abovementioned, there is evidence to suggest that biostimulants and biological control compounds of biotic origin (elicitors) can induce the phenomena of hormesis in plants. Hormesis management by mild stress (eustress) might be a powerful tool in improvement of food nutraceutical quality in crops. In this context, adaptive responses induced by cross talking of stress signals between species (plants and mammalians) can be a powerful tool in improvement of human health.

AUTHOR CONTRIBUTIONS

MV-H was responsible for reviewing of literature and writing the paper. IT-P was responsible for the work team and the conception of the research topic. RG-G the conception of the research topic and reviewing of the literature. IM-B, LA-A, RO-V, ER-G, and SR-G were in charge of reviewing the writing.

ACKNOWLEDGMENTS

Authors thank to FORDECYT 193512, and SEP-CONACYT 178429. MV-H also acknowledges to CONACYT for grant 401146 provided.

REFERENCES

Antonovics, J., Boots, M., Ebert, D., Koskella, B., Poss, M., and Sadd, B. M. (2013). The origin of specificity by means of natural selection: evolved and nonhost resistance in host-pathogen interactions. *Evolution* 67, 1–9. doi: 10.1111/j.1558-5646.2012.01793.x

Aranega-Bou, P., de la O Leyva, M., Finiti, I., García-Agustín, P., and González-Bosch, C. (2014). Priming of plant resistance by natural compounds. Hexanoic acid as a model. *Front. Plant Sci.* 5:488. doi: 10.3389/fpls.2014.00488

Avramova, Z. (2015). Transcriptional 'memory' of a stress: transient chromatin and memory (epigenetic) marks at stress-response genes. *Plant J.* 83, 149–159. doi: 10.1111/tpj.12832

Baenas, N., García-Viguera, C., and Moreno, D. A. (2014). Biotic elicitors effectively increase the glucosinolates content in *Brassicaceae sprouts*. *J. Agric. Food Chem.* 62, 1881–1889. doi: 10.1021/jf404876z

Balasubramaniyam, A. (2015). Hormetic dose response as the paradigm of plant response to stress. *Int. J. Plant Biol. Res.* 3:1037.

Beckers, G. J., Jaskiewicz, M., Liu, Y., Underwood, W. R., He, S. Y., Zhang, S., et al. (2009). Mitogen-activated protein kinases 3 and 6 are required for full priming of stress responses in *Arabidopsis thaliana*. *Plant Cell* 21, 944–953. doi: 10.1105/tpc.108.062158

Belz, R. G., and Cedergreen, N. (2010). Parthenin hormesis in plants depends on growth conditions. *Environ. Exp. Bot.* 69, 293–301. doi: 10.1016/j.envexpbot.2010.04.010

Belz, R. G., and Duke, S. O. (2014). Herbicides and plant hormesis. *Pest Manag. Sci.* 70, 698–707. doi: 10.1002/ps.3726

Bhattacharyya, A., Chattopadhyay, R., Mitra, S., and Crowe, S. E. (2014). Oxidative stress: an essential factor in the pathogenesis of gastrointestinal mucosal diseases. *Physiol. Rev.* 94, 329–354. doi: 10.1152/physrev.00040.2012

Brodeur, J. (2012). Host specificity in biological control: insights from opportunistic pathogens. *Evol. Appl.* 5, 470–480. doi: 10.1111/j.1752-4571.2012.00273.x

Calabrese, E. J. (2008). Hormesis: why it is important to toxicology and toxicologists. *Environ. Toxicol. Chem.* 27, 1451–1474. doi: 10.1897/07-541.1

Calabrese, E. J. (2009). Hormesis: a conversation with a critic. *Environ. Health Perspect.* 117, 1339–1343. doi: 10.1289/ehp.0901002

Calabrese, E. J. (2013). Hormetic mechanisms. *Crit. Rev. Toxicol.* 43, 580–606. doi: 10.3109/10408444.2013.808172

Calabrese, E. J. (2014a). Hormesis: a fundamental concept in biology. *Microb. Cell* 1, 145–149. doi: 10.15698/mic2014.05.145

Calabrese, E. J. (2014b). Hormesis: from mainstream to therapy. *J. Cell Commun. Signal.* 8, 289–291. doi: 10.1007/s12079-014-0255-5

Calabrese, E. J., Bachmann, K. A., Bailer, A. J., Bolger, P. M., Borak, J., Cai, L., et al. (2007). Biological stress response terminology: integrating the concepts of adaptive response and preconditioning stress within a hormetic dose-response framework. *Toxicol. Appl. Pharmacol.* 222, 122–128. doi: 10.1016/j.taap.2007.02.015

Calabrese, E. J., and Blain, R. B. (2005). The occurrence of hormetic dose responses in the toxicological literature, the hormesis database: an overview. *Toxicol. Appl. Pharmacol.* 202, 289–301. doi: 10.1016/j.taap.2004.06.023

Calabrese, E. J., and Mattson, M. P. (2011). Hormesis provides a generalized quantitative estimate of biological plasticity. *J. Cell Commun. Signal.* 5, 25–38. doi: 10.1007/s12079-011-0119-1

Calabrese, V., Giordano, J., Ruggieri, M., Berritta, D., Trovato, A., Ontario, M. L., et al. (2016). Hormesis, cellular stress response, and redox homeostasis in autism spectrum disorders. *J. Neurosci. Res.* 94, 1488–1498. doi: 10.1002/jnr.23893

Cartenì, F., Bonanomi, G., Giannino, F., Incerti, G., Vincenot, C. E., Chiusano, M. L., et al. (2016). Self-DNA inhibitory effects: underlying mechanisms and ecological implications. *Plant Signal. Behav.* 11:e1158381. doi: 10.1080/15592324.2016.1158381

Chapman, P. M. (2001). The implications of hormesis to ecotoxicology and ecological risk assessment. *Hum. Exp. Toxicol.* 20, 499–505. doi: 10.1191/096032701718120337

Cheng, Y., Zhang, H., Yao, J., Wang, X., Xu, J., Han, Q., et al. (2012). Characterization of non-host resistance in broad bean to the wheat stripe rust pathogen. *BMC Plant Biol.* 12:96. doi: 10.1186/1471-2229-12-96

Coers, J. (2013). Self and non-self discrimination of intracellular membranes by the innate immune system. *PLOS Pathog.* 9:e1003538. doi: 10.1371/journal.ppat.1003538

Conrath, U., Beckers, G. J., Langenbach, C. J., and Jaskiewicz, M. R. (2015). Priming for enhanced defense. *Annu. Rev. Phytopathol.* 53, 97–119. doi: 10.1146/annurev-phyto-080614-120132

Costales, D., Falcón-Rodríguez, A., Nápoles, M. C., De Winter, J., Gerbaux, P., Onderwater, R. C. A., et al. (2015). Effect of chitosaccharides in nodulation and growth *in vitro* of inoculated soybean. *Am. J. Plant Sci.* 7, 1380–1391. doi: 10.4236/ajps.2016.79131

Dempsey, D. A., and Klessig, D. F. (2012). SOS - too many signals for systemic acquired resistance? *Trends Plant Sci.* 17, 538–545. doi: 10.1016/j.tplants.2012.05.011

du Jardin, P. (2015). Plant biostimulants: definition, concept, main categories and regulation. *Sci. Hortic.* 196, 3–14. doi: 10.1016/j.scienta.2015.09.021

Frost, C. J., Mescher, M. C., Carlson, J. E., and De Moraes, C. M. (2008). Plant defense priming against herbivores: getting ready for a different battle. *Plant Physiol.* 146, 818–824. doi: 10.1104/pp.107.113027

Gallucci, S., and Maffei, M. E. (2017). DNA sensing across the tree of life. *Trends Immunol.* doi: 10.1016/j.it.2017.07.012 [Epub ahead of print].

García-Mier, L., Guevara-González, R. G., Mondragón-Olguín, V. M., Verduzco-Cuellar, B. D. R., and Torres-Pacheco, I. (2013). Agriculture and bioactives: achieving both crop yield and phytochemicals. *Int. J. Mol. Sci.* 14, 4203–4222. doi: 10.3390/ijms14024203

Garcia-Mier, L., Jimenez-Garcia, S. N., Guevara-González, R. G., Feregrino-Perez, A. A., Contreras-Medina, L. M., and Torres-Pacheco, I. (2015). Elicitor mixtures significantly increase bioactive compounds, antioxidant activity, and quality parameters in sweet bell pepper. *J. Chem.* 2015, 269296. doi: 10.1155/2015/269296

Hasan, M., and Bae, H. (2017). An overview of stress-induced resveratrol synthesis in grapes: perspectives for resveratrol-enriched grape products. *Molecules* 22:E294. doi: 10.3390/molecules22020294

Hasan, S. A., Irfan, M., Masrahi, Y. S., Khalaf, M. A., and Hayat, S. (2016). Growth, photosynthesis, and antioxidant responses of *Vigna unguiculata* L. treated with hydrogen peroxide. *Cogent Food Agric.* 2:1155331. doi: 10.1080/23311932.2016.1155331

Hideg, E., Jansen, M. A., and Strid, A. (2013). UV-B exposure, ROS, and stress: inseparable companions or loosely linked associates? *Trends Plant Sci.* 18, 107–115. doi: 10.1016/j.tplants.2012.09.003

Hong, W., He-Chun, Y. E., Guo-Feng, L. I., Ben-Ye, L., and Kang, C. (2000). Effects of fungal elicitors on cell growth and artemisinin accumulation in hairy root cultures of Artemisia annua. *Acta Bot. Sin.* 42, 905–909.

Hooper, P. L., Hooper, P. L., Tytell, M., and Vígh, L. (2010). Xenohormesis: health benefits from an eon of plant stress response evolution. *Cell Stress Chaperones* 15, 761–770. doi: 10.1007/s12192-010-0206-x

Hornung, V., and Latz, E. (2010). Intracellular DNA recognition. *Nat. Rev. Immunol.* 10, 123–130. doi: 10.1038/nri2690

Howitz, K. T., Bitterman, K. J., Cohen, H. Y., Lamming, D. W., Lavu, S., Wood, J. G., et al. (2003). Small molecule activators of sirtuins extend *Saccharomyces cerevisiae* lifespan. *Nature* 425, 191–196. doi: 10.1038/nature01960

Howitz, K. T., and Sinclair, D. A. (2008). Xenohormesis: sensing the chemical cues of other species. *Cell* 133, 387–391. doi: 10.1016/j.cell.2008.04.019

Jasik, J., and de Klerk, G. (2006). Effect of methyl jasmonate on morphology and dormancy development in lily bulblets regenerated *in vitro*. *J. Plant Growth Regul.* 25, 45–51. doi: 10.1007/s00344-005-0048-4

Jaskiewicz, M., Conrath, U., and Peterhänsel, C. (2011). Chromatin modification acts as a memory for systemic acquired resistance in the plant stress response. *EMBO Rep.* 12, 50–55. doi: 10.1038/embor.2010.186

Ji, L. L. (2014). "Oxidative stress response pathways: role of redox signaling in hormesis," in *Hormesis in Health and Disease*, eds S. I. S. Rattan and É Le Bourg (Boca Raton, FL: CRC Press), 279–302. doi: 10.1201/b17042-17

Jones, J. D. G., and Dangl, J. L. (2006). The plant immune system. *Nature* 444, 323–329. doi: 10.1038/nature05286

Kim, N. H., and Hwang, B. K. (2015). Pepper heat shock protein 70a interacts with the type III effector AvrBsT and triggers plant cell death and immunity. *Plant Physiol.* 167, 307–322. doi: 10.1104/pp.114.253898

Krieg, A. M., Yi, A. K., Matson, S., Waldschmidt, T. J., Bishop, G. A., Teasdale, R., et al. (1995). CpG motifs in bacterial DNA trigger direct B-cell activation. *Nature* 374, 546–549. doi: 10.1038/374546a0

Lamming, D. W., Wood, J. G., and Sinclair, D. A. (2004). MicroReview: small molecules that regulate lifespan: evidence for xenohormesis. *Mol. Microbiol.* 53, 1003–1009. doi: 10.1111/j.1365-2958.2004.04209.x

Liu, J., Liu, Y., Wang, Y., Zhang, Z.-H., Zu, Y.-G., Efferth, T., et al. (2016). The combined effects of ethylene and MeJA on metabolic profiling of phenolic compounds in *Catharanthus roseus* revealed by metabolomics analysis. *Front. Physiol.* 7:217. doi: 10.3389/fphys.2016.00217

Lucas, J. A., García-Cristobal, J., Bonilla, A., Ramos, B., and Gutierrez-Mañero, J. (2014). Beneficial rhizobacteria from rice rhizosphere confers high protection against biotic and abiotic stress inducing systemic resistance in rice seedlings. *Plant Physiol. Biochem.* 82, 44–53. doi: 10.1016/j.plaphy.2014.05.007

Mandal, S., Kar, I., Mukherjee, A. K., and Acharya, P. (2013). Elicitor-induced defense responses in *Solanum lycopersicum* against *Ralstonia solanacearum*. *ScientificWorldJournal* 2013:561056. doi: 10.1155/2013/561056

Martinez-Medina, A., Flors, V., Heil, M., Mauch-Mani, B., Pieterse, C. M., Pozo, M. J., et al. (2016). Recognizing plant defense priming. *Trends Plant Sci.* 21, 818–822. doi: 10.1016/j.tplants.2016.07.009

Martos, G. G., Terán Mdel, M., and Díaz Ricci, J. C. (2015). The defence elicitor AsES causes a rapid and transient membrane depolarization, a triphasic oxidative burst and the accumulation of nitric oxide. *Plant Physiol. Biochem.* 97, 443–450. doi: 10.1016/j.plaphy.2015.10.029

Mattson, M. P. (2008). Hormesis defined. *Ageing Res. Rev.* 7, 1–7. doi: 10.1016/j.arr.2007.08.007

Mazzoleni, S., Cartenì, F., Bonanomi, G., Incerti, G., Chiusano, M. L., Termolino, P., et al. (2014). New perspectives on the use of nucleic acids in pharmacological applications: inhibitory action of extracellular self-DNA in biological systems. *Phytochem. Rev.* 13, 937–946. doi: 10.1007/s11101-014-9386-9

Mazzoleni, S., Cartenì, F., Bonanomi, G., Senatore, M., Termolino, P., Giannino, F., et al. (2015). Inhibitory effects of extracellular self-DNA: a general biological process? *New Phytol.* 206, 127–132. doi: 10.1111/nph.13306

McClure, C. D., Zhong, W., Hunt, V. L., Chapman, F. M., Hill, F. V., and Priest, N. K. (2014). Hormesis results in trade-offs with immunity. *Evolution* 68, 2225–2233. doi: 10.1111/evo.12453

Mejía-Teniente, L., Durán-Flores, F. D. D., Chapa-Oliver, A. M., Torres-Pacheco, I., Cruz-Hernández, A., González-Chavira, M. M., et al. (2013). Oxidative and molecular responses in *Capsicum annuum* L. after hydrogen peroxide, salicylic acid and chitosan foliar applications. *Int. J. Mol. Sci.* 14, 10178–10196. doi: 10.3390/ijms140510178

Ming, Q., Su, C., Zheng, C., Jia, M., Zhang, Q., Zhang, H., et al. (2013). Elicitors from the endophytic fungus *Trichoderma atroviride* promote *Salvia miltiorrhiza* hairy root growth and tanshinone biosynthesis. *J. Exp. Bot.* 64, 5687–5694. doi: 10.1093/jxb/ert342

Mondal, M. M. A., Malek, M. A., Puteh, A. B., and Ismail, M. R. (2013). Foliar application of chitosan on growth and yield attributes of mungbean (*Vigna radiata* (L.) Wilczek). *Bangladesh J. Bot.* 42, 179–183. doi: 10.3329/bjb.v42i1.15910

Naznin, H. A., Kiyohara, D., Kimura, M., Miyazawa, M., Shimizu, M., and Hyakumachi, M. (2014). Systemic resistance induced by volatile organic compounds emitted by plant growth-promoting fungi in *Arabidopsis thaliana*. *PLOS ONE* 9:e86882. doi: 10.1371/journal.pone.0086882

Newman, M.-A., Sundelin, T., Nielsen, J. T., and Erbs, G. (2013). MAMP (microbe-associated molecular pattern) triggered immunity in plants. *Front. Plant Sci.* 4:139. doi: 10.3389/fpls.2013.00139

Paludan, S. R., and Bowie, A. G. (2013). Immune sensing of DNA. *Immunity* 38, 870–880. doi: 10.1016/j.immuni.2013.05.004

Park, C.-J., and Seo, Y.-S. (2015). Heat shock proteins: a review of the molecular chaperones for plant immunity. *Plant Pathol. J.* 31, 323–333. doi: 10.5423/PPJ.RW.08.2015.0150

Peleg-Grossman, S., Melamed-Book, N., and Levine, A. (2012). ROS production during symbiotic infection suppresses pathogenesis-related gene expression. *Plant Signal. Behav.* 7, 409–415. doi: 10.4161/psb.19217

Pieterse, C. M., Zamioudis, C., Berendsen, R. L., Weller, D. M., van Wees, S. C., and Bakker, P. A. (2014). Induced systemic resistance by beneficial microbes. *Annu. Rev. Phytopathol.* 52, 347–375. doi: 10.1146/annurev-phyto-082712-102340

Pisetsky, D. S. (2012). The origin and properties of extracellular DNA: from PAMP to DAMP. *Clin. Immunol.* 144, 32–40. doi: 10.1016/j.clim.2012.04.006

Pohar, J., Lainšèek, D., Fukui, R., Yamamoto, C., Miyake, K., Jerala, R., et al. (2015). Species-specific minimal sequence motif for oligodeoxyribonucleotides activating mouse TLR9. *J. Immunol.* 195, 4396–4405. doi: 10.4049/jimmunol.1500600

Poschenrieder, C., Cabot, C., Martos, S., Gallego, B., and Barceló, J. (2013). Do toxic ions induce hormesis in plants? *Plant Sci.* 212, 15–25. doi: 10.1016/j.plantsci.2013.07.012

Pozo, M. J., van Der Ent, S., van Loon, L. C., and Pieterse, C. M. (2008). Transcription factor MYC2 is involved in priming for enhanced defense during rhizobacteria-induced systemic resistance in *Arabidopsis thaliana*. *New Phytol.* 180, 511–523. doi: 10.1111/j.1469-8137.2008.02578.x

Pradhan, P., Fischer, G., van Velthuizen, H., Reusser, D. E., and Kropp, J. P. (2015). Closing yield gaps: How sustainable can we be? *PLOS ONE* 10:e0129487. doi: 10.1371/journal.pone.0129487

Rodriguez-Salus, M., Bektas, Y., Schroeder, M., Knoth, C., Vu, T., Roberts, P., et al. (2016). The synthetic elicitor 2-(5-bromo-2-hydroxy-phenyl)-thiazolidine-4-carboxylic acid links plant immunity to hormesis. *Plant Physiol.* 170, 444–458. doi: 10.1104/pp.15.01058

Salitxay, T., Phouthaxay, P., Pang, Y. G., Yeong, Y. C., Adhikari, P. B., and Park, C. H. (2016). Effects of elicitors on seedling growth, total polyphenol and chlorophyll content and antioxidant activity of barley (*Hordeum vulgare* L.). *J. Crop Sci.* 61, 33–40. doi: 10.7740/kjcs.2016.61.1.033

Sanabria, N. M., Huang, J.-C., and Dubery, I. A. (2010). Self/nonself perception in plants in innate immunity and defense. *Self Nonself* 1, 40–54. doi: 10.4161/self.1.1.10442

Sewelam, N., Kazan, K., and Schenk, P. M. (2016). Global plant stress signaling: reactive oxygen species at the cross-road. *Front. Plant Sci.* 7:187. doi: 10.3389/fpls.2016.00187

Sharma, P., Jha, A. B., Dubey, R. S., and Pessarakli, M. (2012). Reactive oxygen species, oxidative damage, and antioxidative defense mechanism in plants under stressful conditions. *J. Bot.* 2012, 217037. doi: 10.1155/2012/217037

Shrivastava, G., Ownley, B. H., Augé, R. M., Toler, H., Dee, M., Vu, A., et al. (2015). Colonization by arbuscular mycorrhizal and endophytic fungi enhanced terpene production in tomato plants and their defense against a herbivorous insect. *Symbiosis* 65, 65–74. doi: 10.1007/s13199-015-0319-1

Singh, R., Singh, S., Parihar, P., Mishra, R. K., Tripathi, D. K., Singh, V. P., et al. (2016). Reactive oxygen species (ROS): beneficial companions of plants' developmental processes. *Front. Plant Sci.* 7:1299. doi: 10.3389/fpls.2016.01299

Smoliga, J. M., Baur, J. A., and Hausenblas, H. A. (2011). Resveratrol and health–a comprehensive review of human clinical trials. *Mol. Nutr. Food Res.* 55, 1129–1141. doi: 10.1002/mnfr.201100143

Song, Y., Chen, D., Lu, K., Sun, Z., and Zeng, R. (2015). Enhanced tomato disease resistance primed by arbuscular mycorrhizal fungus. *Front. Plant Sci.* 6:786. doi: 10.3389/fpls.2015.00786

Stebbing, A. R. D. (1987). Growth hormesis—A by-product of control. *Health Phys.* 52, 543–547. doi: 10.1097/00004032-198705000-00003

Stebbing, A. R. D. (1998). A theory for growth hormesis. *Mutat. Res.* 1998, 249–258. doi: 10.1016/S0027-5107(98)00014-1

Stenberg, J. A., Heil, M., Åhman, I., and Björkman, C. (2015). Optimizing crops for biocontrol of pests and disease. *Trends Plant Sci.* 20, 698–712. doi: 10.1016/j.tplants.2015.08.007

Stotz, H. U., Mitrousia, G. K., de Wit, P. J. G. M., and Fitt, B. D. L. (2014). Effector-triggered defense against apoplastic fungal pathogens. *Trends Plant Sci.* 19, 491–500. doi: 10.1016/j.tplants.2014.04.009

Strugala, R., Delventhal, R., and Schaffrath, U. (2015). An organ-specific view on non-host resistance. *Front. Plant Sci.* 6:526. doi: 10.3389/fpls.2015.00526

Tateda, C., Zhang, Z., Shrestha, J., Jelenska, J., Chinchilla, D., and Greenberg, J. T. (2014). Salicylic acid regulates *Arabidopsis* microbial pattern receptor kinase levels and signaling. *Plant Cell* 26, 4171–4187. doi: 10.1105/tpc.114.131938

Tavares, L. C., Rufino, C. A., Oliveira, S., Brunes, A. P., and Villela, F. A. (2014). Treatment of rice seeds with salicylic acid: seed physiological quality and yield. *J. Seed Sci.* 36, 352–356. doi: 10.1590/2317-1545v36n3636

Thakur, M., and Sohal, B. S. (2013). Role of elicitors in inducing resistance in plants against pathogen infection: a review. *ISRN Biochem.* 2013:762412. doi: 10.1155/2013/762412

Tierranegra-García, N., Salinas-Soto, P., Torres-Pacheco, I., Ocampo-Velázquez, R. V., Rico-García, E., Mendoza-Diaz, S. O., et al. (2011). Effect of foliar salicylic acid and methyl jasmonate applications on protection against pill-bugs in lettuce plants (*Lactuca sativa*). *Phytoparasitica* 39, 137–144. doi: 10.1007/s12600-011-0147-7

Wiesel, L., Newton, A. C., Elliott, I., Booty, D., Gilroy, E. M., Birch, P. R. J., et al. (2014). Molecular effects of resistance elicitors from biological origin and their potential for crop protection. *Front. Plant Sci.* 5:655. doi: 10.3389/fpls.2014.00655

Wiktorowska, E., Długosz, M., and Janiszowska, W. (2010). Significant enhancement of oleanolic acid accumulation by biotic elicitors in cell suspension cultures of *Calendula officinalis* L. *Enzyme Microb. Technol.* 46, 14–20. doi: 10.1016/j.enzmictec.2009.09.002

Yakhin, O. I., Lubyanov, A. A., Yakhin, I. A., and Brown, P. H. (2017). Biostimulants in plant science: a global perspective. *Front. Plant Sci.* 26:2049. doi: 10.3389/fpls.2016.02049

Zunun-Pérez, A. Y., Guevara-Figueroa, T., Jimenez-Garcia, S. N., Feregrino-Pérez, A. A., Gautier, F., and Guevara-González, R. G. (2017). Effect of foliar application of salicylic acid, hydrogen peroxide and a Xyloglucan oligosaccharide on capsiate content and gene expression associated with capsinoids synthesis in *Capsicum annuum* L. *J. Biosci.* 42, 245–250. doi: 10.1007/s12038-017-9682-9

Conflict of Interest Statement: The authors declare that the research was conducted in the absence of any commercial or financial relationships that could be construed as a potential conflict of interest.

frontiers in Plant Science

ORIGINAL RESEARCH
published: 06 December 2017
doi: 10.3389/fpls.2017.02072

Biostimulant Effects of Seed-Applied Sedaxane Fungicide: Morphological and Physiological Changes in Maize Seedlings

Cristian Dal Cortivo[1†], Giovanni Battista Conselvan[1†], Paolo Carletti[1], Giuseppe Barion[1], Luca Sella[2] and Teofilo Vamerali[1]*

[1] Department of Agronomy, Food, Natural Resources, Animals and the Environment, University of Padua, Padua, Italy,
[2] Department of Land, Environment, Agriculture and Forestry, University of Padua, Padua, Italy

Most crops are routinely protected against seed-born and soil-borne fungal pathogens through seed-applied fungicides. The recently released succinate dehydrogenase inhibitor (SDHI), sedaxane®, is a broad-spectrum fungicide, used particularly to control *Rhizoctonia* spp., but also has documented growth-enhancement effects on wheat. This study investigates the potential biostimulant effects of sedaxane and related physiological changes in disease-free maize seedlings (3-leaf stage) at increasing application doses (25, 75 and 150 μg a.i. seed^{-1}) under controlled sterilized conditions. We show sedaxane to have significant auxin-like and gibberellin-like effects, which effect marked morphological and physiological changes according to an approximate saturation dose-response model. Maximum benefits were attained at the intermediate dose, which significantly increased root length (+60%), area (+45%) and forks (+51%), and reduced root diameter as compared to untreated controls. Sedaxane enhanced leaf and root glutamine synthetase (GS) activity resulting in greater protein accumulation, particularly in the above-ground compartment, while glutamate synthase (GOGAT) activity remained almost unchanged. Sedaxane also improved leaf phenylalanine ammonia-lyase (PAL) activity, which may be responsible for the increase in shoot antioxidant activity (phenolic acids), mainly represented by *p*-coumaric and caffeic acids. We conclude that, in addition to its protective effect, sedaxane can facilitate root establishment and intensify nitrogen and phenylpropanoid metabolism in young maize plants, and may be beneficial in overcoming biotic and abiotic stresses in early growth stages.

Keywords: biostimulant, hormone-like activity, nitrogen metabolism, phenolic acids, root branching, succinate dehydrogenase inhibitor (SDHI)

OPEN ACCESS

Edited by:
Youssef Rouphael,
University of Naples Federico II, Italy

Reviewed by:
Mostafa Abdelwahed Abdelrahman,
Tohoku University, Japan
Tihana Teklić,
Josip Juraj Strossmayer University of Osijek, Croatia

***Correspondence:**
Paolo Carletti
paolo.carletti@unipd.it

† These authors have contributed equally to this work.

Specialty section:
This article was submitted to Plant Breeding, a section of the journal Frontiers in Plant Science

Received: *19 September 2017*
Accepted: *20 November 2017*
Published: *06 December 2017*

Citation:
Dal Cortivo C, Conselvan GB, Carletti P, Barion G, Sella L and Vamerali T (2017) Biostimulant Effects of Seed-Applied Sedaxane Fungicide: Morphological and Physiological Changes in Maize Seedlings. Front. Plant Sci. 8:2072. doi: 10.3389/fpls.2017.02072

INTRODUCTION

In intensive agriculture, seed coating is a technique of applying several compounds, such as pesticides, fertilizers and biostimulant substances, to the seed surface so they can start to act on the seedlings during germination and/or at the seed-soil interface immediately after sowing (Ehsanfat and Modarres-Sanavy, 2005).

Protecting field crop plants from soil- and seed-borne pathogens during germination and in early growth stages is crucial to ensure safe and fast establishment (Mathre et al., 2001). Fungicides are chemical and biological compounds that kill pathogenic fungi or inhibit fungal spore germination (McGrath, 2004), and, together with insecticides, are the molecules most frequently used in the seed coatings of many crops.

A fungicidal seed treatment is commonly composed of a trace quantity of fungicide evenly distributed among the seeds along with the adhesive substances needed to bind them to the seed surface (Sharma et al., 2015). Modern seed dressing fungicide formulations are often a mixture of several active ingredients with different modes of action (systemic and contact), which broadens the spectrum of control to include a wide range of pathogens and reduces the likelihood of resistance onset (Kitchen et al., 2016). Common fungicide combinations for cereals are triticonazole + prochloraz (Krzyzinska et al., 2005; Vermeulen et al., 2017), both sterol-inhibiting fungicides, and fludioxonil + metalaxyl-M (Mondal, 2004), the former a non-systemic phenylpyrrole, which inhibits transport-associated phosphorylation of glucose, the latter an acilalanine RNA synthesis inhibitor.

Substances on the seed surface can affect germination, as they may vary considerably in the degree to which they attract or repel moisture (Scott, 1989). When applied in high concentrations, fungicides have been reported to have potential direct negative effects on seed germination, rootlet growth, and emergence (Minamor, 2013). In many cases, the effects of seed-applied fungicides on plants vary according to growing conditions: under low pathogen pressure, they do not improve crop emergence and grain yield of wheat, but under high pressure from *Fusarium graminearum* they do (May et al., 2010). Environmental factors may also play a role (Cox and Cherney, 2014). Seed coating is expected to suppress arbuscular mycorrhizal fungi, hindering their colonization of roots and consequently reducing their beneficial effects on plant growth (Chiocchio et al., 2000; Channabasava et al., 2015).

In the search for highly effective active ingredients, attention is currently focused on useful secondary effects of fungicides on seedling development, regardless of genotype and growing conditions. Several fungicides have been found to have positive side-effects on plant physiology (Berdugo et al., 2012): The ubiquinol oxidase inhibitor (Qol) Strobilurin family is known to increase several morphological traits of maize, such as leaf number and area, and shoot and root biomasses (Lazo and Ascencio, 2014). Strobilurins have also been found to increase tolerance to abiotic stresses, as they can delay senescence of the photosynthetic leaf area, change the balance of the phytohormones, and increase CO_2 assimilation in wheat (Wu and von Tiedemann, 2001; Köhle et al., 2002). The azole fungicide class also influences the physiology of treated plants by increasing the chlorophyll content in winter wheat plants, delaying leaf senescence, and protecting plants from several abiotic stresses (Fletcher et al., 2010).

Recent studies have demonstrated the influence of pyrazole-carboxamide succinate dehydrogenase inhibitors (SDHIs) on plant physiology (Ajigboye et al., 2014, 2016). These comprise a relatively new class of fungicide (since 2000), and now include various active ingredients, such as boscalid, bixafen, isopyrazam and sedaxane, which can disrupt fungal respiration causing a breakdown in energy/ATP production (Avenot and Michailides, 2010). The SDHI sedaxane (Syngenta Crop Protection, Basel, Switzerland) has recently been released for use as a treatment for local and systemic protection of cereal seeds, seedlings and roots against pathogenic fungi, both seed-borne (*Ustilago nuda, Tilletia caries, Monographella nivalis, Pyrenophora graminea*) and soil-born (*Rhizoctonia solani, R. cerealis, Gaeumannomyces graminis*, *Typhula incarnata*) (Zeun et al., 2013; Ajigboye et al., 2016). When sedaxane moves from the seed to the soil and into the plant tissues, it has been found to improve the development of the roots and lower stems of cereals (Swart, 2011). Previous research has described wheat responding positively to sedaxane in terms of greater biomass, better growth and drought resistance (Ajigboye et al., 2016). These morpho-physiological reactions are also known to be induced by biostimulants (Calvo et al., 2014), defined as substances that at low doses are able to enhance hormone biosynthesis, nutrient uptake from the soil, resistance to biotic/abiotic stresses, crop quality, and root growth (Kauffman et al., 2007).

Given all this, the present study aimed to investigate the potential biostimulant activity of seed-applied sedaxane on maize plants, and the possible physiological mechanisms underlying the morphological changes. To this end, we: (i) carried out a bioassay (Audus test) to determine the biostimulant activity of sedaxane, (ii) measured the morphological variations in pot-cultivated, disease-free maize plants at increasing fungicide doses, and (iii) studied the response of the enzymes involved in nitrogen and phenylpropanoid metabolism, and the protein, sugar and total phenol contents in the leaves and roots of the same plants.

MATERIALS AND METHODS

Characteristics of Sedaxane

In this study, we used the fungicide formulation Vibrance® 500 FS, a commercial flowable concentrate for seed treatment containing 500 g sedaxane® L^{-1}, i.e., 43.7% w/w of AI (density 1.17 g mL^{-1}; pH 6.39). Sedaxane is the ISO common name for a mixture of two *cis*-isomers, 2′-[(1RS,2RS)-1,1′-bicycloprop-2-yl]-3-(difluoromethyl)-1-methylpyrazole-4-carboxanilide and two *trans*-isomers 2′-[(1RS,2SR)-1,1′-bicycloprop-2-yl]-3-(difluoromethyl)-1-methylpyrazole-4-carboxanilide (IUPAC). Its minimum purity is 960 g kg^{-1}, with ranges of 820–890 g kg^{-1} for the 2 *trans*-isomers (SYN508210 – 50:50 mixture of enantiomers), and 100–150 g kg^{-1} for the 2 *cis*-isomers (SYN508211 – 50:50 mixture of enantiomers) (EFSA, 2012).

Pot Trial Set-Up and Plant Analysis

Plants of the maize hybrid Hydro (Syngenta, Basel, Switzerland) were grown in cylindrical PVC pots (50 cm high, 9 cm diameter, 3.1 L volume) in a greenhouse in the L. Toniolo experimental farm of the University of Padua (Legnaro, NE Italy). The pots were filled with a sterilized mixture (36 h in an oven at 120°C) of silty-loam soil collected from a field on the experimental farm

(pH 8.4), and fine sand (1:1 w/w) to facilitate water drainage and root collection, to which was added a standard dose of pre-sowing fertilizer (about 100 kg N ha^{-1}, 150 kg P_2O_5 ha^{-1} and 300 kg K_2O ha^{-1}). Maize seeds were treated with three increasing doses of sedaxane: 25, 75, and 150 μg AI $seed^{-1}$, corresponding to label doses of 2.5, 7.5, and 15 mL of the commercial product Vibrance® 500 FS (500 g AI L^{-1}) in 50,000 seeds. Plants grown from treated seeds were compared with untreated controls. The experimental design was completely randomized with 6 replicates.

Three seeds per pot were sown at the end of June, and immediately after emergence plants were thinned to one per pot. At harvest, growth measurements were taken from three pots/plants, and enzymatic activity assays were carried out with a further three.

Water stress was avoided throughout the experiment by regularly watering the plants. Before plant harvest, which took place 20 days after sowing (DAS) at the 3-leaf stage, SPAD (Soil Plant Analysis Development) was measured in the last fully developed leaf with a 502 chlorophyll meter (Konica-Minolta, Hong Kong). Fresh and dry (oven-dried for 24 h at 105°C) weights were measured on three replicate samples of shoots, and roots were collected, gently washed of soil, and stored in a 15% v/v ethanol solution until morphological characterization. Root length, surface area, diameter, and number of tips and forks were measured by analysis of 1-bit 400-DPI images of the roots acquired with a flatbed scanner (Epson Expression 11000XL, Epson, Suwa, Japan) using the WinRhizo software (Regent Instruments, Ville de Québec, QC, Canada).

Three replicates were stored at −80°C until analysis, then shoot and root tissue samples were taken from them for enzymatic activity assays. Each enzymatic assay ($n = 9$) was carried out in triplicate on each plant.

A further trial was performed following the same procedure and timing of the main experiment, and using the same sand-soil mixture (1:1 w/w), but this time it was not sterilized. We took SPAD readings, and measured fresh and dry weights, and root morphological parameters of plants grown in unsterilized soil, as reported above (Supplementary Table S1).

Bioassay to Test the Biological Activity of Sedaxane

In order to investigate the biological activity of sedaxane, we measured the reduction in root growth in the model plant watercress (*Lepidium sativum* L.) to assess auxin-like activity, and the increase in shoot length in lettuce (*Lactuca sativa L.*) to assess gibberellin-like activity (Audus, 1972).

Watercress and lettuce seeds were surface-sterilized by immersion in 8% hydrogen peroxide for 15 min. After rinsing 5 times with sterile distilled water, 20 seeds were aseptically placed on filter paper in a Petri dish. In the case of watercress, the filter paper was moistened with 1.2 mL of H_2O (controls), or with 1.2 mL of 0.1, 1, 10 and 20 mg L^{-1} indoleacetic acid (IAA, natural auxin) (Sigma–Aldrich, St. Louis, MO, United States) to obtain the calibration curve, or with 1.2 mL of a serial dilution of the tested product Vibrance containing 500 g L^{-1} of AI sedaxane. The experimental design for lettuce was the same as for watercress, except that the sterile filter paper was moistened with 1.4 mL of the above solutions, while the calibration curve was a progression of 0.0001, 0.001, 0.01, and 0.1 mg L^{-1} gibberellic acid (GA) (Sigma–Aldrich, St. Louis, MO, United States).

Seeds were germinated in the dark at 25°C. After 48 h, the watercress seedlings were removed from the dishes and the roots measured with a digital gauge; after 72 h, the lettuce seedlings were removed and the shoots measured with a digital gauge.

A linear regression model ($y = a + bx$) was used to describe the dose-response relationship after logarithmic transformation of IAA, GA and the sedaxane doses, where x was the sedaxane concentration (g L^{-1}) and y the root or shoot length (mm) (Conselvan et al., 2017).

Protein Extraction and Determination

Fresh leaf and root samples, previously stored at −80°C, were ground to a homogenous powder with liquid N_2. Proteins were extracted by homogenizing 0.5 g of root or shoot materials with 5 mL of 38 mM KH_2PO_4 and 62 mM K_2HPO_4, pH 7, at 4°C. After 2 min, the extract was filtered through three layers of muslin and centrifuged at 15,000 *g* for 20 min at 4°C. A 50-μL supernatant sample was incubated with 50 μL of Milli-Q water and 2.5 mL of 0.00117 M Bradford reagent. After 15 min, the protein concentration in the extract was determined according to Bradford (1976), using a Jasco V-530 UV/Vis spectrophotometer (Jasco Corporation, Tokyo, Japan) at 595 nm wavelength. The protein concentration was expressed as mg of protein per g of fresh root or shoot.

Enzyme Extraction and Assay Conditions

To extract the enzymes involved in N reduction and assimilation pathways, fresh shoot and root samples were ground to a homogeneous powder with liquid N_2. Each activity assay was carried out in triplicate and with 3 biological repetitions using specific buffers for enzyme extraction.

Glutamine synthetase (GS; EC 6.3.1.2) was extracted by homogenizing 0.6 g of root or shoot material at 4°C with 2.4 mL of a solution of 1 mM Tris(hydroxymethyl)aminomethane HCl (Tris-HCl), 25 mM KH_2PO_4, 10 mM L-cysteine hydrochloride monohydrate and 3% (w/v) bovine serum albumin at pH 7.8 (Baglieri et al., 2014). After 10 min, the extract was filtered through two layers of gauze and centrifuged at 15,000 *g* for 25 min at 4°C. A 200-μL sample of supernatant was incubated with 200 μL of reaction buffer (50 mM Tris-HCl, 20 mM $MgSO_4$, 80 mM L-glutamate, 30 mM NH_2OH, 24 mM ATP, pH 7.8) at 37°C for 25 min. Reaction was blocked with a stopping solution (0.5 mL of 370 mM $FeCl_2 \cdot 6H_2O$ and 670 mM HCl). Samples were centrifuged at 15,000 g for 15 min. The amount of γ-glutamyl hydroxamate in the supernatant was determined photometrically (wavelength 540 nm) against an immediately stopped parallel sample (Jezek et al., 2015). A standard curve was made using authentic γ-glutamyl hydroxamate (GHA) proportional to absorbance intensity. Enzyme activity was expressed as μmol of GHA produced per g of fresh root or leaf tissue per minute (Conselvan et al., 2017).

Glutamate synthase (GOGAT; EC 1.4.7.1) was extracted by homogenizing 0.5 g of root or shoot material with 2 mL of a

solution of 100 mM Tris-HCl, pH 8.2, 10 mM $MgCl_2 \cdot 6H_2O$, 2 mM β-mercaptoethanol, 10% (v/v) glycerol and 1 mM Na_2EDTA. After 15 min, the extract was filtered through two layers of gauze and centrifuged at 15,000 *g* for 30 min at 4°C. The supernatant was centrifuged a second time at 15,000 *g* for 15 min at 4°C. For the enzyme assay, 100 μL of extract was added to 900 μL of reaction buffer (41.6 mM HEPES, pH 7.5, 1 mM NADH, 10 mM EDTA, 20 mM glutamine) and 300 μL (for leaf extract) or 900 μL (for root extract) of 10 mM α-ketoglutaric acid. The reaction time was 2 min for the shoot extract, and 1.5 min for the root extract at 30°C. GOGAT was assayed spectrophotometrically by monitoring NADH oxidation at wavelength 340 nm according to Avila et al. (1987). GOGAT activity was expressed as nmol NADH reduced per g of fresh root or shoot per minute.

For the phenylalanine ammonia-lyase (PAL; EC 4.3.1.5) assay, 1 g of shoot material was homogenized with 0.1 g of poly(vinylpolypyrrolidone) (PVPP) and 5 mL of 100 mM potassium phosphate buffer (pH 8.0) containing 1.4 mM β-mercaptoethanol. After 10 min, the extract was filtered through two layers of gauze and centrifuged at 15,000 *g* for 20 min at 4 °C. A 60 μL sample of supernatant was incubated with 400 μL of 100 mM Tris-HCl buffer (pH 8.8), 140 μL of 100 mM phosphate buffer and 200 μL of 40 mM phenylalanine, at 37°C for 30 min. Reaction was stopped with 200 μL 6N HCl (El-Shora, 2002). After centrifuging at 10,000 *g* for 15 min, the absorbance of the supernatant was measured at 280 nm against an immediately stopped parallel sample. A standard curve was made using authentic cinnamic acid at increasing dilutions. PAL activity was expressed as nmol cinnamic acid produced per mg of protein in the sample per minute.

Soluble Phenol Extraction and Determination

Soluble phenolic acids were extracted by homogenizing 200 mg of leaf material with 600 mL of pure methanol. The extract was kept on ice for 30 min then centrifuged at 15,000 *g* for 30 min at 4°C. Total phenols were measured according to the procedure described by Arnaldos et al. (2001). In brief, 1 mL of 2% Na_2CO_3 and 75 μL of Folin-Ciocalteau reagent (Sigma–Aldrich, St. Louis, MO, United States) were added to 50 μL of the phenolic extract. After incubation in the dark for 15 min at 25°C, absorbance was measured at 725 nm. A standard curve was made using authentic gallic acid. The soluble phenol content was expressed as mg of gallic acid equivalent (GAE) per g of fresh shoot material.

Free phenolic acid concentrations were revealed on 0.1 g shoot samples treated with 5 mL 80% (v/v) acetonitrile (ACN) in 10-mL tubes for 5 min at room temperature with agitation (70 rpm). After centrifugation (5 min, 10,000 RCF), clear supernatant was filtered at 0.2 μm (Acrodisc syringe filters with GHP membranes) and kept in clean tubes at −20°C until processing. HPLC analysis was carried out according to the method described by Adom et al. (2003) with modifications. Samples were manually shaken, then 200 μL was extracted and placed in vials for HPLC autosampling. The mobile phase was 0.25% (v/v) trifluoroacetic acid (TFA, solvent A) and pure ACN (solvent B). The HPLC gradient was linear: after 2 μL sample injection, solvent B was kept at 4% for 1.16 min, then increased gradually to 12% over 1.16 min, to 23% over 4.63 min, to 95% over 1.85 min, and to 100% over 1.16 min, with final rate maintained for a further 2.78 min. The duration of the analysis was 11.58 min at a solvent flow rate of 1.1 mL min^{-1}. The HPLC equipment (Shimadzu, Kyoto, Japan) had a UV diode array detector (SPD-M20A) at wavelength 282 nm, and an Ultra Tech sphere C18 analytical column (33 mm × 4.6 mm i.d., 1.5 μm particle size; Cil Cluzeau, Sainte-Foy-La-Grande, France) kept at 36°C. Control sample solutions of shoots containing known phenolic acid concentrations were analyzed at the beginning of each new batch analysis, and measurement accuracy was verified by checking expected concentrations.

Each peak was identified by analyzing the retention time and absorbance spectrum of each pure compound (i.e., *p*-Coumaric, caffeic, syringic, vanillic and *t*-ferulic acids; Supplementary Figure S1). The coefficients of determination of all calibration curves were >99%.

Quantitative Determination of Sugars

Shoots (5 g) were homogenized in methanol (20 mL) with an Ultra Turrax T25 at 13,500 rpm for 30 s until they attained uniform consistency. Samples were filtered once through filter paper (589 Schleicher), and a second time through cellulose acetate syringe filters (0.45 μm). The extract was then ready for HPLC analysis, for which we used a Jasco X-LC liquid chromatography system (Jasco Inc., Easton, MD, United States) consisting of a PU-2080 pump, an MD-2015 multiwavelength detector, an AS-2055 autosampler, and a CO-2060 column oven interfaced to a PC using the ChromNAV chromatography data system software (Jasco Inc., Easton, MD, United States).

Sugars were separated in a HyperRez XP Carbohydrate Pbþþ analytical column (7.7 mm × 300 mm; ThermoFisher Scientific, Waltham, MA, United States), operating at 80°C. Isocratic elution was carried out with water at a flow rate of 0.6 mL min^{-1}. D-(β)-glucose and D-(β)- fructose were quantified by a calibration method. Standards were dissolved in water and the calibration curves were generated with concentrations ranging from 100 mg L^{-1} to 1,000 mg L^{-1} (Nicoletto et al., 2013).

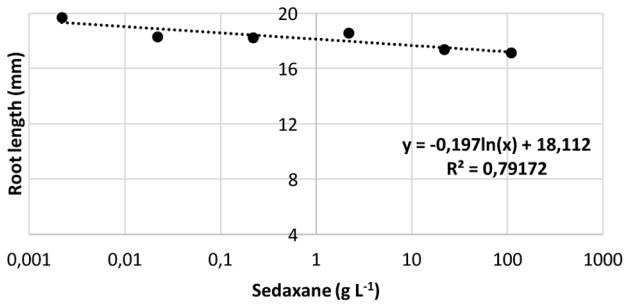
Statistical Analysis

The data are the means of measurements from three different pots per treatment. The analysis of variance (ANOVA) was performed in the SPSS 23 (IBM Corp) software, and was followed by pairwise *post hoc* analyses (Student–Newman–Keuls test) to determine significant differences among means at $P \leq 0.05$.

RESULTS

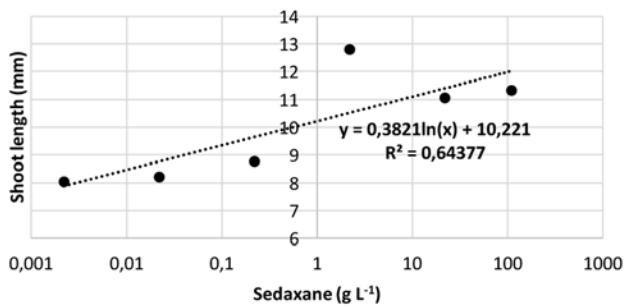
Audus Test and Effects of Sedaxane on Shoot and Root Growth

Ahead of the analysis, the Audus test was used to determine the biostimulant properties of the active ingredient sedaxane. As



	R^2	b	*P*
IAA	0.99	-1.76	0.00
Sedaxane	0.79	-0.197	0.02

FIGURE 1 | Audus test: auxin-like activity of sedaxane measured as variations in root length in watercress. Linear regression analysis (below) performed on 20 samples and averaged over 5 replicates. Note that the x axis has a logarithmic scale.



	R^2	b	*P*
GA	0.93	0.72	0.04
Sedaxane	0.644	0.382	0.05

FIGURE 2 | Audus test: gibberellin-like activity of sedaxane measured as variations in shoot length in lettuce. Linear regression analysis (below) was performed on 20 samples and averaged over five replicates. Note that the *x*-axis has a logarithmic scale.

with the natural auxin IAA, which reduces root elongation in the model plant watercress and is dose-proportional, increasing concentrations of sedaxane led to a progressive reduction in root length, suggesting an auxin-like effect (**Figure 1**). We also found sedaxane to exhibit gibberellin-like activity, as it enhanced the shoot growth of lettuce and had a similar dose-proportionality to exogenous gibberellic acid (**Figure 2**). Both regression curves were significant ($P < 0.02$ for root responses, $P \leq 0.05$ for shoot responses), revealing the hormone-like activity of this fungicide.

Under sterile conditions, fungicide treatment did not significantly enhance plant growth, although the medium dose of sedaxane (75 μg seed^{-1}) appreciably increased shoot (+21%) and root (+10%) biomasses as compared to untreated controls (**Table 1**). The effects of the seed treatments were more evident on other root features: Root length increased by 60% and root area by 45% at the intermediate fungicide dose. While root diameter was slightly smaller ($P > 0.05$), the number of root tips and forks increased, most noticeably with the intermediate (tips +27%, forks +51%) and maximum doses (tips +17%, forks +48%), although only the root branching increase was significant. These results show that root stimulation by sedaxane may be dose-dependent up to saturation.

Effects of Sedaxane on SPAD, Protein and Sugar Contents

Leaf greenness, measured in terms of SPAD values, was very stable across treatments at the end of the trial (**Table 2**), while protein content was significantly influenced by sedaxane ($P < 0.001$), with an increase of 14% at the intermediate and highest AI doses (**Table 2**). A similar effect was found in the roots, with protein content increasing significantly at the highest AI dose (+20% *vs.* untreated controls).

Fungicide treatment did not affect the shoot and root glucose content, the former having an average concentration of 3374 μg g^{-1} FW, the latter 3766 μg g^{-1} FW. The only variation found with regard to fructose was that it was significantly reduced in the shoot at the lowest and highest sedaxane doses (−21% and −15%, respectively, *vs.* untreated controls) (**Table 2**).

TABLE 1 | Main shoot and root parameters (mean ± SE; $n = 3$) in *Zea mays* at 20 days after sowing (DAS) in sterilized pot soil under increasing seed-applied doses of sedaxane.

Sedaxane dose (μg seed^{-1})	Shoot	Root					
	DW (g plant^{-1})	DW (g plant^{-1})	Length (m plant^{-1})	Area (m^2 plant^{-1})	Diameter (mm)	Tips (n plant^{-1})	Forks (n plant^{-1})
0	0.50 ± 0.07^{a}	0.24 ± 0.03^{a}	130.2 ± 35^{b}	0.23 ± 0.04^{b}	1.81 ± 0.13^{a}	6657 ± 1769^{a}	10594 ± 2280^{a}
25	0.52 ± 0.04^{a} (+3)	0.25 ± 0.03^{a} (+3)	167.4 ± 6^{ab} (+29)	0.26 ± 0.01^{ab} (+14)	1.54 ± 0.03^{a} (−15)	6180 ± 957^{a} (−7)	12649 ± 1681^{ab} (+19)
75	0.61 ± 0.04^{a} (+21)	0.26 ± 0.01^{a} (+10)	208.0 ± 24^{a} (+60)	0.33 ± 0.03^{a} (+45)	1.59 ± 0.10^{a} (−12)	7784 ± 994^{a} (+17)	15985 ± 1849^{b} (+51)
150	0.53 ± 0.04^{a} (+6)	0.24 ± 0.01^{a} (+1)	184.9 ± 16^{ab} (+42)	0.31 ± 0.02^{ab} (+37)	1.68 ± 0.10^{a} (−7)	8467 ± 405^{a} (+27)	15711 ± 718^{b} (+48)

Letters indicate significant differences among treatments within the same parameter (Student–Newman–Keuls test, $P \leq 0.05$). In brackets: % variation vs. untreated controls.

Variations in GS and GOGAT Activities with Sedaxane

Glutamine synthetase (GS) activity and glutamate synthase (GOGAT) activity were, respectively, 3.8 and 2.1 times higher, on average, in the shoots than in the roots. Seed treatment with sedaxane significantly increased GS activity in the shoots ($P < 0.01$) at the lowest (+145% *vs.* controls) and intermediate AI doses (+45%), and in the roots ($P < 0.001$), particularly at the intermediate and highest AI doses (both +66%, $P \leq 0.05$) (**Figure 3**).

Sedaxane treatments did not affect GOGAT activity in the shoots, while slight, but insignificant, reductions were observed in the roots (**Figure 3**).

Effect of Sedaxane on Leaf Phenylpropanoid Metabolism

A significant increase in soluble phenolic acids in the shoots was observed at the lowest sedaxane dose (+14% *vs.* untreated controls), while values similar to controls were detected at greater AI doses ($P \leq 0.05$) (**Table 3**). However, when individual compounds were analyzed, large differences among treatments were detected for caffeic acid, and, to a lesser extent, for syringic and *p*-coumaric acids ($P \leq 0.05$). Significantly higher concentrations of caffeic acid were found in all treated plants compared with untreated controls ($P \leq 0.05$). Sedaxane increased caffeic acid by 41–58%, depending on the dose, and *p*-coumaric acid, the most abundant phenolic compound, by 23% at the lowest and 19% at the intermediate dose. There were only slight differences in the vanillic and *t*-ferulic acid contents in treated plants as compared with controls ($P > 0.05$).

The ANOVA revealed a significant increase ($P \leq 0.05$) in PAL enzyme activity in the shoots with the lowest and highest fungicide doses (+29% and +43%, respectively) as compared with untreated controls (**Table 3**).

DISCUSSION

Sedaxane belongs to the new class of succinate dehydrogenase inhibitors, and is currently used as a seed-coating fungicide on various crops in several countries, with registration approval being increasingly granted worldwide. It is a broad-spectrum antifungal agent, and is of particular interest in combatting *Rhizoctonia solani* and *Mycosphaerella reliana* in maize.

In light of previous results on root stimulation in wheat (Barchietto et al., 2012), we investigated the side-effects of sedaxane in maize over and above its protective capacity, and found that seed treatment significantly modified morphological traits and physiological activities in disease-free plants grown in sterile soil.

The Audus test is considered to be the most reliable bioassay in terms of reproducibility and repeatability for verifying and quantifying the biostimulant activity of molecules in plants, and can be used to ascertain whether an exogenous compound has auxin- and/or gibberellin-like activity (Conselvan et al., 2017). Auxin (IAA) is the most important hormone in plants, and is

TABLE 2 | Leaf SPAD values, shoot and root protein, glucose, and fructose contents (mean ± SE; $n = 9$) in *Zea mays* at 20 days after sowing (DAS) in sterilized pot soil under increasing seed-applied doses of sedaxane.

Sedaxane dose (µg seed^{-1})	Shoot				Root		
	SPAD	Protein (mg g^{-1} FW)	Glucose (µg g^{-1} FW)	Fructose (µg g^{-1} FW)	Protein (mg g^{-1} FW)	Glucose (µg g^{-1} FW)	Fructose (µg g^{-1} FW)
0	34.6 ± 0.8^{a}	5.6 ± 0.1^{b}	3328 ± 170^{a}	1005 ± 24^{a}	1.5 ± 0^{b}	3819 ± 128^{a}	1322 ± 56^{a}
25	34.1 ± 0.2^{a} (−1)	5.9 ± 0.1^{ab} (+5)	3310 ± 33^{a} (−1)	792 ± 35^{b} (−21)	1.5 ± 0.1^{b}	3724 ± 163^{a} (−2)	1418 ± 109^{a} (+7)
75	34.7 ± 0.9^{a} (+0.5)	6.4 ± 0.2^{a} (+14)	3340 ± 113^{a}	1012 ± 59^{a} (+1)	1.5 ± 0.1^{b}	3665 ± 140^{a} (−4)	1476 ± 125^{a} (+12)
150	34.3 ± 0.8^{a} (−0.5)	6.4 ± 0.2^{a} (+14)	3518 ± 70^{a} (+6)	855 ± 14^{b} (−15)	1.8 ± 0^{a} (+20)	3859 ± 384^{a} (+1)	1313 ± 191^{a} (−1)

Letters indicate significant differences among treatments within the same parameter (Student–Newman–Keuls test, $P \leq 0.05$). In brackets: % variation vs. untreated controls.

involved in several plant growth and development phases, such as embryogenesis, organogenesis, tissue patterning and tropisms (Davies, 2010). Molecular genetic studies have brought to light the central role of auxin in primary root elongation, lateral root initiation, and root hair development (De Smet et al., 2006; Overvoorde et al., 2010). The phytohormone gibberellin (GA) also modulates plant development by lengthening roots and stems, and expanding leaves (Fleet and Sun, 2005). We used an Audus bioassay to demonstrate that sedaxane has both auxin- and gibberellin-like activity, as the confirmation of its biostimulant properties.

Although the improvements in aerial and root biomasses detected in this trial were not significant, we found that root length and area, and the number of root tips and branches increased almost in proportion to the dose of sedaxane, consistent with results reported by Colla et al. (2014) on maize coleoptile elongation with protein hydrolysates. All these root morphology modifications are known responses to biostimulant compounds (Calvo et al., 2014). Root development is essential for plant survival as it plays a crucial role in water and nutrient acquisition for growth, the synthesis and accumulation of secondary metabolites, and interaction with soil organisms (Saini et al., 2013).

The data collected from this trial are consistent with Barchietto et al. (2012) regarding stimulation of wheat shoots and roots by seed-applied sedaxane. At 30 days after sowing (DAS), they observed significant increases in root length in treated plants as compared with controls, and no differences in root biomass, as in our case study at 20 DAS. Interestingly, they also found that at 60 DAS root length was unaffected by sedaxane seed treatment, whereas root biomass increased significantly (+39–87%, according to variety).

In the sterile soil conditions of our trial, the SPAD value was very stable across treatments, but this was not the case in the supplementary trial we carried out in unsterile soil conditions to investigate the potential effect of sedaxane in field-like conditions, where we found a slight but significant increase in SPAD (up to 7%) (Supplementary Table S1). This result is in line with practical expectations in the field given the correlation between SPAD and photosynthetic activity, the N status of the plant and protein contents (Prost and Jeuffroy, 2007; Sim et al., 2015).

It should be noted that sedaxane may affect not only fungal mitochondria but also the SDH complex II of plants, partially inhibiting its activity (Avenot and Michailides, 2010). Fuentes et al. (2011) reported better photosynthetic performance in *Arabidopsis* plants with compromised expression of the flavoprotein subunit of SDH than in wild-type plants. Inhibition of the SDH subunit also resulted in an increase in the number and aperture of leaf stomata, which significantly increased CO_2 assimilation, in turn enhancing growth and protein production. Araújo et al. (2011) obtained similar results with tomato plants with antisense inhibition of the iron-sulfur subunit of SDH. However, the higher SPAD values of sedaxane-treated maize observed in our supplementary study with unsterilized soil may also be related to a slowing down of chlorophyll molecule degradation, as reported for fungicides of the Strobilurin class (Grossmann and Retzlaff, 1997; Xu and Huang, 2009). However,

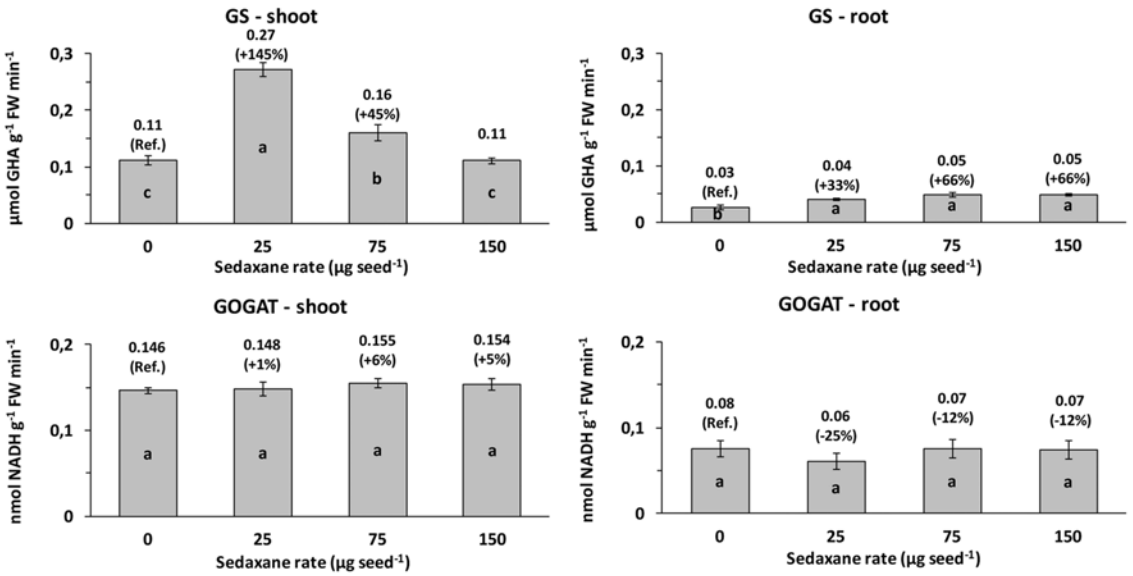


FIGURE 3 | Shoot and root glutamine synthetase (GS) and glutamate synthase (GOGAT) activities (mean ± SE; $n = 9$) in *Zea mays* at 20 days after sowing (DAS) in sterilized pot soil under increasing seed-applied doses of sedaxane. Letters indicate significant differences among treatments within the same parameter (Student–Newman–Keuls test, $P \leq 0.05$). In brackets: % variation *vs*. untreated controls.

this hypothesis needs to be confirmed by studying SDH activity and chlorophyll content in sedaxane-treated plants.

The higher protein content in sedaxane-treated seedlings may be ascribed to better nitrogen metabolism through the activity of the enzymes involved. In fact, the GS/GOGAT metabolic pathway is the main route of N assimilation in higher plants (Mokhele et al., 2012), allowing ammonium taken up directly or originating from nitrate to be assimilated into amino acids (Xu et al., 2012). The GS enzyme is also critical for re-assimilation of the NH_4^+ constantly released in large amounts via photorespiration, phenylalanine consumption for lignin biosynthesis, and protein catabolism (Lea and Miflin, 2011). GS activity, which increased significantly following sedaxane application, therefore plays a pivotal role in many aspects of plant development (Seabra and Carvalho, 2014), as it is a key component in nitrogen use efficiency (NUE) and plant yield (Thomsen et al., 2014).

GS and GOGAT enzyme activities have been previously reported to be affected by biostimulants (Baglieri et al., 2014). Our data are consistent with those of Ajigboye et al. (2016), who found that improvements in the photosynthetic efficiency, growth, and biomass of sedaxane-treated wheat plants were associated with up- or down-regulation changes in gene expression, and consequent modifications of physiological processes, particularly under drought stress conditions. In particular, sedaxane is reported to induce transcriptional regulation of genes and transcriptional factors, altering the flavonoid and phenolic metabolism (Ajigboye et al., 2016). Our study confirmed that sedaxane stimulates phenylpropanoid metabolism in maize as we found an increase in PAL enzyme activity, although, unexpectedly, the effect was not observed at the intermediate dose. The PAL enzyme catalyzes the first metabolic step from primary to secondary metabolism (Douglas, 1996), deaminating phenylalanine to produce cinnamic acid. As a consequence, there was an increase in the total content of phenolic compounds in shoot tissues from seedlings treated with the lowest concentration of sedaxane, but not at the highest dose. However, there were substantial changes in the concentrations of individual phenolic acids in relation to fungicide application: In particular, there was a considerable increase in caffeic acid in treated plants, which may be of interest in view of its weak auxin-like effect (Lavee et al., 1986; Ishikura et al., 2001; Nagasawa et al., 2016). The main precursor of lignin in the cell wall of gramineous plants is *p*-coumaric acid, and a greater abundance of it in sedaxane-treated plants could contribute to more intense cell activity and division. Vanillic and *p*-coumaric acids are also reported to be antifungal phenols, meaning that sedaxane may also contribute indirectly to plant defense (Lattanzio et al., 2006; Zabka and Pavela, 2013; Pusztahelyi et al., 2015). Stimulation of the secondary metabolism may also be explained by enhanced primary metabolism activity, as evidenced by the protein and sugar contents (**Table 2**).

As with other SDHIs studied in wheat, all the physiological changes brought about by sedaxane may also delay senescence, and improve the yield and protein content of maize plants (Bayles, 1999; Dimmock and Gooding, 2002; Zhang et al., 2010; Abdelrahman et al., 2017), but this requires further investigation in current field conditions.

CONCLUSION

Sedaxane has a considerable effect on rooting power of maize, particularly on the length, surface area and number of lateral roots. This study found that sedaxane exhibits biostimulant

TABLE 3 | Shoot phenylalanine ammonia-lyase activity (PAL), soluble phenol content and phenolic acid profiles (mean ± SE; $n = 9$) of *Zea mays* at 20 days after sowing (DAS) in sterilized pot soil under increasing seed-applied doses of sedaxane.

Sedaxane dose (μg seed^{-1})	PAL (nmol cinn. acid mg^{-1} prot. min^{-1})	Soluble phenols (as mg gallic acid g^{-1} FW)	Vanillic acid (μg g^{-1} FW)	Caffeic acid (μg g^{-1} FW)	Syringic acid (μg g^{-1} FW)	*p*-coumaric acid (μg g^{-1} FW)	*t*-ferulic acid (μg g^{-1} FW)
0	3.1 ± 0.12^{b}	36.4 ± 1.5^{b}	0.78 ± 0.06^{a}	2.88 ± 0.24^{b}	11.7 ± 0.6^{b}	21 ± 0.9^{b}	0.72 ± 0.04
25	3.99 ± 0.28^{a} (+29)	41.4 ± 0.8^{a} (+14)	0.63 ± 0.06^{a} (−19)	4.14 ± 0.24^{a} (+44)	14.6 ± 0.9^{a} (+25)	25.8 ± 1.9^{a} (+23)	0.83 ± 0.07 (+15)
75	3.08 ± 0.22^{b} (−1)	35.1 ± 1^{b} (−4)	0.63 ± 0.03^{a} (−19)	4.55 ± 0.43^{a} (+58)	13.8 ± 0.6^{ab} (+18)	24.9 ± 0.6^{a} (+19)	0.82 ± 0.03 (+14)
150	4.42 ± 0.26^{a} (+43)	35.2 ± 2^{b} (−3)	0.63 ± 0.05^{a} (−19)	4.06 ± 0.34^{a} (+41)	12.4 ± 0.6^{ab} (+6)	21.9 ± 1^{b} (+4)	0.74 ± 0.03 (+3)

Letters indicate significant differences among treatments within the same parameter (Student–Newman–Keuls test, $P \leq 0.05$). In brackets: % variation vs. untreated controls.

activity in maize seedlings due to its hormone-like activities, corroborated by the fact that most of the observed effects are saturated at moderate doses, as with phytohormones. We have high expectations that seed treatment with this fungicide will facilitate plant establishment, and may provide particular benefits under adverse soil and climatic conditions. Stimulation of the enzyme activities involved in N assimilation and phenylpropanoid metabolism is in agreement with previous findings on this active ingredient and other SDHI fungicides, and is consistent with improved N status and antioxidant activity.

As the fungicide doses tested here are within the recommended label range, the biostimulant activity of sedaxane is an additional benefit, over and above its protective role against seed- and soil-borne diseases, which could be exploited in the cultivation of maize. Although further studies are needed to see whether these improvements also influence final growth and yield, our preliminary results suggest that, as things currently stand, roots may be enhanced in the early growth stages, even in non-sterile soil.

AUTHOR CONTRIBUTIONS

CDC oversaw the greenhouse trial, assisted with the laboratory analyses, collected and analyzed the data, and wrote the first draft of the manuscript. GBC performed all the enzymatic and biological assays, and also collected and analyzed the data, carried out the literature research and improved the manuscript content. GB performed the HPLC analysis and assisted with analysis of the statistical data. PC helped design the experiment, analyzed the data and improved the manuscript content. LS helped revise the text. TV conceived the research idea, and corrected and arranged the final version of this work. All authors contributed to the interpretation and discussion of the results.

FUNDING

The authors gratefully thank Syngenta Crop Protection (Basel, Switzerland) for funding this research. GBC was funded by a Ph.D. grant from MIUR under "Law no. 170", and PC received DOR1692027/16 funding.

ACKNOWLEDGMENTS

The authors thank Adriano Massignan for technical assistance in the greenhouse trial, Dr. Michael Feitknecht for valuable revision of the text, and Tessa Say for revising the English text.

SUPPLEMENTARY MATERIAL

The Supplementary Material for this article can be found online at: https://www.frontiersin.org/articles/10.3389/fpls.2017.02072/full#supplementary-material

REFERENCES

Abdelrahman, M., El-Sayed, M., Jogaiah, S., Burritt, D. J., and Tran, L.-S. P. (2017). The "STAY-GREEN" trait and phytohormone signaling networks in plants under heat stress. *Plant Cell Rep.* 36, 1009–1025. doi: 10.1007/s00299-017-2119-y

Adom, K. K., Sorrells, M. E., and Liu, R. H. (2003). Phytochemical profiles and antioxidant activity of wheat varieties. *J. Agric. Food Chem.* 51, 7825–7834. doi: 10.1021/jf030404l

Ajigboye, O. O., Lu, C., Murchie, E. H., Schlatter, C., Swart, G., and Ray, R. V. (2016). Altered gene expression by sedaxane increases PSII efficiency, photosynthesis and growth and improves tolerance to drought in wheat seedlings. *Pestic. Biochem. Physiol.* 137, 49–61. doi: 10.1016/j.pestbp.2016.09.008

Ajigboye, O. O., Murchie, E., and Ray, R. V. (2014). Foliar application of isopyrazam and epoxiconazole improves photosystem II efficiency, biomass and yield in winter wheat. *Pestic. Biochem. Physiol.* 114, 52–60. doi: 10.1016/j.pestbp.2014.07.003

Araújo, W. L., Nunes-Nesi, A., Osorio, S., Usadel, B., Fuentes, D., Nagy, R., et al. (2011). Antisense inhibition of the iron-sulphur subunit of succinate dehydrogenase enhances photosynthesis and growth in tomato via an organic acid–mediated effect on stomatal aperture. *Plant Cell* 23, 600–627. doi: 10.1105/tpc.110.081224

Arnaldos, T. L., Munoz, R., Ferrer, M. A., and Calderon, A. A. (2001). Changes in phenol content during strawberry (*Fragaria x ananassa*, cv. Chandler) callus culture. *Physiol. Plant.* 113, 315–322. doi: 10.1034/j.1399-3054.2001.1130303.x

Audus, L. J. (1972). *Plant Growth Substances*. London: Leonard Hill.

Avenot, H. F., and Michailides, T. J. (2010). Progress in understanding molecular mechanisms and evolution of resistance to succinate dehydrogenase inhibiting (SDHI) fungicides in phytopathogenic fungi. *Crop Prot.* 29, 643–651. doi: 10.1016/j.cropro.2010.02.019

Avila, C., Botella, J. R., Cánovas, F. M., de Castro, I. N., and Valpuesta, V. (1987). Different characteristics of the two glutamate synthases in the green leaves of lycopersicon esculentum. *Plant Physiol.* 85, 1036–1039. doi: 10.1104/pp.85.4.1036

Baglieri, A., Cadili, V., Mozzetti Monterumici, C., Gennari, M., Tabasso, S., Montoneri, E., et al. (2014). Fertilization of bean plants with tomato plants hydrolysates. Effect on biomass production, chlorophyll content and N assimilation. *Sci. Hortic.* 176, 194–199. doi: 10.1016/j.scienta.2014.07.002

Barchietto, T., Prévot, C., Rambach, O., Petit, M., Seng, J. M., and Schlatter, C. (2012). Sedaxane: towards a new concept in plant protection? *Phytoma* 653, 7–10.

Bayles, R. (1999). The interaction of strobilurin fungicides with cereal varieties. *Plant Var. Seeds* 12, 129–140.

Berdugo, C. A., Steiner, U., Dehne, H. W., and Oerke, E. C. (2012). Effect of bixafen on senescence and yield formation of wheat. *Pestic. Biochem. Physiol.* 104, 171–177. doi: 10.1016/j.pestbp.2012.07.010

Bradford, M. (1976). A rapid and sensitive method for the quantitation of microgram quantities of protein utilizing the principle of protein-dye binding. *Anal. Biochem.* 72, 248–254. doi: 10.1016/0003-2697(76)90527-3

Calvo, P., Nelson, L., and Kloepper, J. W. (2014). Agricultural uses of plant biostimulants. *Plant Soil* 383, 3–41. doi: 10.1007/s11104-014-2131-8

Channabasava, A., Lakshman, H. C., and Jorquera, M. A. (2015). Effect of fungicides on association of arbuscular mycorrhiza fungus *Rhizophagus fasciculatus* and growth of *Proso millet* (*Panicum miliaceum* L.). *J. Soil Sci. Plant Nutr.* 15, 35–45. doi: 10.4067/S0718-95162015005000004

Chiocchio, V., Venedikian, N., Martinez, A., Menendez, A., Ocampo, J., and Godeas, A. (2000). Effect of the fungicide benomyl on spore germination and hyphal length of the arbuscular mycorrhizal fungus *Glomus mosseae*. *Int. Microbiol.* 3, 173–175.

Colla, G., Rouphael, Y., Canaguier, R., Svecova, E., and Cardarelli, M. (2014). Biostimulant action of a plant-derived protein hydrolysate produced through enzymatic hydrolysis. *Front. Plant Sci.* 5:448. doi: 10.3389/fpls.2014.00448

Conselvan, G. B., Pizzeghello, D., Francioso, O., Di Foggia, M., Nardi, S., and Carletti, P. (2017). Biostimulant activity of humic substances extracted from leonardites. *Plant Soil* 420, 119–134. doi: 10.1007/s11104-017-3373-z

Cox, W. J., and Cherney, J. H. (2014). Soybean seed treatments interact with locations for populations, yield, and partial returns. *Agron. J.* 106, 2157–2162. doi: 10.2134/agronj14.0074

Davies, P. J. (2010). "The plant hormones: their nature, occurrence, and functions," in *Plant Hormones: Biosynthesis, Signal Transduction, Action!*, ed. P. J. Davies (Dordrecht: Springer), 1–15. doi: 10.1007/978-1-4020-2686-7

De Smet, I., Vanneste, S., Inzé, D., and Beeckman, T. (2006). Lateral root initiation or the birth of a new meristem. *Plant Mol. Biol.* 60, 871–887. doi: 10.1007/s11103-005-4547-2

Dimmock, J., and Gooding, M. J. (2002). The effects of fungicides on rate and duration of grain filling in winter wheat in relation to maintenance of flag leaf green area. *J. Agric. Sci.* 138, 1–16. doi: 10.1017/S0021859601001666

Douglas, C. J. (1996). Phenylpropanoid metabolism and lignin biosynthesis: from weeds to trees. *Trends Plant Sci.* 1, 171–178. doi: 10.1016/1360-1385(96)10019-4

EFSA (2012). Conclusion on the peer review of the pesticide risk assessment of the active substance sedaxane. *EFSA J.* 10:2823.

Ehsanfat, S., and Modarres-Sanavy, S. A. M. (2005). Crop protection by seed coating. *Commun. Agric. Appl. Biol. Sci.* 70, 225–229.

El-Shora, H. M. (2002). Properties of phenylalanine ammonia-lyase from marrow cotyledons. *Plant Sci.* 162, 1–7. doi: 10.1016/s0168-9452(01)00471-x

Fleet, C. M., and Sun, T.-P. (2005). A DELLAcate balance: the role of gibberellin in plant morphogenesis. *Curr. Opin. Plant Biol.* 8, 77–85. doi: 10.1016/j.pbi.2004.11.015

Fletcher, R. A., Gilley, A., Sankhla, N., and Davis, T. D. (2010). "Triazoles as plant growth regulators and stress protectants," in *Horticultural Reviews*, ed. J. Janick (Oxford: John Wiley & Sons), 55–138.

Fuentes, D., Meneses, M., Nunes-Nesi, A., Araújo, W. L., Tapia, R., Gómez, I., et al. (2011). A deficiency in the flavoprotein of arabidopsis mitochondrial complex II results in elevated photosynthesis and better growth in nitrogen-limiting conditions. *Plant Physiol.* 157, 1114–1127. doi: 10.1104/pp.111.183939

Grossmann, K., and Retzlaff, G. (1997). Bioregulatory effects of the fungicidal strobilurin kresoxim-methyl in wheat (*Triticum aestivum*). *Pest Manag. Sci.* 50, 11–20. doi: 10.1002/(SICI)1096-9063(199705)50:1<11::AID-PS556>3.0.CO;2-8

Ishikura, Y., Kojima, Y., and Terazawa, M. (2001). Effects of phenolic compounds on seed germination of shirakamba birch, *Betula platyphylla* var. japonica. *Eur. J. For. Res.* 2, 17–25.

Jezek, M., Geilfus, C. M., and Muhling, K. H. (2015). Glutamine synthetase activity in leaves of *Zea mays* L. as influenced by magnesium status. *Planta* 242, 1309–1319. doi: 10.1007/s00425-015-2371-8

Kauffman, G. L., Kneivel, D. P., and Watschke, T. L. (2007). Effects of a biostimulant on the heat tolerance associated with photosynthetic capacity, membrane thermostability, and polyphenol production of perennial ryegrass. *Crop Sci.* 47, 261–267. doi: 10.2135/cropsci2006.03.0171

Kitchen, J. L., van den Bosch, F., Paveley, N. D., Helps, J., and van den Berg, F. (2016). The evolution of fungicide resistance resulting from combinations of foliar-acting systemic seed treatments and foliar-applied fungicides: a modeling analysis. *PLOS ONE* 11:e0161887. doi: 10.1371/journal.pone.0161887

Köhle, H., Grossmann, K., Jabs, T., Gerhard, M., Kaiser, W., Glaab, J., et al. (2002). "Physiological effects of the strobilurin fungicide F 500 on plants," in *Modern Fungicides and Antifungal Compounds III*, eds W. H. Dehne, H. B. Deising, U. Gisi, K. H. Kuck, P. E. Russell, and H. Lyr (Bonn: Agroconcept), 61–74.

Krzyzinska, B., Glazek, M., and Maczynska, A. (2005). Seed treatment for control leaf spot diseases of spring wheat. *Acta Agrobot.* 58, 37–43. doi: 10.5586/aa.2005.006

Lattanzio, V., Lattanzio, V. M., and Cardinali, A. (2006). "Role of phenolics in the resistance mechanisms of plants against fungal pathogens and insects," in *Phytochemistry: Advances in Research*, ed. F. Imperato (Trivandrum: Research Signpost), 23–67.

Lavee, S., Harshemesh, H., and Avidan, N. (1986). Phenolic acids - possible involvement in regulating growth and alternate fruiting in olive trees. *Acta Hortic.* 179, 317–328. doi: 10.17660/ActaHortic.1986.179.46

Lazo, J. V., and Ascencio, J. (2014). Some morphometric and physiological responses induced by fungicide Opera (R) (Pyraclostrobin plus Epoxiconazole) in corn (*Zea mays* L.). *Rev. Fac. Agron.* 31, 39–59.

Lea, P. J., and Miflin, B. J. (2011). Nitrogen assimilation and its relevance to crop improvement. *Annu. Plant Rev.* 42, 1–40.

Mathre, D. E., Johnston, R. H., and Grey, W. E. (2001). *Small Grain Cereal Seed Treatment*. Saint Paul, MN: American Phytopathological Society.

May, W. E., Fernandez, M. R., and Lafond, G. P. (2010). Effect of fungicidal seed treatments on the emergence, development, and grain yield of *Fusarium graminearum*-infected wheat and barley seed under field conditions. *Can. J. Plant Sci.* 90, 893–904. doi: 10.4141/cjps09173

McGrath, M. T. (2004). *What are Fungicides*. Available at: http://www.apsnet.org/edcenter/intropp/topics/Pages/fungicides.aspx

Minamor, A. A. (2013). Effect of two fungicides (Caocobre and Ridomil) on germination and radicle elongation of cocoa (*Theobroma cacao* L) seedlings. *Int. J. Pure Appl. Sci. Technol.* 15, 79–86.

Mokhele, B., Zhan, X., Yang, G., and Zhang, X. (2012). Review: nitrogen assimilation in crop plants and its affecting factors. *Can. J. Plant Sci.* 92, 399–405. doi: 10.4141/cjps2011-135

Mondal, K. K. (2004). Evaluation of seed-dressing fungicides against sclerotinia root rot of buckwheat. *Fagopyrum* 21, 105–107.

Nagasawa, K., Wang, B., Nishiya, K., Ushijima, K., Zhu, Q., Fukushima, M., et al. (2016). Effects of humic acids derived from lignite and cattle manure on antioxidant enzymatic activities of barley root. *J. Environ. Sci. Health B* 51, 81–89. doi: 10.1080/03601234.2015.1080516

Nicoletto, C., Santagata, S., Tosini, F., and Sambo, P. (2013). Qualitative and healthy traits of different Italian typical artichoke genotypes. *CYTA J. Food* 11, 108–113. doi: 10.1080/19476337.2012.700951

Overvoorde, P., Fukaki, H., and Beeckman, T. (2010). Auxin control of root development. *Cold Spring Harb. Perspect. Biol.* 2:a001537. doi: 10.1101/cshperspect.a001537

Prost, L., and Jeuffroy, M.-H. (2007). Replacing the nitrogen nutrition index by the chlorophyll meter to assess wheat N status. *Agron. Sustain. Dev.* 27, 321–330. doi: 10.1051/agro:2007032

Pusztahelyi, T., Holb, I. J., and Pócsi, I. (2015). Secondary metabolites in fungus-plant interactions. *Front. Plant Sci.* 6:573. doi: 10.3389/fpls.2015.00573

Saini, S., Sharma, I., Kaur, N., and Pati, P. K. (2013). Auxin: a master regulator in plant root development. *Plant Cell Rep.* 32, 741–757. doi: 10.1007/s00299-013-1430-5

Scott, J. M. (1989). Seed coating and treatments and their effects on plant establishment. *Adv. Agron.* 42, 43–83. doi: 10.1016/s0065-2113(08)60523-4

Seabra, A. R., and Carvalho, H. G. (2014). Glutamine synthetase in *Medicago truncatula*, unveiling new secrets of a very old enzyme. *Front. Plant Sci.* 6:578. doi: 10.3389/fpls.2015.00578

Sharma, K. K., Singh, U. S., Sharma, P., Kumar, A., and Sharma, L. (2015). Seed treatments for sustainable agriculture - A review. *J. Nat. Appl. Sci.* 7, 521–539.

Sim, C., Zaharah, A., Tan, M., and Goh, K. (2015). Rapid determination of leaf chlorophyll concentration, photosynthetic activity and NK concentration of *Elaeis guineensis* via correlated SPAD-502 chlorophyll index. *Asian J. Agric. Res.* 9, 132–138. doi: 10.3923/ajar.2015.132.138

Swart, G. M. (2011). *Root Health - The Key to Improving Yield*. Basel: Syngenta Crop Protection AG.

Thomsen, H. C., Eriksson, D., Møller, I. S., and Schjoerring, J. K. (2014). Cytosolic glutamine synthetase: a target for improvement of crop nitrogen use efficiency? *Trends Plant Sci.* 19, 656–663. doi: 10.1016/j.tplants.2014.06.002

Vermeulen, P., Flémal, P., Pigeon, O., Dardenne, P., Fernández Pierna, J., and Baeten, V. (2017). Assessment of pesticide coating on cereal seeds by near infrared hyperspectral imaging. *J. Spectral Imaging* 6, 1–7. doi: 10.1255/jsi.2017.a1

Wu, Y. X., and von Tiedemann, A. (2001). Physiological effects of azoxystrobin and epoxiconazole on senescence and the oxidative status of wheat. *Pestic. Biochem. Physiol.* 71, 1–10. doi: 10.1006/pest.2001.2561

Xu, G., Fan, X., and Miller, A. J. (2012). Plant nitrogen assimilation and use efficiency. *Annu. Rev. Plant Biol.* 63, 153–182. doi: 10.1146/annurev-arplant-042811-105532

Xu, Y., and Huang, B. (2009). Effects of foliar-applied ethylene inhibitor and synthetic cytokinin on creeping bentgrass to enhance heat tolerance. *Crop Sci.* 49, 1876–1884. doi: 10.2135/cropsci2008.07.0441

Zabka, M., and Pavela, R. (2013). Antifungal efficacy of some natural phenolic compounds against significant pathogenic and toxinogenic filamentous fungi. *Chemosphere* 93, 1051–1056. doi: 10.1016/j.chemosphere.2013.05.076

Zeun, R., Scalliet, G., and Oostendorp, M. (2013). Biological activity of sedaxane–a novel broad-spectrum fungicide for seed treatment. *Pest Manag. Sci.* 69, 527–534. doi: 10.1002/ps.3405

Zhang, Y.-J., Zhang, X., Chen, C.-J., Zhou, M.-G., and Wang, H.-C. (2010). Effects of fungicides JS399-19, azoxystrobin, tebuconazloe, and carbendazim on the physiological and biochemical indices and grain yield of winter wheat. *Pestic. Biochem. Physiol.* 98, 151–157. doi: 10.1016/j.pestbp.2010.04.007

Conflict of Interest Statement: The research was funded by Syngenta Crop Protection (Basel, Switzerland), which also provided the seeds and fungicide. Collection of data, analysis and interpretation, as well as article writing was carried out by the authors independently. The authors have the full data set available.

The authors declare that the research was conducted in the absence of any commercial or financial relationships that could be construed as a potential conflict of interest.

frontiers in Microbiology

ORIGINAL RESEARCH
published: 21 December 2017
doi: 10.3389/fmicb.2017.02593

Contribution of Zinc Solubilizing Bacteria in Growth Promotion and Zinc Content of Wheat

*Sana Kamran[1], Izzah Shahid[1], Deeba N. Baig[1], Muhammad Rizwan[2], Kauser A. Malik[1] and Samina Mehnaz[1]**

[1] Department of Biological Sciences, Forman Christian College (A Chartered University), Lahore, Pakistan, [2] Department of Chemistry, Government College Township, Lahore, Pakistan

Zinc is an imperative micronutrient required for optimum plant growth. Zinc solubilizing bacteria are potential alternatives for zinc supplementation and convert applied inorganic zinc to available forms. This study was conducted to screen zinc solubilizing rhizobacteria isolated from wheat and sugarcane, and to analyze their effect on wheat growth and development. Fourteen exo-polysaccharides producing bacterial isolates of wheat were identified and characterized biochemically as well as on the basis of 16S rRNA gene sequences. Along these, 10 identified sugarcane isolates were also screened for zinc solubilizing ability on five different insoluble zinc sources. Out of 24, five strains, i.e., EPS 1 (*Pseudomonas fragi)*, EPS 6 (*Pantoea dispersa)*, EPS 13 (*Pantoea agglomerans)*, PBS 2 (*E. cloacae)* and LHRW1 (*Rhizobium* sp.) were selected (based on their zinc solubilizing and PGP activities) for pot scale plant experiments. $ZnCO_3$ was used as zinc source and wheat seedlings were inoculated with these five strains, individually, to assess their effect on plant growth and development. The effect on plants was analyzed based on growth parameters and quantifying zinc content of shoot, root and grains using atomic absorption spectroscopy. Plant experiment was performed in two sets. For first set of plant experiments (harvested after 1 month), maximum shoot and root dry weights and shoot lengths were noted for the plants inoculated with *Rhizobium* sp. (LHRW1) while *E. cloacae* (PBS 2) increased both shoot and root lengths. Highest zinc content was found in shoots of *E. cloacae* (PBS 2) and in roots of *P. agglomerans* (EPS 13) followed by zinc supplemented control. For second set of plant experiment, when plants were harvested after three months, *Pantoea dispersa* (EPS 6), *P. agglomerans* (EPS 13) and *E. cloacae* (PBS 2) significantly increased shoot dry weights. However, significant increase in root dry weights and maximum zinc content was recorded for *Pseudomonas fragi* (EPS 1) inoculated plants, isolated from wheat rhizosphere. While maximum zinc content for roots was quantified in the control plants indicating the plant's inability to transport zinc to grains, supporting accelerated bioavailability of zinc to plant grains with zinc solubilizing rhizobacteria.

Keywords: PGPR, zinc solubilization, atomic absorption spectroscopy, zinc quantification, grain zinc content, exopolysaccharides

OPEN ACCESS

Edited by:
Giuseppe Colla,
Università degli Studi della Tuscia, Italy

Reviewed by:
Jay Prakash Verma,
Banaras Hindu University, India
Mohammad Oves,
King Abdulaziz University, Saudi Arabia

***Correspondence:**
Samina Mehnaz
saminamehnaz@fccollege.edu.pk

Specialty section:
This article was submitted to Plant Microbe Interactions, a section of the journal Frontiers in Microbiology

Received: *21 October 2017*
Accepted: *12 December 2017*
Published: *21 December 2017*

Citation:
Kamran S, Shahid I, Baig DN, Rizwan M, Malik KA and Mehnaz S (2017) Contribution of Zinc Solubilizing Bacteria in Growth Promotion and Zinc Content of Wheat. Front. Microbiol. 8:2593. doi: 10.3389/fmicb.2017.02593

INTRODUCTION

Despite the growing industrial and technological advancement, agriculture is still the most significant sector of Pakistan, contributing one-fifth of the total GDP. Wheat is considered to be one of the chief food crops in Pakistan, contributing 10.3% to the agricultural sector and having annual production of 25.3 million tons, calculated in 2013–2014 (Mirza et al., 2015). Zinc, one of the domineering micronutrients, is required in small amount for the proper growth and development of living organisms (Hafeez et al., 2013). In plants, specifically, it is involved in carbohydrate metabolism (Alloway, 2008), auxin metabolism (Alloway, 2004) and acts as a significant anti-oxidant. Zn-finger transcription factors play an important role in the normal development of floral tissues, flowering, fertilization and fruiting (Epstein and Bloom, 2005). Zinc deficiency in plants leads to retarded shoot growth, chlorosis, reduced leaf size (Alloway, 2004), susceptibility to heat, light and fungal infections, as well as affects grain yield, pollen formation, root development, water uptake and transport (Tavallali et al., 2010). Zinc deficiency in wheat leads to yellowing of leaves and stunted growth. Consuming zinc deficient wheat can lead to zinc deficiency in humans as well.

Plants can uptake zinc as divalent cation (Kabata-Pendias and Pendias, 2001) but only a very minor portion of total zinc is present in soil solution as soluble form. Rest of the zinc is in the form of insoluble complexes and minerals (Alloway, 2008). Due to unavailability of zinc in soil, zinc deficiency occurs which is one of the most widespread micronutrient deficiency. To alleviate zinc deficiency, various methods have been applied since long. Zinc fertilizers in the form of zinc sulfate (White and Broadly, 2005) or Zn-EDTA (Karak et al., 2005) have been used, but their usage puts an economical and environmental pressure and these are transformed into insoluble complex forms within 7 days of fertilizer application (Rattan and Shukla, 1991). Regular crop rotation and intercropping has been used in various areas (Gunes et al., 2007; Zuo and Zhang, 2009) to promote zinc uptake by plants. Other methods include conventional breeding (Cakmak et al., 2010), transgenic approaches and genetic engineering (Gustin et al., 2009; Mhatre et al., 2011; Tan et al., 2015). However, these approaches are expensive, laborious and slower. A better alternative to all these approaches is the use of zinc solubilizing rhizobacteria.

Plant growth promoting rhizobacteria (PGPR) are soil borne bacteria that colonize the rhizosphere, multiply and compete with other bacteria to promote plant growth (Kloepper and Okon, 1994). PGPR promote plant growth either by solubilizing and assisting nutrient acquisition or by releasing phytohormones or biocontrol agents to protect plant from various pathogens (Glick, 2012). Various PGPR have found to be effective zinc solubilizers. These bacteria improve the plant growth and development by colonizing the rhizosphere and by solubilizing complex zinc compounds into simpler ones, thus making zinc available to the plants.

Zinc solubilizing microorganisms solubilize zinc through various mechanisms, one of which is acidification. These microbes produce organic acids in soil which sequester the zinc cations and decrease the pH of the nearby soil (Alexander, 1997). Moreover, the anions can also chelate zinc and enhance zinc solubility (Jones and Darrah, 1994). Other mechanisms possibly involved in zinc solubilization include production of *siderophores* (Saravanan et al., 2011) and proton, oxido-reductive systems on cell membranes and chelated ligands (Wakatsuki, 1995; Chang et al., 2005). Various PGPR have shown enhanced growth and zinc content when inoculated in plants. These include *Pseudomonas, Rhizobium* strains (Deepak et al., 2013; Naz et al., 2016), *Bacillus aryabhattai* (Ramesh et al., 2014), *Bacillus* sp. (Hussain et al., 2015), and *Azospirillum.* Bacterial strains that have been reported to show zinc solubilization on lab scale include *Pseudomonas aeruginosa* (Fasim et al., 2002), *Gluconacetobacter diazotrophicus* (Saravanan et al., 2007), *Bacillus* sp., *Pseudomonas striata*, *Pseudomonas fluorescence*, *Burkholderia cenocepacia* (Pawar et al., 2015), *Serratia liquefaciens*, *S. marcescens,* and *Bacillus thuringiensis* (Abaid-Ullah et al., 2015). Prospective zinc solubilizing bacteria for enhanced nutrition and zinc uptake in *Zea mays* L., zinc solubilizing *Bacillus* strains that modulate growth, yield and zinc biofortification of soybean and wheat have also been characterized by researchers (Khande et al., 2017). These strains have been reported for increasing zinc content of straw and grains in soybean and wheat, enhancing food efficacy and coping with zinc deficiency. Vaid et al. (2014) have reported rice growth promotion and 42.7% increased zinc nutrition of grains when inoculated with zinc solubilizing bacteria.

Keeping in view the above facts, this study was designed to identify and characterize pre-isolated bacteria from wheat and sugarcane for plant growth promoting (PGP) abilities, zinc solubilizing ability using plate assays and to evaluate the contribution (if any), of zinc solubilizing strains on growth and zinc content of wheat plants, through pot experiments.

MATERIALS AND METHODS

Bacterial Isolates

In this study, 14 un-identified exopolysaccharide (EPS) producing bacterial isolates, previously isolated from wheat (Mehnaz, unpublished) and 10 identified strains from sugarcane: PBS1, PBS2, QS2, QST-W1, LHR-Sterilized, LHST-IN-W1, LHR-W1, LHR-W2, LS1-a, and LS1-b (Mehnaz et al., 2010) were used and maintained on LB agar plates (Bertani, 1952) at 28°C. Colony morphology of these isolates was observed on LB agar and RCV-sucrose agar media (Weaver et al., 1975) by incubating at 28°C for 24–48 h.

Biochemical and Molecular Characterization of Bacterial Isolates

Gram staining of the unidentified wheat isolates was performed using the standard procedure described by Vincent (1970) and biochemical tests were performed using bacterial miniaturized identification kits QTS-24 (DESTO Laboratories, Karachi, Pakistan) following manufacturer's instructions.

Genomic DNA of unidentified isolates was extracted using Genomic DNA purification kit (ThermoScientific^{TM} GeneJET^{TM} USA). 16S rRNA gene for each bacterial isolate was amplified in 50 µl reaction mixture containing 25 µl of PCR Dream *Taq* master mix; Taq DNA polymerase (0.05 U/µl), reaction buffer, $MgCl_2$ (4 mM) and dNTPs, 0.4 mM each (Thermo Fisher Scientific, USA; Catalog Number: EP0701), 5 µl template DNA, 12 µl diH_2O and 8 µl of 20 pmol P15 (5′-CGGGATCCAGAGTCAGAACGAACGCT-3′and P65- 5′CGGGATCCTACGGACGACTTCACCCC-3′) universal primers (Tan et al., 1997). Initial denaturation was provided at 94°C for 2 min following 35 cycles of 94°C for 1 min, 55°C for 1 min, 72°C for 3 min and final extension at 72°C for 10 min (Chun and Goodfellow, 1995). The amplified (1.5 kb) 16S rRNA gene products were analyzed using gel electrophoresis by running DNA on 1% agarose gel at 90 V for 40–50 min. and concentration was determined by Nanodrop UV spectrophotometer (ThermoScientific, USA; Model no: E4-106-50-0001-S). Gene products were purified with PCR Purification Kit (Thermo Fisher Scientific, USA), commercially sequenced (Eurofins, Germany) and sequences were BLAST (Basic Local Alignment Search Tool) for homology.

Multiple Sequence alignment of all bacterial isolates was carried out using CLUSTALW (ver. 1.83). *Planctomycete* was used as out-group to construct Phylogenetic Tree and evolutionary history was created using neighbor-joining method. Percentage of replicate trees in which associated taxa clustered together in the bootstrap test (1,000 replicates) are shown next to the branches. The evolutionary distances were calculated using the maximum composite likelihood method and were in the units of number of base substitutions per site. All positions containing gaps and missing data were eliminated from the dataset using the complete deletion option. There were a total of 1,408 positions in the final dataset. Phylogenetic analysis was conducted with MEGA4 software. These sequences were deposited in the GenBank database and accession numbers were obtained (i.e., KY848812 to KY848824 and MF150305).

Plant Growth Promoting (PGP) Ability Assays

Indole-3- Acetic Acid (IAA) Production Assay

Single bacterial colonies of individual strains were inoculated in 100 ml LB broth containing 0.1% L-tryptophan as a precursor for IAA production and incubated at 120 rpm, 28°C for 7 days. Qualitative assessment was done by harvesting cells from 500 µl culture in sterile eppendorf tubes at 10,000 rpm for 10 min and adding 1 ml Salkowski's reagent (Gordon and Weber, 1950) in supernatant. Samples were kept in dark for 15–20 min to observe color change.

For quantitative assessment, cells from 7 days old, 100 ml bacterial culture grown in LB with 0.1% L-tryptophan, were harvested at 10,000 rpm for 15–20 min. The pH of the supernatant was adjusted at 2.8 using hydrochloric acid and it was extracted twice with equal volumes of ethyl acetate (Tien et al., 1979) following evaporation using rotary evaporator and was resuspended in 1 ml methanol. The samples were analyzed by high-performance liquid chromatography (HPLC) on Waters HPLC System (e2995, separations module) with 2998 photodiode-array (PDA) detector using a Nucleosil C18 column (4.6 × 250 mm, 5 µM; Macherey-Nagel, Germany). The mobile phase was a mixture of methanol/acetic acid/water (30:1:70, v/v/v) and the flow rate was adjusted at 1.2 ml /min (Rasul et al., 1998). Pure indole-3-acetic acid (Sigma) was used to prepare standard solutions.

Siderophore and Hydrocyanic Acid (HCN) Production Assay

For qualitative assessment of siderophore production, O-CAS method as described by Perez-Miranda et al. (2007) was used. Each bacterial culture was spot inoculated on LB agar plate and incubated at 28°C for 1–2 days. CAS medium was prepared as described by Schwyn and Neilands (1987). PIPES buffer medium and CAS dye solution were mixed and applied to 48 h grown bacterial cultures, as overlay. The plates were kept at 28°C for 20 min to 24 h.

Qualitative production of HCN was determined by streaking bacterial colonies on LB agar plates. Filter papers dipped in alkaline picrate solution (0.25% picric acid and 1.25% sodium carbonate) were placed on the lids of petri plates (Miller and Higgins, 1970), sealed with parafilm and incubated at 28°C for 4-5 days.

Extracellular Enzyme Assays

Overnight grown bacterial cultures were transferred aseptically by spot inoculation on 1% Tween-20 LB agar plates to detect lipase production (Sierra, 1957). National Botanical Research Institute's Phosphate Growth medium (NBRIP; Nautiyal, 1999) plates were used for detection of P solubilization ability by isolates. LB agar plates supplemented with 1% carboxymethyl cellulose (CMC) for cellulase production (Lin et al., 2012) and skim milk agar plates (Kumar et al., 2005), were used to detect the production of proteases. Fresh bacterial cultures were spot inoculated and the plates were incubated at 28°C for 3–4 days for lipase and protease and 14 days for phosphatase production ability. Clear zones around colonies indicated respective activity. For cellulase, plates were stained with 0.1% congo red for 15 min, following de-staining (1M NaCl for 15 min). Formation of yellowish zones around bacterial growth indicated positive results.

In Vitro Zinc Solubilization Assessment Using Plate Assay

All bacterial strains were screened for their zinc solubilizing ability for five insoluble zinc compounds viz. zinc sulfate ($ZnSO_4$), zinc oxide (ZnO), zinc chloride ($ZnCl_2$), zinc phosphate $Zn_3(PO_4)_2$ and zinc carbonate ($ZnCO_3$). Overnight grown single colonies were transferred aseptically by inoculating as spot on respective zinc medium plates (Sharma et al., 2012). These plates were covered with aluminum foil and incubated in dark at 28°C for 14 days. Zinc solubilizing strains produced clear zones around colonies. The diameter of these zones was recorded.

Plant Experiments

To analyze the effect of zinc solubilizing bacteria on wheat growth and development, plant experiments were carried out. Based on zinc solubilizing ability, five strains; *Pseudomonas fragi* (EPS 1), *Pantoea dispersa* (EPS 6), *Pantoea agglomerans* (EPS 13), *Enterobacter cloacae* (PBS 2), and *Rhizobium* sp. (LHRW1) were selected for plant experiments and zinc carbonate ($ZnCO_3$) was used as zinc source. In total, seven treatments with 10 replicates each, were designed as follows: Control (without zinc source + without bacteria), Control+$ZnCO_3$ (without bacteria), $ZnCO_3$ + EPS 1, $ZnCO_3$ + EPS 6, $ZnCO_3$ + EPS 13, $ZnCO_3$ + PBS 2 and $ZnCO_3$ + LHRW1. Seventy plastic pots of 11.5 cm diameter and 10.5 cm height were labeled and 500 g autoclaved oven-dried sand was added per pot. One percent $ZnCO_3$, i.e., 5 g $ZnCO_3$ per 500 g sand was added in each of the 60 pots excluding 10 pots of the control treatment. For seed sterilization, 100 seeds of wheat variety, i.e., Faisalabad-2008 were soaked in 100 ml of 10% bleach solution for 15 min, and four successive washes of 10 min each were given using 100 ml autoclaved water (Cheng et al., 1997). Using sterile forceps the seeds were carefully transferred to 1% water-agar plates and sealed plates were incubated at 28°C for 3 days to allow germination.

Zinc solubilizing isolates were individually inoculated in 60 ml LB broth and grown in shaking incubator at 120 rpm, 28°C. After 24 h, the optical density of each strain was adjusted at 1.0 at the wavelength of 600 nm. Cells were harvested from 50 ml culture at 6,000 rpm for 20 min and resuspended in 0.85% saline, containing 10^8 cells/ml for inoculum. Fifty milliliter sterile full strength, nitrogen containing Hoagland's solution (Hoagland and Arnon, 1950) was added in each pot. Using sterilized forceps, 3-days old seedlings were transferred in pots and 1 ml bacterial inoculum was given to each respective pot. Pots were placed in a climate room and arranged in a Randomized Complete Block Design (RCBD). The temperature in climate room was maintained at 20 ± 2°C; with light source of 6,000 ± 500 FLUX and light period of 10 ± 1 h. To provide moisture, plants were watered at alternate days using autoclaved distilled water. Two sets of experiments were carried out. For first set, Plants were harvested after 1 month. This experiment was repeated twice. However, for second set, plants were harvested after 3 months (at grain level). Roots were washed using tap water and separated from shoots. Lengths, fresh, and dry weights of all samples including shoots and roots were measured. Data was statistically analyzed using the Statistical Package for the Social Sciences (SPSS) software (IBM Statistics 23.0).

Analysis of Zinc Content Using Atomic Absorption Spectroscopy

Roots, shoots and grains of three plant samples per treatment were analyzed for zinc estimation. 0.1 g plant material was digested in di-acid mixture containing 25 ml concentrated nitric acid and 10 ml concentrated sulphuric acid. The mixture was heated at 70°C until complete digestion and diluted using 50 ml distilled water. Extract was filtered through a Whatman's filter paper (Jepkoech et al., 2013) and samples were analyzed for total zinc content using atomic absorption spectrophotometer (Varian AA 240 F.S., USA).

RESULTS

Biochemical and Molecular Characterization of Bacterial Isolates

Based on gram staining, cell morphology and biochemical tests, wheat isolates were characterized according to Bergey's manual of determinative bacteriology (**Table 1**). Based on 16S rRNA sequence analysis, two isolates were identified as *Pseudomonas fragi* (EPS 1, EPS 15), five isolates as different species of *Pantoea*, i.e., *P. dispersa* (EPS 6), *Pantoea sp.* (EPS 4) and *P. agglomerans* (EPS 2, EPS 17, EPS 13), respectively; three isolates as *Acinetobacter johnsonii* (EPS 5, EPS 10, EPS 11), one as *Kosakonia oryzae* (EPS 7), one as *Enterobacter cloacae* (EPS 14), EPS 12 as *Microbacterium sp.*, and one as *Bacillus pumilus* (EPS 16). Phylogenic tree is shown in **Figure 1**. Sequence lengths, accession numbers and percentage homology of each strain is summarized in **Table 2**.

Plant Growth Promoting Assays

Indole-3-Acetic Acid (IAA) Production

For qualitative assessment of IAA, all isolates except *Bacillus pumilus* (EPS 16), displayed color change ranging from light pink to reddish, on the addition of Salkowski's reagent, indicating a positive result for IAA (Figure S1). Quantification through HPLC showed maximum indole acetic acid production by *Enterobacter cloacae* (EPS 14), i.e., 12.125 μg/ml, followed by *Pantoea agglomerans* (EPS 17) which produced 8.449 μg/ml of IAA (Figure S2). Among all isolates, *Acinetobacter johnsonii* (EPS 11) showed minimum indole acetic acid production of 0.066 μg/ml (**Table 3**).

Siderophore Assay and HCN Production

Four isolates were siderophore positive showing color change from greenish blue to yellow. Among these four, *Kosakonia oryzae* (EPS 7) showed maximum siderophore production and produced biggest halo around bacterial colony. All isolates were found negative for HCN production as color change in filter paper was not observed (**Table 3**).

Extracellular Enzyme Assays

Five isolates i.e., *Pantoea agglomerans* (EPS 2), *Acinetobacter johnsonii* (EPS 5), *Pantoea dispersa* (EPS 6), *Bacillus pumilus* (EPS 16), and *Pantoea agglomerans* (EPS 17) showed whitish translucent zones around colonies indicating a positive lipase result while *Acinetobacter johnsonii* (EPS 10) and *Pantoea agglomerans* (EPS 13) showed very light zones and were weak positive for the test. For protease test, six isolates showed halo zone around the bacterial colonies and were positive for the assay while nine isolates didn't produce protease. Six isolates showed phosphate solubilization on NBRIP medium by forming a halo zone around their colonies (Figure S3). However, nine isolates did not solubilize phosphate. Among 14 bacterial isolates, three isolates i.e., *Pantoea agglomerans* (EPS 2 and EPS 13) and *Pantoea dispersa* (EPS 6) formed a light yellow/whitish zone around their colonies and hence were cellulase positive (**Table 3**).

TABLE 1 | Biochemical Characterization of EPS isolates using QTS-24 identification kits and Bergey's Manual of Identification.

Sr. No.	1	2	3	4	5	6	7	8	9	10	11	12	13	14	15	16	17	18	19	20	21	Identification Bergey's Manual
Test/Bacterial Isolates	**ONPG**	**CIT**	**MALO**	**ADH**	**ODC**	**H_2S**	**UREA**	**VP**	**GEL**	**NO_3/N_2**	**MR**	**MOT**	**OX**	**GLU**	**Suc**	**RHAM**	**SORB**	**MEL**	**RAF**	**FRUC**	**MANS**	
EPS 1	−	+	+	+	+	+	+	+	−	−/−	+	+	+	+	−	−	−	−	−	−	−	*Pseudomonas fragi*
EPS 2	+	−	+	−	−	−	−	+	−	+/+	+	+	−	+	+	−	−	+	−	w	+	*Pantoea* sp.
EPS 4	+	−	+	−	−	−	−	−	+	−/−	−	−	−	w	W	−	−	+	+	+	−	*Pantoea* sp.
EPS 5	−	−	−	−	−	+	−	+	−	+/−	−	−	+	+	+	+	+	+	−	−	−	*Acinetobacter* sp.
EPS 6	+	−	−	−	−	−	−	+	−	−/−	+	+	+	+	+	+	−	+	−	+	+	*Pantoea dispersa*
EPS 7	+	−	+	−	−	+	−	+	−	+/−	+	+	+	w	+	+	+	−	−	+	W	*Kosakonia* sp.
EPS 10	−	+	+	+	+	+	+	−	−	+/−	+	−	+	+	+	+	+	+	+	+	−	*Acinetobacter johnsonii*
EPS 11	+	−	+	−	−	−	−	−	+	+/−	−	+	−	+	+	W	+	w	−	−	−	*Acinetobacter* sp.
EPS 12	−	−	+	−	−	−	−	+	−	−/−	−	−	−	−	+	−	+	+	−	+	−	*Microbacterium* sp.
EPS 13	+	−	+	−	−	−	−	+	−	−/−	+	+	+	+	−	W	−	+	−	+	+	*Pantoea vagans*
EPS 14	+	+	−	+	−	−	−	+	−	−/−	+	+	+	+	+	+	+	+	+	+	+	*Enterobacter* sp.
EPS 15	−	−	−	+	−	+	−	+	−	+/−	+	+	+	−	+	−	−	+	−	+	−	*Pseudomonas fragi*
EPS 16	−	−	−	−	−	−	−	+	+	−/−	−	+	+	+	+	−	+	+	−	−	−	*Bacillus* sp.
EPS 17	+	−	+	−	−	−	−	+	+	+/−	+	+	−	+	+	+	+	+	+	w	+	*Pantoea agglomerans*

(+): activity, (−): no activity, (w): weak activity.

Symbols: ONPG, Ortho-Nitrophenyl-β-galactosidase; CIT, sodium citrate; MALO, malonate; ADH, arginine dihydrolase; ODC, ornithine decarboxylase; H_2S, hydrogen sulfide; UREA, urease; VP, voges-proskauer; GEL, gelatin hydrolysis; NO_3, nitrate reduction; N_2, nitrogen gas production; GLU, glucose; SUC, sucrose; RHAM, rhamnose; SORB, sorbitol; MEL, melibiose; RAF, raffinose; FRUC, fructose; MANS, mannose.

All strains were positive for mannitol, (MANN) and arabinose (ARAB) and negative for inositol (INOS), indole (IND) and adonitol (ADO). All strains were found positive for maltose (MALT) except EPS-1 while only EPS-10 and EPS-15 were found positive for lysine decarboxylase (LDC) and tryptophan deaminase (TDA) respectively.

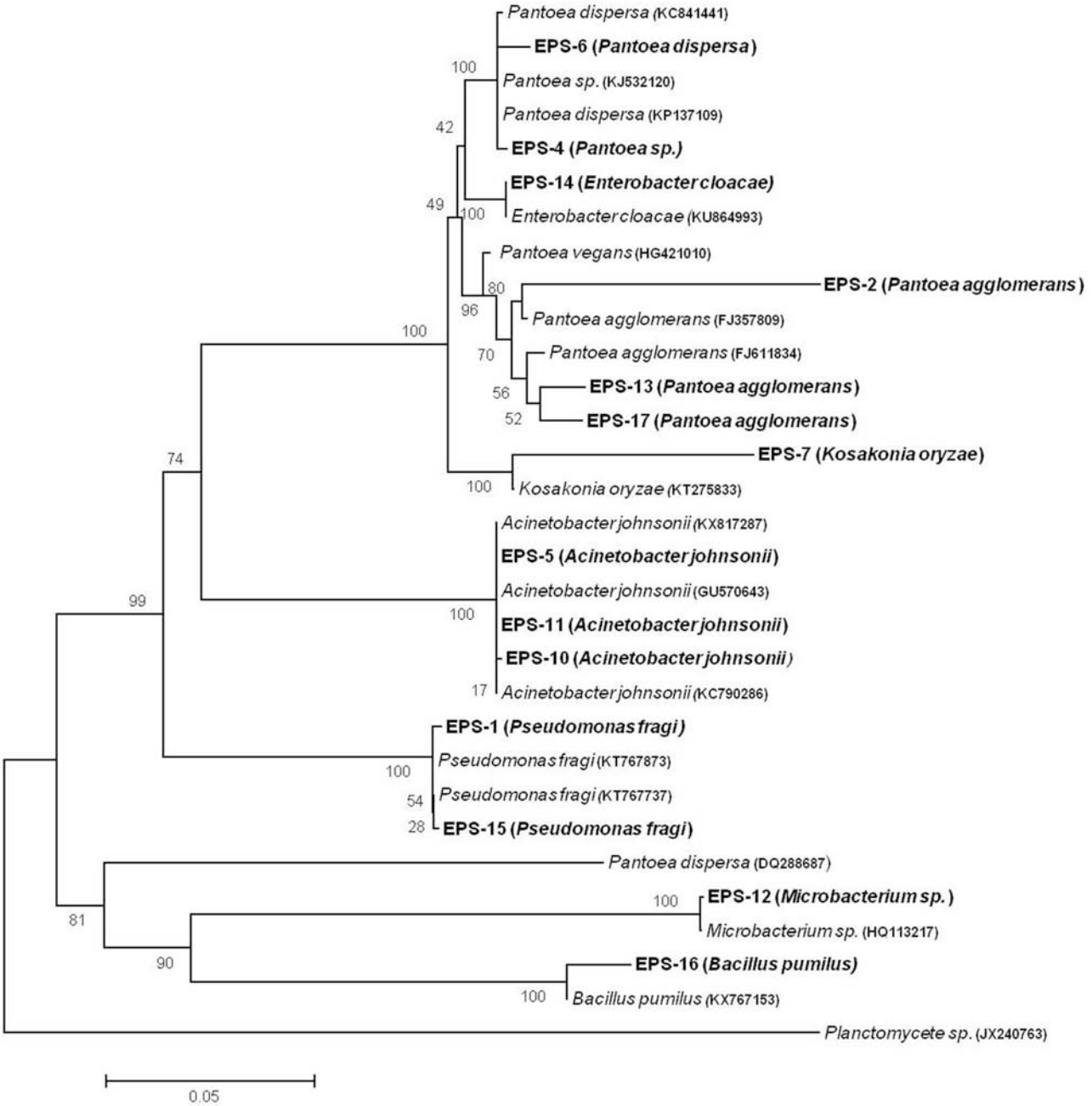


FIGURE 1 | Neighbor-joining tree of 16S rRNA gene sequences of wheat EPS strains. Sequences of closest members were obtained from database and accession numbers are mentioned. There were total of 1,408 positions in final dataset. The percentage of replicate trees in which the associated taxa clustered together in the bootstrap test (1,000 replicates) is shown next to the branches. The evolutionary distances were computed using the maximum composite likelihood method and are in units of number of base substitutions per site.

Zinc Solubilization Assay

Zinc solubilization ability of the bacterial strains was evaluated by determining the zone diameter. Among all 24 wheat and sugarcane isolates, six i.e., *Pseudomonas fragi* (EPS 1), *Pantoea dispersa* (EPS 6), *Pantoea agglomerans* (EPS 13), *Enterobacter cloacae* (PBS 2 and PBS 1), and *Rhizobium* sp. (LHRW1) showed zinc solubilization zones on $ZnCO_3$ medium. Maximum zone of 1.8 cm was observed for *Rhizobium* sp. (LHRW1). On ZnO medium, six isolates *Pseudomonas fragi* (EPS 1), *Pantoea dispersa* (EPS 6), *Enterobacter cloacae* (PBS 1 and PBS 2), *Rhizobium* sp. (LHRW1) and *Pantoea* sp. (LS1-b) showed solubilization. On $Zn_3(PO_4)_2$ medium, 20 strains

TABLE 2 | Sequence length and % homology of EPS isolates based on BLAST results.

Sr. No.	Strain	Sequence Length	Query Cover (%)	E value	% Identity	Sequences ID	Accession Number	Identified As
1	EPS 1	1,425	100	0.0	98	KT767873	KY848812	*Pseudomonas fragi*
2	EPS 2	1,395	100	0.0	95	FJ357809	KY848813	*Pantoea agglomerans*
3	EPS 4	1,389	100	0.0	99	KJ532120	KY848814	*Pantoea* sp.
4	EPS 5	1,139	100	0.0	99	KX817287	KY848815	*Acinetobacter johnsonii*
5	EPS 6	1,386	100	0.0	99	KP137109	KY848816	*Pantoea dispersa*
6	EPS 7	1,356	100	0.0	96	KT275833	KY848817	*Kosakonia oryzae*
7	EPS 10	1,406	100	0.0	99	KC790286	KY848818	*Acinetobacter johnsonii*
8	EPS 11	1,393	100	0.0	99	GU570643	KY848819	*Acinetobacter johnsonii*
9	EPS 12	1,434	100	0.0	97	HQ113217	KY848820	*Microbacterium* sp.
10	EPS 13	1,385	100	0.0	100	EU240963	MF150305	*Pantoea agglomerans*
11	EPS 14	1,354	100	0.0	100	KU864993	KY848821	*Enterobacter cloacae*
12	EPS 15	1,348	100	0.0	99	KT767737	KY848822	*Pseudomonas fragi*
13	EPS 16	1,390	100	0.0	99	KX767153	KY848823	*Bacillus pumilus*
14	EPS 17	1,025	100	0.0	99	FJ611834	KY848824	*Pantoea agglomerans*

TABLE 3 | Plant growth promoting traits by EPS bacterial strains.

Wheat isolates	Protease assay	Lipase assay	Cellulase assay	Phosphatase assay	IAA production (μg/ml)	Siderophore assay
EPS 1	–	–	–	+	0.25	+
EPS 2	W	+	+	w	0.312	–
EPS 4	–	–	–	–	0.71	–
EPS 5	–	+	–	w	0.394	+
EPS 6	–	+	+	w	0.934	–
EPS 7	–	–	–	–	0.413	++
EPS 9	+	–	–	–	0.324	–
EPS 10	–	W	–	–	0.108	–
EPS 11	+	–	–	–	0.066	–
EPS 12	+	–	–	–	0.472	–
EPS 13	–	W	+	+	1.134	–
EPS 14	–	–	–	–	12.125	+
EPS 15	–	–	–	+	0.912	–
EPS 16	+	+	–	–	–	–
EPS 17	+	+	–	–	8.449	–

–, no activity; w, weak activity; ++, very good activity.

showed weak solubilization and four isolates, i.e., *Pantoea* sp. (EPS 4), *Acinetobacter johnsonii* (EPS 11, EPS 10) and *Klebsiella oxytoca* (LHRW2) did not show any solubilization. *Pseudomonas fragi* (EPS 15) showed maximum diameter of 1 cm on $Zn_3(PO_4)_2$ medium. All 24 isolates did not solubilize zinc on $ZnCl_2$ and $ZnSO_4$ supplemented medium (Figures S4–S6, Table S1).

Plant Experiments

Among zinc sources, zinc carbonate was maximum solubilized by bacterial isolates as compared to other zinc salts, hence it was selected for zinc supplementation in plant experiments. Based on zinc carbonate solubilization ability, five strains were selected for plant experiments. These strains were also positive for indole-3-acetic acid production and phosphate solubilization. The results described below are based on average values of the ten replicates of each treatment.

A. Plants harvested after 4 weeks (First set)

Growth parameters: The results of first set of plant experiments manifested that two of these strains; PBS 2 and LHRW1, considerably increased dry weights of roots and shoots as compared with un-inoculated plants. Significant increase in shoots dry weights was only seen for the plants inoculated with *Rhizobium* sp. (LHRW1). Plants inoculated with all other strains and un-inoculated showed almost similar shoot dry weights. No significant difference was observed in fresh root and shoot weights for inoculated and un-inoculated plants (**Figure 2A**).

Significantly high as well as maximum dry root weight was recorded for the plants inoculated with *Enterobacter cloacae* (PBS 2) and it was followed by *Rhizobium* sp. (LHRW1) inoculated plants. Non-significant increase was found in dry root weights of plants inoculated with all other strains as compared with un-inoculated control plants (**Figure 2B**, Figures S7, S8).

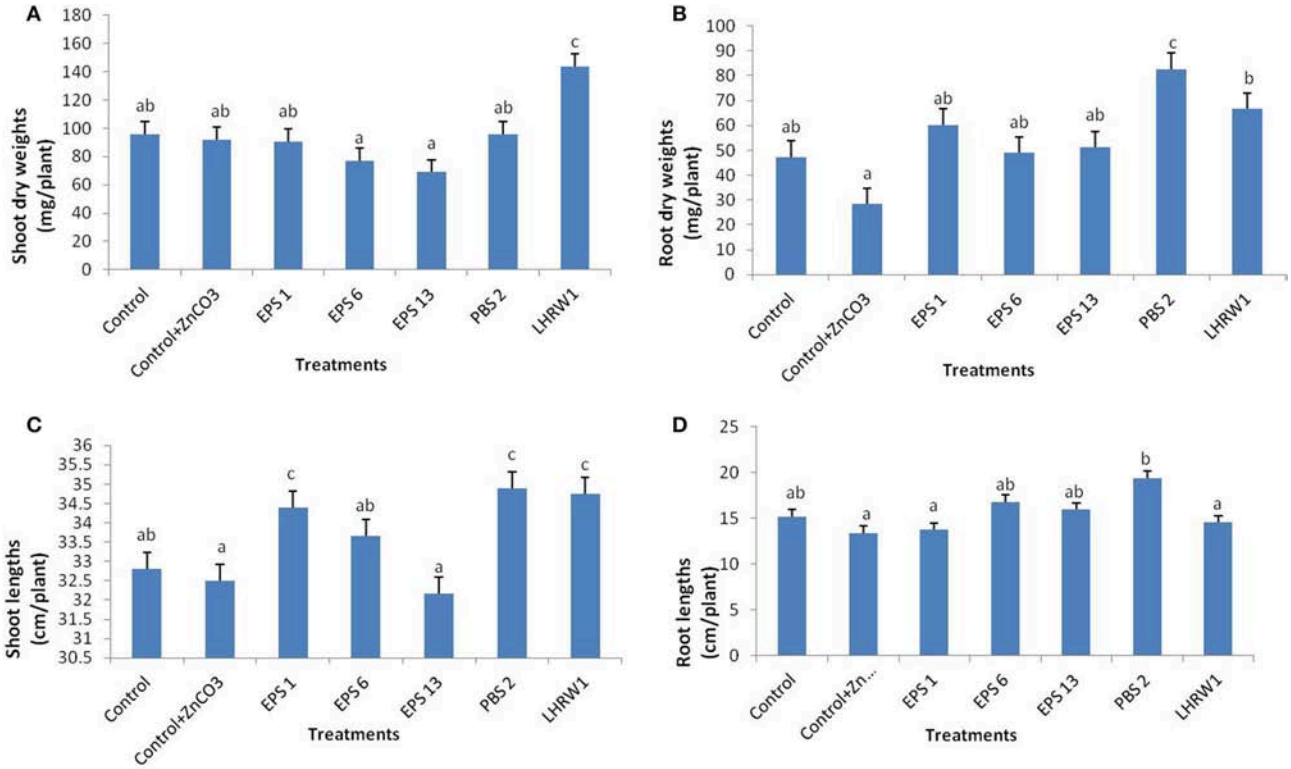


FIGURE 2 | Effect of bacterial inoculation on growth parameters of plants harvested after 4 weeks (first set). **(A)** Dry weight of shoots, **(B)** Dry weight of roots, **(C)** Shoot lengths, **(D)** Root lengths in comparison with un-inoculated control. *All inoculated treatments were supplemented with 1% $ZnCO_3$. Alphabets (a–c) represent the significant and non-significant difference among datasets. [a]Indicates no significant difference among dataset labeled with a on them. [b]Shows a slight difference from (a) and same group category for dataset labeled with (b). [c]Shows significant difference from a and b datasets and same data group labeled with c.

Enterobacter cloacae (PBS 2) also significantly contributed to increase lengths of roots and shoots while *Pseudomonas fragi* (EPS 1) and *Rhizobium* sp. (LHRW1) increased lengths of shoots. The maximum shoot length was recorded for *Enterobacter cloacae* (PBS 2) inoculated plants; *Pseudomonas fragi* (EPS 1) and *Rhizobium* sp. (LHRW1) followed it with minor difference. No other strain was observed to significantly increase root and shoot lengths with respect to un-inoculated controls (**Figures 2C,D**).

Zinc Content: Significant increase was found in zinc content of shoots and roots of inoculated plants as compared to control. The zinc content of control shoots was found to be 4.25 mg/kg. Maximum zinc content, i.e., 18.25 mg/kg was found in shoots of *Enterobacter cloacae* (PBS 2) inoculated plants, followed by *Pantoea agglomerans* (EPS 13) which showed zinc content of 17.85 mg/kg. Zinc content in shoots of other treatments ranged from ∼ 12 to 15 mg/kg for inoculated plants and ∼ 10 mg/kg for un-inoculated plants provided with $ZnCO_3$ (**Figure 3A**).

Control plants that were not given any zinc also showed zinc presence in roots and shoots; 4.25 mg/kg of zinc in shoots and 3.74 mg/kg in roots. This zinc can be correlated with the amount of zinc provided to the plants in Hoagland's solution that contains 0.05 mg/l zinc. Zinc content of the shoots of control plants supplemented with $ZnCO_3$ was also higher that of the control plants. This higher amount of zinc in $ZnCO_3$ supplemented control is actually associated with the ability of the plant itself to mobilize available nutrients and use them for their growth.

The zinc content of roots of inoculated plants showed even more significant increase as compared to control plants. *Pantoea agglomerans* (EPS 13) showed maximum zinc content of 42.96 mg/kg in roots, followed by *Rhizobium* sp. (LHRW1) inoculated plants which showed zinc content of 40.77 mg/kg. Zinc content in roots of other inoculated treatments ranged from 26 to 34 mg/kg. However, difference was non-significant as compared to roots of un-inoculated plants provided with $ZnCO_3$ (**Figure 3B**). Analysis of zinc content of roots and shoots demonstrate that plants can take up available zinc to their roots but its transport to the shoots can be facilitated by inoculating the plants with PGPR that can also colonize inside the plants as endophytes and make zinc bio-available to the plants.

B: Plants harvested after 3 months (second set)

Growth parameters: Plants were harvested after 3 months and data for shoots and roots weights and lengths was recorded. Highest fresh weights for shoots were recorded for plants inoculated with *Rhizobium* sp. (LHRW1) followed by *E. cloacae* (PBS 2). All other strains showed insignificant difference in shoot fresh weights when compared with un-inoculated controls (**Figure 4A**). None of the strains showed significant increase

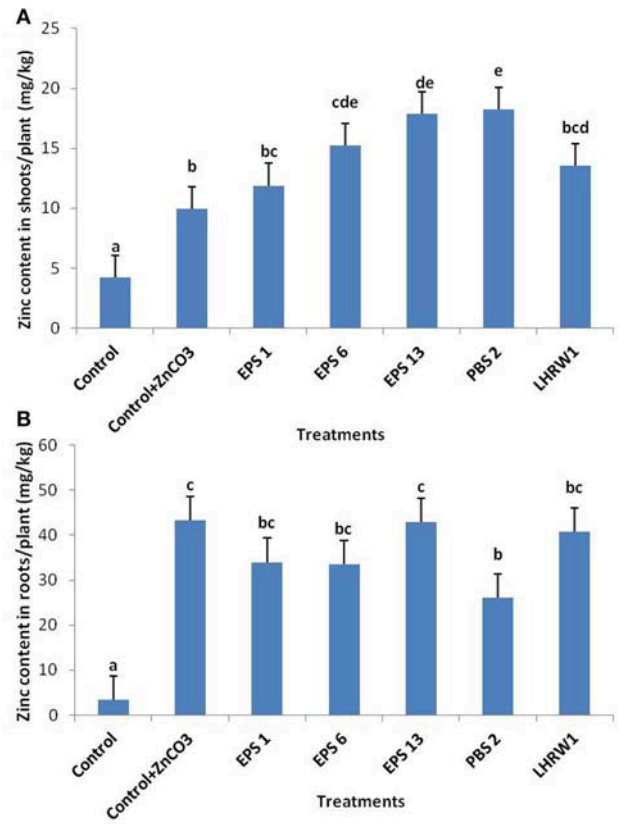


FIGURE 3 | Estimation of Zinc content in **(A)** shoots and **(B)** roots of inoculated and un-inoculated plants, harvested after 4 weeks. Small letters (a–e) represent the significance and non-significance of data. All bars with same alphabet on them indicate the same group and non-significance with each other. While bars with different alphabets indicate significant difference with other groups.

in fresh weights of roots when compared with control plants (**Figure 4B**).

Most of these strains significantly increased dry weights of roots and shoots as compared with un-inoculated controls. Significant increase in shoots dry weights was seen for the plants inoculated with strains *P. dispersa* (EPS 6), *P. agglomerans* (EPS 13) and *E. cloacae* (PBS 2). Maximum dry shoot weights were recorded for the plants inoculated with *E. cloacae* (PBS 2). Plants inoculated with all other strains and un-inoculated showed almost similar shoot dry weights (**Figure 4C**).

Pseudomonas fragi (EPS 1), *E. cloacae* (PBS 2) and *Rhizobium* sp. (LHRW1) also significantly increased root dry weights as compared to controls. Plants inoculated with *Pseudomonas fragi* (EPS 1) showed highest dry root weights followed by *Rhizobium* sp. (LHRW1) and *E. cloacae* (PBS 2) when compared with un-inoculated plants. *Rhizobium* sp. (LHRW1) and *E. cloacae* (PBS 2) also significantly increased dry weights of roots in plants harvested after 4 weeks. (**Figure 4D**).

Rhizobium sp. (LHRW1) significantly increased shoot lengths while all other strains showed insignificant increase with respect to un-inoculated ones (**Figure 4E**). *Rhizobium* sp. (LHRW1) plants showed similar results for plants harvested after 4 weeks. *P. agglomerans* (EPS 13) inoculated plants showed maximum increase in root length. Unlike previous experiment, increase in root length of *E. cloacae* (PBS 2) inoculated plants was insignificant (**Figure 4F**).

Zinc Content Significant increase in zinc content of grains was observed for the plants inoculated with *Pseudomonas fragi* (EPS 1), *Pantoea dispersa* (EPS 6) and *Pantoea agglomerans* (EPS 13). Highest amounts of zinc were detected in the grains of *Pseudomonas fragi* (EPS 1) inoculated plants with 6.96 mg/kg of zinc followed by *Pantoea dispersa* (EPS 6) and *Pantoea agglomerans* (EPS 13) which had 6.3 and 4.64 mg/kg of zinc, respectively. Plants inoculated with strains *E. cloacae* (PBS 2) and *Rhizobium* sp. (LHRW1) showed insignificant increase in grain zinc content as compared to un-inoculated controls, i.e., 2.66 mg/kg and 2.68 mg/kg in control+$ZnCO_3$ (**Figure 5A**). These results indicate the efficacy of three PGPR strains; *Pseudomonas fragi* (EPS 1), *Pantoea dispersa* (EPS 6) and *Pantoea agglomerans* (EPS 13) that they enhanced the bioavailability of zinc and mobilized it toward wheat grains.

Comparison of control and inoculated plants showed considerable increase in zinc content of shoots of inoculated ones. Highest zinc content in shoots was seen for the plants inoculated with strain *P. fragi* (EPS 1); 8.83 mg/kg followed by *P. agglomerans* (EPS 13) and *P. dispersa* (EPS 6) which showed zinc content of 5.73 and 4.46 mg/kg, respectively. Though, *E. cloacae* (PBS 2) and *Rhizobium* sp. (LHRW1) also showed increase in zinc content of shoots but it was non-significant (**Figure 5B**).

Interestingly, control plants showed significant difference in zinc content of roots as compared to all inoculated treatments and highest zinc content was recorded for control plants i.e., 47.97 mg/kg followed by *P. fragi* (EPS 1) that was 42.53 mg/kg. Minimum zinc content was seen for the roots of the plants inoculated with strain *E. cloacae* (PBS 2) that was 18.207 mg/kg. All other strains showed non-significant difference of zinc content in comparison to $ZnCO_3$ supplemented control plants (**Figure 5C**).

DISCUSSION

Zinc is the key constituent of plants and is very crucial for their development. Zinc deficiency is the most common micronutrient deficiency in crops worldwide and results in substantial losses in crop yields. Use of zinc fertilizers may not be cost effective in alleviating zinc deficiency and increasing crop yield. In addition to this, zinc fertilizers are underutilized in many countries like Pakistan, despite the widespread occurrence of zinc-deficient soils. These zinc deficient soils hamper the growth of many plants including staple foods such as wheat, rice, corn and sugarcane. Wheat yield is critically affected by zinc deficiency and local studies have shown the use of zinc fertilizers to overcome this (Khan et al., 2009; Ahmad et al., 2012; Joy et al., 2017). But use of chemical fertilizers threatens public health and environment and also puts farmer's livelihood in jeopardy. Therefore, use of chemical fertilizers has declined in many countries and growers are returning to organic farming.

Rhizobacteria play a vital role in environmental cycling processes such as solubilization of metals into soluble forms that

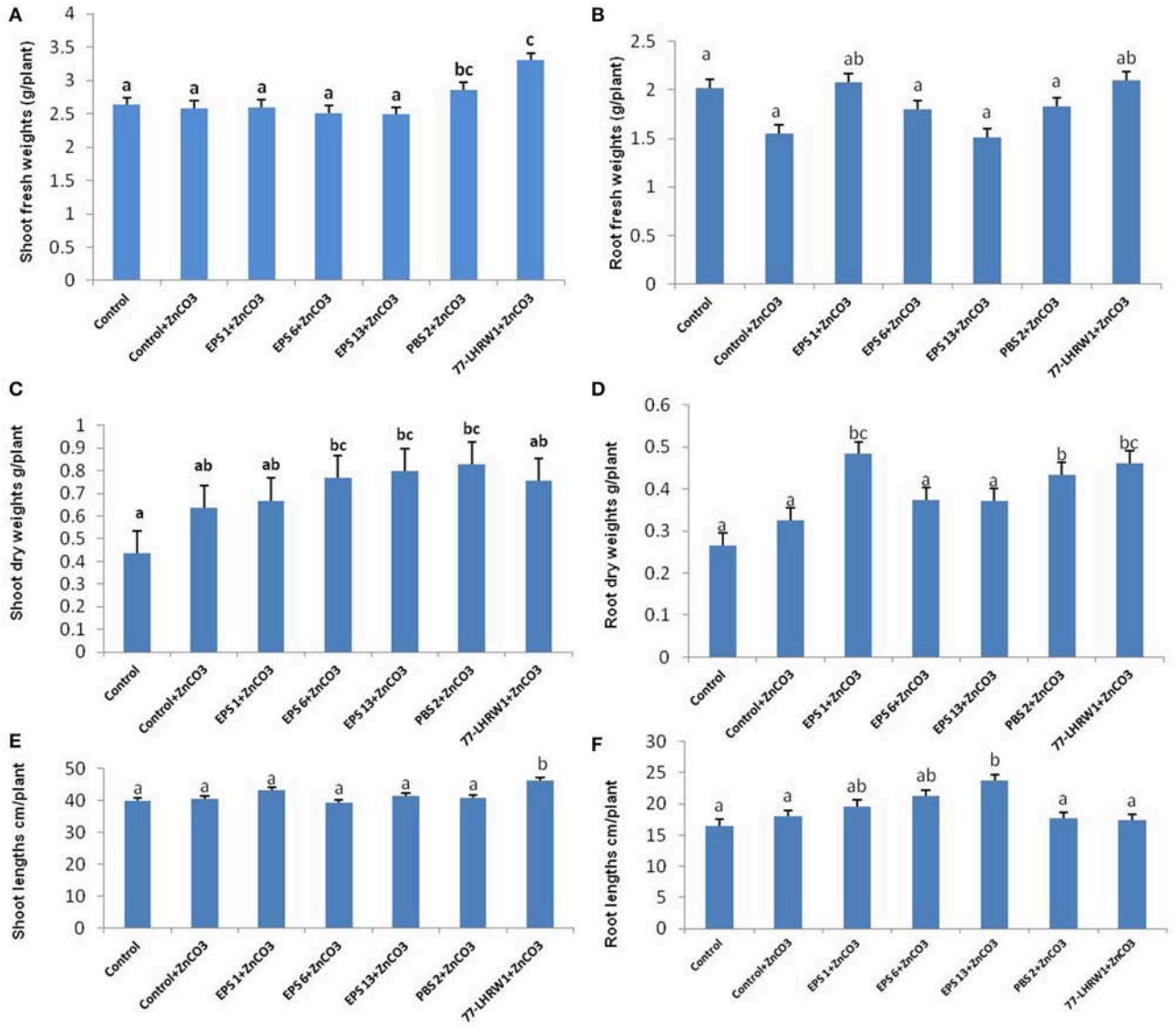


FIGURE 4 | Effect of bacterial inoculation on growth parameters of plants harvested after 3 months (second set). **(A)** Fresh weight of shoots, **(B)** Fresh weight of roots, **(C)** Dry weight of shoots, **(D)** Dry weights of roots, **(E)** Shoot lengths, and **(F)** Root length. Alphabets (a–c) represent the significant and non-significant difference among datasets. [a]Indicates no significant difference among dataset labeled with a on them. [b]Shows a slight difference from (a) and same group category for dataset labeled with (b). [c]Shows significant difference from a and b datasets and same data group labeled with c.

are suitable for plant uptake. These ions and metals significantly improve plant growth and nutrition. The main focus of this study was to identify and characterize the strains that have potential to be used as zinc-biofertilizers. Based on zinc solubilization, three EPS producing strains of wheat and two of sugarcane, were selected. One of these strains was identified as *Pseudomonas fragi* (EPS 1), two as *Pantoea dispersa* (EPS 6) and *Pantoea agglomerans* (EPS13), one sugarcane strain as *Enterobacter cloacae* (PBS 2), and one as *Rhizobium* sp. (LHRW1). These bacterial genera are known to colonize rhizosphere of wheat and sugarcane and increasing plant growth (Baig et al., 2012; Verma et al., 2015). Large body of literature describes the potential of these PGPR genera to increase plant growth and crop yield.

Various species of *Pseudomonas* have extensively been studied in relation to plant growth promotion. *P. fluorescens* has shown indole acetic acid production resulting in enhanced growth of onion (Reetha et al., 2014) as well as phosphate solubilization (Oteino et al., 2015). Zinc solubilization (Pawar et al., 2015), HCN production and biocontrol properties of *Pseudomonas* sp. have also been reported (Tank and Saraf, 2009). However, few reports are available for the beneficial effect of *P. fragi* strains on plants. Selvakumar et al. (2009) reported P solubilization, IAA and HCN production, enhanced germination rate, plant biomass and uptake of nutrients by wheat plants upon inoculation with *P. fragi* strain. However, reports of zinc solubilization by *P. fragi* were not found. Species belonging to *Pantoea* have been extensively

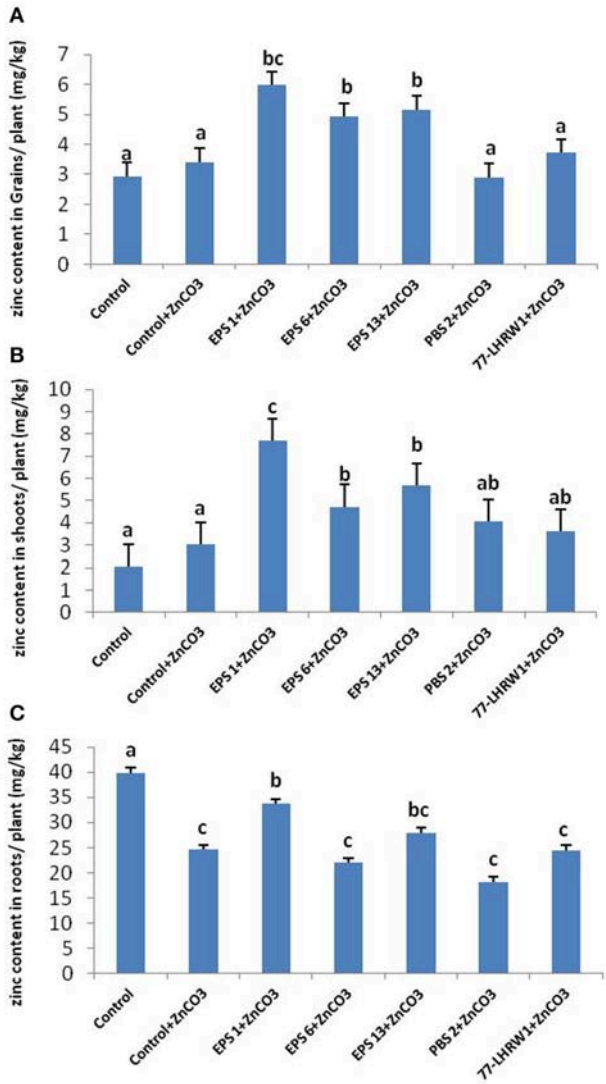


FIGURE 5 | Quantification of Zinc content in **(A)** Grains, **(B)** Shoots, and **(C)** Roots of inoculated and un-inoculated plants. Alphabets (a–c) represent the significant and non-significant difference among datasets. [a]Indicates no significant difference among dataset labeled with a on them. [b]Shows a slight difference from (a) and same group category for dataset labeled with (b). [c]Shows significant difference from a and b datasets and same data group labeled with c.

studied and reported to have many plant growth promoting abilities. *Pantoea agglomerans* has been reported to solubilize phosphorus and produce IAA, siderophores as well as ammonia. On inoculation with *P. agglomerans*, jute plants have shown significant increase in plant height, weight, chlorophyll content and total carbohydrate content (Majumdar and Chakraborty, 2015). Plant growth promoting abilities of *Enterobacter* species have been reported extensively. *Enterobacter asburiae* has shown IAA, HCN, ammonia and siderophore production, as well as phosphate solubilization (Ahemad and Khan, 2010). *Rhizobium* sp. and *E. cloacae* have been reported to produce phytohormones like acetoin and other bioactive compounds. It has also shown to solubilize phosphate. Inoculation of *Pisum sativum*, with these strains resulted in a significant increase in growth of the plant (Khalifa et al., 2016). In this study too, *E. cloacae* (EPS 14) and *Pantoea agglomerans* (EPS 17) have also shown highest IAA production with 12.125 and 8.449 μg/ml. These genera have also previously been reported for producing high amounts of indole-3-acetic acid; 23.006 μg/ml (Mohite, 2013).

Our findings are consistent with previously reported literature and use of Zinc solubilizing *Pseudomonas fragi*, *Pantoea agglomerans*, *E. cloacae,* and *Rhizobium* sp. showed encouraging results. When inoculated with these strains, wheat plants showed enhanced shoot and root length and weight as well as zinc content. Most of the strains used in this study are not yet reported for zinc solubilizing ability and for their effect on wheat growth. Significant difference was seen in root and shoot zinc content with all inoculated plants as compared to un-inoculated controls. *Pseudomonas fragi* (EPS 1), *Pantoea dispersa* (EPS 6) and *Pantoea agglomerans* (EPS 13) showed promising results when the grain zinc content of wheat was analyzed after 3 months. *Pseudomonas fragi* (EPS 1) showed highest grain zinc content followed by *Pantoea agglomerans* (EPS 13) and *Pantoea dispersa* (EPS 6). Though *Rhizobium* sp. (LHRW1) and *E. cloacae* (PBS2) significantly increased biomass of the plant in all three experiments but highest root dry weight was observed by *Pseudomonas fragi* strain EPS 1. Plants harvested after 3 months, also showed the maximum amount of zinc in roots of un-inoculated control plants when compared with inoculated ones which is evidence that these PGPR genera have successfully helped the plant in zinc solubilization and uptake. It supports that inoculation with this strain accelerated the bioavailability of zinc to the roots of the plants and provided it with more solubilized zinc as compared to un-inoculated controls.

Increased zinc content of roots and shoots as compared to un-inoculated plants is supported by the previous reports where inoculation of plants with PGPR has resulted in increased yield, enhanced plant growth and improved nutrition and many effective strains have been formulated as biofertilizers in this regard. The PGPR strains identified so far mainly belong to genra *Pseudomonas, Ochrobacterum, Bacillus, Azospirillum, Azotobacter, Rhizobium, Stenotrophomonas, Serratia*, and *Enterobacteria* (Maleki et al., 2011). In addition to increase the overall yield of the plant, PGPR have extensively been reported in combating nutrient deficiencies of the plants and has been given attention to be used as biofertilizers. For example, Ramesh et al. (2014) has reported increased mobilization of zinc by zinc solubilizing *Bacillus aryabhattai* in wheat and soybean. Recent studies have also revealed 7–12% enhanced zinc translocation toward wheat grains by certain strains of *Serratia* sp., *Bacillus* sp. *Pseudomonas* sp. and many others as compared to chemical zinc supplementation to the plant (Lefèvre et al., 2014). In addition to increase biomass, zinc solubilizing bacteria used in this study, have also significantly enhanced zinc content of shoots and roots in comparison to un-inoculated plants which is a well documented phenomenon and has been explained in many earlier research studies. Previous studies have demonstrated that application of PGPR or PGPE has enhanced zinc translocation toward rice and wheat grains and this ability of rhizobacteria

or plant growth promoting endophytes (PGPE) is related with their capacity of executing successful plant microbe interactions such as induction of physiological processes, mineralization and solubilization (Lucas et al., 2014; Wang et al., 2014). Plants apoplasts are known for offering different growth conditions and hence, many rhizosphere bacterial strains can be the effective endophytic colonizers of the plants (Khan et al., 2015). Bacterial genera used in this study have also been reported to colonize plant roots as endophytes and thus significantly solubilize minerals and available nutrients. Research studies have also shown that plants, when supplied with different nutrients as chemical fertilizers or through biofortification, triggered many physiological changes that helped them to take up the nutrients from the soil (Chattha et al., 2017).

In addition to this, all five strains used in plant experiments could solubilize phosphate and produced indole-3-acetic acid. IAA can also be a contributing factor in increasing plant growth and biomass. Though, no source of insoluble P was present in soil, but P solubilization is an important parameter in improving plant growth and cannot be neglected.

Zinc solubilization by PGPR is relatively a newer approach and most of the strains have not yet been tested for this activity. This study indicates the potential of *Pantoea, E. cloacae* and especially *Pseudomonas fragi* to be used as bio-fertilizer and overcome zinc deficiency in countries like Pakistan where zinc fertilizers are under-used and are not cost effective.

AUTHOR CONTRIBUTIONS

SK: Conducted major experiments; IS: manuscript writing and data analysis; DB: molecular analysis; MR: conducted HPLC analysis; KM: provided the basic lab infrastructure, Dean for Research and Postgraduate Studies; SM: edited manuscript, guided in whole experiment plan.

ACKNOWLEDGMENTS

Authors gratefully acknowledge the support of Professor Dr. Dildar Ahmed (Department of Chemistry, Forman Christian College; A Chartered University, Lahore, Pakistan) for kindly providing access to atomic absorption spectroscopy.

SUPPLEMENTARY MATERIAL

The Supplementary Material for this article can be found online at: https://www.frontiersin.org/articles/10.3389/fmicb.2017.02593/full#supplementary-material

REFERENCES

Abaid-Ullah, M., Hassan, M. N., Jamil, M., Brader, G., Shah, M. K. N., Sessitsch, A., et al. (2015). Plant growth promoting rhizobacteria: an alternate way to improve yield and quality of wheat (*Triticum aestivum*). *Int. J. Agric. Biol.* 17, 51–60.

Ahemad, M., and Khan, M. S. (2010). Plant growth promoting activities of phosphate-solubilizing *Enterobacter asburiae* as influenced by fungicides. *Eurasia. J. Biosci.* 4, 88–95. doi: 10.5053/ejobios.2010.4.0.11

Ahmad, M., Zahir, Z. A., Asghar, H. N., and Arshad, M. (2012). The combined application of rhizobial strains and plant growth promoting rhizobacteria improves growth and productivity of mung bean (*Vigna radiata* L.) under salt-stressed conditions. *Ann. Microbiol.* 62, 1321–1330. doi: 10.1007/s13213-011-0380-9

Alexander, M. (1997). *Introduction to Soil Microbiology*. New York, NY: John Wiley and Sons.

Alloway, B. J. (2004). *Zinc in Soil and Crop Nutrition*. Belgium: International Zinc Association.

Alloway, B. J. (2008). *Zinc in Soils and Plant Nutrition*. Belgium: International Zinc Association.

Baig, K. S., Arshad, M., Shaharoona, B., Khalid, A., and Ahmed, I. (2012). Comparative effectiveness of *Bacillus* spp. possessing either dual or single growth-promoting traits for improving phosphorus uptake, growth and yield of wheat (*Triticum aestivum L.*). *Ann. Microbiol.* 62, 1109–1119. doi: 10.1007/s13213-011-0352-0

Bertani, G. (1952). Studies on lysogenesis. I. The mode of phage liberation by lysogenic *Escherichia coli*. *J. Bacteriol.* 62, 293–300.

Cakmak, I., Pfeiffer, W. H., and McClafferty, B. (2010). Biofortification of durum wheat with zinc and iron. *Cereal Chem.* 87, 10–20. doi: 10.1094/CCHEM-87-1-0010

Chang, H. B., Lin, C. W., and Huang, H. J. (2005). Zinc induced cell death in rice (*Oryza sativa* L.) roots. *Plant Growth Regul.* 46, 261–266. doi: 10.1007/s10725-005-0162-0

Chattha, M. U., Hassan, M. U., Khan, I., Chattha, M. B., Mahmood, A., Chattha, M. U., et al. (2017). Biofortification of wheat cultivars to combat zinc deficiency. *Front. Plant Sci.* 8:281. doi: 10.3389/fpls.2017.00281

Cheng, M., Fry, J. E., Pang, S., Zhou, H., Hironaka, C. M., Duncan, D. R., et al. (1997). Genetic transformation of wheat mediated by *Agrobacterium tumefaciens*. *Plant Physiol.* 115, 971–980. doi: 10.1104/pp.115.3.971

Chun, J., and Goodfellow, M. (1995). A phylogenetic analysis of the genus *Nocardia* with 16S rRNA gene sequences. *Int. J. Syst. Bacteriol.* 45, 240–245. doi: 10.1099/00207713-45-2-240

Deepak, J., Geeta, N., Sachin, V., and Anita, S. (2013). Enhancement of wheat growth and Zn content in grains by zinc solubilizing bacteria. *Int. J. Agr. Environ. Biotechnol.* 6, 363–370. doi: 10.5958/j.2230-732X.6.3.004

Epstein, E., and Bloom, A. J. (2005). *Mineral Nutrition of Plants: Principles and Perspectives*. California: Sinauer Associates.

Fasim, F., Ahmed, N., Parsons, R., and Gadd, G. M. (2002). Solubilization of zinc salts by a bacterium isolated from the air environment of a tannery. *FEMS Microbiol. Lett.* 213, 1–6. doi: 10.1111/j.1574-6968.2002.tb11277.x

Glick, B. (2012). *Plant Growth-Promoting Bacteria: Mechanisms and Applications*. New York, NY: Hindawi Publishing Corporation.

Gordon, A. S., and Weber, R. P. (1950). Colorimetric estimation of indole acetic acid. *Plant Physiol.* 26, 192–195. doi: 10.1104/pp.26.1.192

Gunes, A., Inal, A., Adak, M. S., Alpaslan, M., Bagci, E. G., Erol, T., et al. (2007). Mineral nutrition of wheat, chickpea and lentil as affected by mixed cropping and soil moisture. *Nutr. Cycl. Agroecosyst.* 78, 83–96. doi: 10.1007/s10705-006-9075-1

Gustin, J. L., Loureiro, M. E., Kim, D., Na, G., Tikhonova, M., and Salt, D. E. (2009). MTP1-dependent Zn sequestration into shoot vacuoles suggests dual roles in Zn tolerance and accumulation in Zn-hyperaccumulating plants. *Plant J.* 57, 1116–1127. doi: 10.1111/j.1365-313X.2008.03754.x

Hafeez, B., Khanif, Y. M., and Saleem, M. (2013). Role of zinc in plant nutrition-A review. *Am. J. Exp. Agric.* 3, 374–391. doi: 10.9734/AJEA/2013/2746

Hoagland, D. R., and Arnon, D. I. (1950). *The Water-culture Method for Growing Plants without Soil*. Berkley, CA: California Agricultural Experimental Station Publications; College of Agriculture; University of California.

Hussain, A., Arshad, M., Zahir, Z. A., and Asghar, M. (2015). Prospects of zinc solubilizing bacteria for enhancing growth of maize. *Pak. J. Agri. Sci.* 52, 915–922.

Jepkoech, J. K., Simiyu, G. M., and Arusei, M. (2013). Selected heavy metals in water and sediments and their bioconcentrations in plant (*Polygonum*

pulchrum) in Sosiani River, Uasin Gishu County, Kenya. *J. Environ. Protect.* 4, 796–802. doi: 10.4236/jep.2013.48093

Jones, D. L., and Darrah, P. R. (1994). Role of root derived organic acids in the mobilization of nutrients from the rhizosphere. *Plant Soil* 166, 247–257. doi: 10.1007/BF00008338

Joy, E. J. M., Ahmad, W., Zia, M. H., Kumssa, D. B., Young, S. D., Anders, E. L., et al. (2017). Valuing increased zinc (Zn) fertiliser-use in Pakistan. *Plant Soil* 411:139. doi: 10.1007/s11104-016-2961-7

Kabata-Pendias, A., and Pendias, H. (2001). *Trace Elements in Soils and Plants*. London: CRC Press.

Karak, T., Singh, U. K., Das, S., Das, D. K., and Kuzyakov, Y. (2005). Comparative efficacy of $ZnSO_4$ and Zn-EDTA application for fertilization of rice (*Oryza sativa* L.). *Arch. Agron. Soil Sci.* 51, 253–264. doi: 10.1080/03650340400026701

Khalifa, A. Y. Z., Alsyeeh, A. M., Almalki, M. A., and Saleh, F. A. (2016). Characterization of the plant growth promoting bacterium, *Enterobacter cloacae* MSR1, isolated from roots of non-nodulating *Medicago sativa*. *Saudi J. Biol. Sci.* 23, 79–86. doi: 10.1016/j.sjbs.2015.06.008

Khande, R., Sushil, K. S., Ramesh, A., and Mahaveer, P. S. (2017). Zinc solubilizing *Bacillus* strains that modulate growth, yield and zinc biofortification of soybean and wheat. *Rhizosphere* 4, 126–138. doi: 10.1016/j.rhisph.2017.09.002

Khan, R., Gurmani, A. R., Khan, M. S., and Gurmani, A. H. (2009). Residual, direct and cumulative effect of zinc application on wheat and rice yield under rice wheat system. *Soil Environ*. 28, 24–28.

Khan, M. U., Sessitsch, A., Harris, M., Fatima, K., Imran, A., and Arslan, M. (2015). Cr-resistant and endophytic bacteria associated with *Prosopisjuli flora* and their potential as phytoremediation enhancing agents in metal-degraded soils. *Front. Plant Sci.* 5:755. doi: 10.3389/fpls.2014.00755

Kloepper, J. W., and Okon, Y. (1994). "Plant growth-promoting rhizobacteria (other systems)," in *Azospirillum/Plant Associations,* ed Y. Okon (Boca Raton: CRC Press), 111–118.

Kumar, R. S., Ayyadurai, N., Pandiaraja, P., Reddy, A. V., Venkatesvarlu, Y., Prsakash, O., et al. (2005). Characterization of antifungal metabolite produced by a new strain *Pseudomonas aeruginosa* PUPa3 that exhibits broad spectrum antifungal activity and biofertilizing traits. *J. Appl. Microbiol.* 98, 145–154. doi: 10.1111/j.1365-2672.2004.02435.x

Lefèvre, I., Vogel-Mikuš, K., Jeromel, L., Vavpetič, P., Planchon, S., Arčon, I., et al. (2014). Differential cadmium and zinc distribution in relation to their physiological impact in the leaves of the accumulating *Zygophyllum fabago* L. *Plant Cell Environ.* 37, 1299–1320. doi: 10.1111/pce.12234

Lin, L., Kan, X., Yan, H., and Wang, D. (2012). Characterization of extracellular cellulose degrading enzymes from *Bacillus thuringiensis* strains. *Electron. J. Biotechnol.* 15, 1–7. doi: 10.2225/vol15-issue3-fulltext-1

Lucas, J. A., García-Cristobal, J., Bonilla, A., Ramos, B., and Gutierrez-Manero, J. (2014). Beneficial rhizobacteria from rice rhizosphere confers high protection against biotic and abiotic stress inducing systemic resistance in rice seedlings. *Plant Physiol. Biochem*. 82, 44–53. doi: 10.1016/j.plaphy.2014.05.007

Majumdar, S., and Chakraborty, U. (2015). Phosphate solubilizing rhizospheric *Pantoea agglomerans* Acti-3 promotes growth in jute plants. *World J. Agric. Sci.* 11, 401–410. doi: 10.5829/idosi.wjas.2015.11.6.1893

Maleki, M., Mokhtarnejad, L., and Mostafaee, S. (2011). Screening of rhizobacteria for biological control of cucumber root and crown rot caused by *Phytophthora drechsleri*. *Plant Pathol. J.* 27, 78–84. doi: 10.5423/PPJ.2011.27.1.078

Mehnaz, S., Baig, D. N., and Lazarovits, G. (2010). Genetic and phenotypic diversity of plant growth promoting rhizobacteria isolated from sugarcane plants growing in Pakistan. *J. Microbiol. Biotechnol.* 20, 1614–1623. doi: 10.4014/jmb.1005.05014

Mhatre, M., Srinivas, L., and Ganapathi, T. R. (2011). Enhanced iron and zinc accumulation in genetically engineered pineapple plants using soybean Ferritin gene. *Biol. Trace Elem. Res.* 144, 1219–1228. doi: 10.1007/s12011-011-9092-z

Miller, R. L., and Higgins, V. J. (1970). Association of cyanide with infection of birdsfoot trefoil by *Stemphylium loti*. *Phytopathology* 60, 104–110. doi: 10.1094/Phyto-60-104

Mirza, F. M., Najam, N., Mehdi, M., and Ahmad, B. (2015). Determinants of technical efficiency of wheat farms in Pakistan. *Pak. J. Agri. Sci.* 52, 565–570.

Mohite, B. (2013). Isolation and characterization of indole acetic acid (IAA) producing bacteria from rhizospheric soil and its effect on plant growth. *J. Soil Sci. Plant Nutr*. 13, 638–649. doi: 10.4067/S0718-95162013005000051

Nautiyal, C. S. (1999). An efficient microbiological growth medium for screening phosphate solubilizing microorganisms. *FEMS Microbiol. Lett.* 170, 265–270. doi: 10.1111/j.1574-6968.1999.tb13383.x

Naz, I., Ahmad, H., Khokhar, S. N., Khan, K., and Shah, A. H. (2016). Impact of zinc solubilizing bacteria on zinc contents of wheat. *Am. Euras. J. Agric. Environ. Sci.* 16, 449–454. doi: 10.5829/idosi.aejaes.2016.16.3.12886

Oteino, N., Lally, R. D., Kiwanuka, S., Lloyd, A., Ryan, D., Germaine, K. J., et al. (2015). Plant growth promotion induced by phosphate solubilizing endophytic *Pseudomonas* isolates. *Front. Microbiol.* 6:745. doi: 10.3389/fmicb.2015.00745

Pawar, A., Ismail, S., Mundhe, S., and Patil, V. D. (2015). Solubilization of insoluble zinc compounds by different microbial isolates in vitro condition. *Int. J. Trop. Agric*. 33, 865–869.

Perez-Miranda, S., Cabirol, N., George-Tellez, R., Zamudio-Rivera, L. S., and Fernandez, F. J. (2007). O-CAS, a fast and universal method for siderophore detection. *J. Microbiol. Methods* 70, 127–131. doi: 10.1016/j.mimet.2007.03.023

Ramesh, A., Sharma, S. K., Sharma, M. P., Yadav, N., and Joshi, O. P. (2014). Inoculation of zinc solubilizing *Bacillus aryabhattai* strains for improved growth, mobilization and biofortification of zinc in soybean and wheat cultivated in vertisols of central India. *Agric. Ecosyst. Environ. Appl. Soil Ecol.* 73, 87–96. doi: 10.1016/j.apsoil.2013.08.009

Rasul, G., Mirza, M. S., Latif, F., and Malik, K. A. (1998). "Identification of plant growth hormones produced by bacterial isolates from rice, wheat and kallar grass," in *Nitrogen Fixation with Non-legumes,* eds K. A. Malik, M. S. Mirza, and J. K. Ladha (Dordrecht: Kluwer Academic Publishers), 25–37.

Rattan, R. K., and Shukla, L. M. (1991). Influence of different zinc carriers on the utilization of micronutrients by rice. *J. Indian Soc. Soil Sci.* 39, 808–810.

Reetha, S., Bhuvaneswari, G., Thamizhiniyan, P., and Mycin, T. R. (2014). Isolation of indole acetic acid (IAA) producing rhizobacteria of *Pseudomonas fluorescens* and *Bacillus subtilis* and enhance growth of onion (*Allim cepa. L). Int. J. Curr. Microbiol. App. Sci.* 3, 568–574.

Saravanan, V. S., Madhaiyan, M., and Thangaraju, M. (2007). Solubilization of zinc compounds by the diazotrophic, plant growth promoting bacterium *Gluconacetobacter diazotrophicus*. *Chemosphere* 66, 1794–1798. doi: 10.1016/j.chemosphere.2006.07.067

Saravanan, V. S., Kumar, M. R., and Sa, T. M. (2011). "Microbial zinc solubilization and their role on plants," in *Bacteria in Agrobiology: Plant Nutrient Management*, ed D. K. Maheshwari (Berlin: Springer), 47–63.

Schwyn, B., and Neilands, J. B. (1987). Universal chemical assay for the detection and determination of siderophores. *Anal. Biochem.* 160, 47–56. doi: 10.1016/0003-2697(87)90612-9

Selvakumar, G., Joshi, P., Nazim, S., Mishra, P. K., Bisht, J. K., and Gupta, H. S. (2009). Phosphate solubilization and growth promotion by *Pseudomonas fragi* CS11RH1 (MTCC 8984), a psychrotolerant bacterium isolated from a high altitude Himalayan rhizosphere. *Biologia* 64, 239–245. doi: 10.2478/s11756-009-0041-7

Sharma, S. K., Sharma, M. P., Ramesh, A., and Joshi, O. P. (2012). Characterization of zinc solubilizing *Bacillus* isolates and their potential to influence zinc assimilation in soybean seeds. *J. Microbiol. Biotechnol.* 22, 352–359. doi: 10.4014/jmb.1106.05063

Sierra, G. (1957). A simple method for the detection of lipolytic activity of micro-organisms and some observations on the influence of the contact between cells and fatty acid substrates. *Anton. Leeuw. Int. J. G.* 23, 15–22. doi: 10.1007/BF02545855

Tan, S., Han, R., Li, P., Yang, G., Li, S., Zhang, P., et al. (2015). Over-expression of the MxIRT1 gene increases iron and zinc content in rice seeds. *Transgenic Res.* 24, 109–122. doi: 10.1007/s11248-014-9822-z

Tan, Z. Y., Xu, X. D., Wang, E. T., Gao, J. I., Romero, E. M., and Chen, W. X. (1997). Phylogenetic and genetic relationships of *Mesorhizobium tinshanense* and related rhizobia. *Int. J. Syst. Bacteriol.* 47, 874–879. doi: 10.1099/00207713-47-3-874

Tank, N., and Saraf, M. (2009). Enhancement of plant growth and decontamination of nickel-spiked soil using PGPR. *J. Basic Microbiol.* 49, 195–204. doi: 10.1002/jobm.200800090

Tavallali, V., Rahemi, M., Eshghi, S., Kholdebarin, B., and Ramezanian, A. (2010). Zinc alleviates salt stress and increases antioxidant enzyme activity in the leaves of pistachio (*Pistacia vera* L. *'Badami')* seedlings. *Turk. J. Agr. Forest*. 34, 349–359. doi: 10.3906/tar-0905-10

Tien, T. M., Gaskins, M. H., and Hubbel, D. H. (1979). Plant growth substances produced by *Azospirillum brasilense* and their effect on the growth of pearl millet (*Pennisetum americanum* L.). *Appl. Environ. Microbiol.* 37, 1016–1027.

Vaid, S. K., Kumar, B., Sharma, A., Shukla, A. K., and Srivastava, P. C. (2014). Effect of zinc solubilizing bacteria on growth promotion and zinc nutrition of rice. *J. Soil Sci. Plant Nut*. 14, 889–910. doi: 10.4067/S0718-95162014005000071

Verma, P., Yadav, A. N., Khannam, K. S., Panjiar, N., Kumar, S., Saxena, A. K., et al. (2015). Assessment of genetic diversity and plant growth promoting attributes of psychrotolerant bacteria allied with wheat (*Triticum aestivum*) from the northern hills zone of India. *Ann. Microbiol.* 65, 1885–1899. doi: 10.1007/s13213-014-1027-4

Vincent, J. M. (1970). *A Manual for the Practical Study of the Root-nodule Bacteria*. Great Britain: Burgess and Son Ltd.

Wakatsuki, T. (1995). Metal oxidoreduction by microbial cells. *J. Ind. Microbiol.* 14, 169–177. doi: 10.1007/BF01569900

Wang, Y., Yang, X., Zhang, X., Dong, L., Zhang, J., Wei, Y., et al. (2014). Improved plant growth and Zn accumulation in grains of rice *(Oryza sativa* L.) by inoculation of endophytic microbes isolated from a Zn hyperaccumulator, *Sedum alfredii* H. *J. Agric. Food Chem*. 62, 1783–1791. doi: 10.1021/jf404152u

Weaver, P. F., Wall, J. D., and Gest, H. (1975). Characterization of *Rhodopseudomonas capsulata*. *Arch. Microbiol*. 105, 207–216.

White, P. J., and Broadly, M. R. (2005). Biofortifying crops with essential mineral elements. *Trends Plant Sci*. 10, 586–593. doi: 10.1016/j.tplants.2005.10.001

Zuo, Y., and Zhang, F. (2009). Iron and zinc biofortification strategies in dicot plants by intercropping with gramineous species. A review. *Agron. Sustain. Dev*. 29, 63–71. doi: 10.1051/agro:2008055

Conflict of Interest Statement: The authors declare that the research was conducted in the absence of any commercial or financial relationships that could be construed as a potential conflict of interest.

frontiers in Plant Science

REVIEW
published: 22 December 2017
doi: 10.3389/fpls.2017.02202

Biostimulant Action of Protein Hydrolysates: Unraveling Their Effects on Plant Physiology and Microbiome

Giuseppe Colla[1], Lori Hoagland[2], Maurizio Ruzzi[3], Mariateresa Cardarelli[4], Paolo Bonini[5], Renaud Canaguier[6] and Youssef Rouphael[7]*

[1] Department of Agricultural and Forestry Sciences, University of Tuscia, Viterbo, Italy, [2] Department of Horticulture and Landscape Architecture, Purdue University, West Lafayette, IN, United States, [3] Department for Innovation in Biological, Agrofood and Forest Systems, University of Tuscia, Viterbo, Italy, [4] Consiglio per la Ricerca in Agricoltura e l'Analisi dell'Economia Agraria, Centro di Ricerca Orticoltura e Florovivaismo, Pontecagnano, Italy, [5] NGA Laboratory, Tarragona, Spain, [6] Nixe, Valbonne, France, [7] Department of Agricultural Sciences, University of Naples Federico II, Portici, Italy

OPEN ACCESS

Edited by:
Angeles Calatayud, Instituto Valenciano de Investigaciones Agrarias, Spain

Reviewed by:
Sergio Mugnai, Erasmus University Rotterdam, Netherlands
Ana Quiñones, Instituto Valenciano de Investigaciones Agrarias, Spain

***Correspondence:**
Giuseppe Colla
giucolla@unitus.it

Specialty section:
This article was submitted to Crop and Product Physiology, a section of the journal Frontiers in Plant Science

Received: *02 November 2017*
Accepted: *14 December 2017*
Published: *22 December 2017*

Citation:
Colla G, Hoagland L, Ruzzi M, Cardarelli M, Bonini P, Canaguier R and Rouphael Y (2017) Biostimulant Action of Protein Hydrolysates: Unraveling Their Effects on Plant Physiology and Microbiome. Front. Plant Sci. 8:2202. doi: 10.3389/fpls.2017.02202

Plant-derived protein hydrolysates (PHs) have gained prominence as plant biostimulants because of their potential to increase the germination, productivity and quality of a wide range of horticultural and agronomic crops. Application of PHs can also alleviate the negative effects of abiotic plant stress due to salinity, drought and heavy metals. Recent studies aimed at uncovering the mechanisms regulating these beneficial effects indicate that PHs could be directly affecting plants by stimulating carbon and nitrogen metabolism, and interfering with hormonal activity. Indirect effects could also play a role as PHs could enhance nutrient availability in plant growth substrates, and increase nutrient uptake and nutrient-use efficiency in plants. Moreover, the beneficial effects of PHs also could be due to the stimulation of plant microbiomes. Plants are colonized by an abundant and diverse assortment of microbial taxa that can help plants acquire nutrients and water and withstand biotic and abiotic stress. The substrates provided by PHs, such as amino acids, could provide an ideal food source for these plant-associated microbes. Indeed, recent studies have provided evidence that plant microbiomes are modified by the application of PHs, supporting the hypothesis that PHs might be acting, at least in part, via changes in the composition and activity of these microbial communities. Application of PHs has great potential to meet the twin challenges of a feeding a growing population while minimizing agriculture's impact on human health and the environment. However, to fully realize the potential of PHs, further studies are required to shed light on the mechanisms conferring the beneficial effects of these products, as well as identify product formulations and application methods that optimize benefits under a range of agro-ecological conditions.

Keywords: abiotic stress, amino acids, enzymatic hydrolysis, microbial inoculants, peptides, product quality, physiological mechanisms, sustainable agriculture

INTRODUCTION

In the coming years, agriculture must meet the twin challenge of feeding a growing global population, while simultaneously minimizing agriculture's impact on human health and the environment (Searchinger, 2013). To meet global demand several solutions have been proposed, that focus on breeding varieties with greater yield potential, however, this one-size-fits-all solution leads to limited benefits, especially given that limits of the genetic potential of staple crops have almost been reached. Alternatively, it has been hypothesized that to increase the reliability and stability of agricultural crop yield, optimizing crop management and improving resource use efficiency (i.e., fertilizers and water) under different agro-ecological conditions, holds the key to sustainably increase yield across different environments and years. In other words produce *'more with less.'*

An innovative technology with promising application potential in confronting these critical challenges entails the use of protein hydrolysates (PHs). Application of PHs as biostimulants on a wide range of horticultural and agronomic crops has been acclaimed. PHs are *'mixtures of polypeptides, oligopeptides and amino acids that are manufactured from protein sources using partial hydrolysis'* (Schaafsma, 2009). They are available as liquid extracts or in soluble powder and granular form, and may be side-dressed near the root or applied as foliar sprays (Colla et al., 2015a). PHs are mainly produced by chemical (acid and alkaline hydrolysis), thermal and enzymatic hydrolysis of a wide range of both animal wastes and plant biomass (Colla et al., 2015a; du Jardin, 2015; Halpern et al., 2015). Animal residues include animal epithelial or connective tissues such as leather by-products, blood meal, fish by-products, chicken feathers and casein, whereas biomass of plant origin includes legume seeds, alfalfa hay, corn wet-milling and vegetable by-products (Colla et al., 2015a). In particular, PHs coming from by-products of vegetables and the corn wet-milling industry are gaining popularity among the scientific community and commercial enterprises, since they could represent a sustainable, economical and eco-friendly solution to the problem of waste disposal (Pecha et al., 2012; Baglieri et al., 2014). Currently, most of the market for PHs biostimulants accounts for animal-derived proteins procured through acid hydrolysis, with the remainder coming from enzymatic hydrolysis of plant-derived proteins (Colla et al., 2015a). On a global scale, most of the PHs for agricultural use are produced from companies located in Italy, Spain, United States, China and India. Some of these companies in Europe and East Asia were developed by leather/meat industries as a way to valorize their by-products through the production of biostimulants and fertilizers. Moreover, in the last years some companies introduced plant-derived PHs in the United States, European and Asian market; these plant-derived PHs are gaining greater acceptance by farmers due to their richness in bioactive compounds and their great efficacy in enhancing crop performances.

In many cases, PHs have been demonstrated to play key roles as biostimulants through the modulation of plant molecular and physiological processes that trigger growth, increase yield and alleviate the impact of abiotic stress on crops (Calvo et al., 2014; Yakhin et al., 2017). These include salinity, heavy metal, thermal, nutrient stress, and water stress (Botta, 2013; Cerdán et al., 2013; Colla et al., 2013, 2014; Ertani et al., 2013; Lucini et al., 2015; Rouphael et al., 2017a). Direct effects of PHs on plants include stimulation of carbon and nitrogen metabolism, as well as regulation of N uptake mediated by key enzymes involved in the N assimilation process and regulation of the activity of three enzymes involved in the tricarboxylic acid cycle (citrate synthase, isocitrate dehydrogenase and malate dehydrogenase) (Colla et al., 2015a; du Jardin, 2015; Nardi et al., 2016). PHs could also interfere with hormonal activities, due to the presence of bioactive peptides (Colla et al., 2014, 2015a). Several studies have demonstrated that many commercial products obtained from PHs elicited hormone-like activities (auxin and gibberellins), promoting root and shoot growth, and thus crop productivity (Ertani et al., 2009; Matsumiya and Kubo, 2011; Colla et al., 2014; Lucini et al., 2015).

In addition, to the direct effect of PHs, indirect effects on growth and plant nutrition have been also demonstrated when PHs were applied to soils and plants (du Jardin, 2015). Foliar and root applications have been shown to enhance the uptake and use efficiency of both macro and micronutrients (Ertani et al., 2009; Colla et al., 2015a; Halpern et al., 2015). Improved nutrient uptake performance of PH-treated plants has been mostly associated with modifications of root architecture (density, length and number of lateral roots), as well as to an increase of nutrient availability in the soil solution resulting from complexation of nutrients by peptides and amino acids, and enhanced microbial activity (Colla et al., 2015a; du Jardin, 2015). In addition to the positive effects of PH-treated plants, there are several authors (Ruiz et al., 2000; Cerdán et al., 2009; Lisiecka et al., 2011) reporting phytotoxicity effects as well as suppression of growth related to the use of animal-derived PHs of fruiting crops. This phenomenon is known as 'general amino acid inhibition' (Bonner and Jensen, 1997) and it is due to excessive leaf uptake of free-amino acids, which causes intracellular amino acid imbalance, energy drain due to active transport of amino acids, inhibition of nitrate uptake, and increase of cell susceptibility to apoptosis.

Despite the efforts of scientists to understand the biostimulant properties of PHs, knowledge about target metabolic pathways, mechanisms of action elicited by the application of PHs is far from unraveled. Moreover, the biostimulant action of PHs can vary depending on their origin and characteristics, species, cultivars, phenological stages, growing conditions, concentration, time and mode (leaf versus root) of application, solubility, and leaf permeability (Colla et al., 2015a). The penetration of active ingredients (amino acids and peptides) into internal structures of PH-treated plants is crucial since PHs based biostimulant are usually foliarly applied (Colla et al., 2015a; Yakhin et al., 2017).

The biostimulant activity observed in response to the application of PHs could be acting, at least in part, indirectly through a microbially mediated enhancement of plant health (Colla et al., 2014). It is now commonly accepted that microbes can improve plant fitness by altering physiological and development processes, resulting in greater nutrient and water uptake as well as enhanced resilience against environmental stressors (Philippot et al., 2013). Many of these interactions have

been found to occur in the rhizosphere, which encompasses a limited soil volume confined to and affected by the root system. More recently, microbes that promote growth and help plants to withstand biotic and abiotic stress have also been found in the phyllosphere, which covers plant leaf surfaces. It has been estimated that the number of microbial cells living on and within plant tissues outnumbers plant cells, and this community of microorganisms is now commonly referred to as part of the 2nd genome of the plant, or its microbiome (Berendsen et al., 2012). The organic molecules in PHs could be adopted as a source of carbon and nitrogen for the microbes residing in the rhizosphere and phyllosphere of plants. Moreover, microbes are generally more competitive for amino acids than plants (Moe, 2013), indicating that much of the organic materials provided by PHs could be utilized or altered by microbes before they can directly influence or be taken up by plants. If this proves to be correct, understanding how to modify the plant microbiome with PHs has potential to enhance their benefits and further improve plant productivity.

The aim of the current review is to provide an updated scientific overview of the effects of PHs on growth, productivity, and quality of agricultural commodities; moreover, it sheds light on the possible modes of action and mechanisms mediating these effects. The impact of PHs application on the primary and secondary metabolism, and physiology, the resilience to adverse chemical soil conditions and environmental stresses, as well as the effects of PHs on the plant microbiome are also covered.

CHEMICAL CHARACTERISTICS

Protein hydrolysates contain mainly peptides and free amino acids (Calvo et al., 2014). PHs can also contain carbohydrates and negligible quantities of mineral elements, phenols, phytohormones and other organic compounds (Ertani et al., 2014; Colla et al., 2015a). Chemical characteristics of PHs vary depending on source of proteins (e.g., collagen from leather by-products, fish by-products, legume seeds, alfa-alfa biomass), and production process (chemical and/or enzymatic hydrolysis). Collagen-derived PHs composition is dominated by amino acids like glycine and proline as well as aspartic and glutamic acids in legume-derived and fish-derived PHs (Ertani et al., 2009, 2013; Chalamaiah et al., 2012; Colla et al., 2015a). Moreover, collagen derived-PHs typically contain significant amounts of hydroxyproline and hydroxylysine, which can be used as markers for this type of PHs (Colla et al., 2015a).

Animal derived-PHs are usually produced through chemical hydrolysis with the use of acids (hydrochloric and sulphuric acid) at high temperature (>121°C) and pressure (>220.6 kPa). Because acid hydrolysis is very aggressive, the resulting product is composed of a large amount of free amino acids and to a lesser extent by soluble peptides. During the acid hydrolysis some amino acids like tryptophan, cysteine, serine and threonine are partially or totally destroyed and many other amino acids are converted from the L-form to D-form (racemisation) thus losing their biological activity (Colla et al., 2015a). Since plant derived-PHs are produced through a more gentle method (enzymatic hydrolysis using proteolytic enzymes and temperature below 60°C), the resulting PHs contain higher peptides:free amino acids ratio, and proportion of L-amino acids in comparison with those obtained by chemical hydrolysis. The peptides molecular weight varies from several hundred to thousands of Daltons, with low molecular weight peptides being more biologically active (Quartieri et al., 2002). Biologically active peptides have been isolated and chemically characterized from PHs, especially those derived from plant materials. For instance, a short peptide (12 amino acids) called 'root hair promoting peptide' has been identify in a soybean-derived PHs (Matsumiya and Kubo, 2011) and in the commercial legume derived-PHs 'Trainer®'. Many other bioactive peptides acting as signaling molecules in plant defense, growth, and development have been discovered in plant tissues (e.g., systemins, phytosulfokines, clavata3) (Ryan et al., 2002), and may also been present in PHs.

EFFECTS ON PHYSIOLOGICAL AND AGRONOMIC TRAITS OF CROPS

Germination and Seedling Growth

Several technologies have been proposed to enhance sowing and seedling establishment under a wide range of environmental conditions. These technologies include seed conditioning and priming as well as seed coating. Seed coated with hydrophilic materials and hydro-absorbers can protect young seedlings from pests, diseases, fungi and low temperature (Gorim and Asch, 2012). Seed coatings may also contain macro and micronutrients (Farooq et al., 2012), herbicides (Rushing et al., 2013), growth regulators (Halmer, 2004) and beneficial microorganisms (Colla et al., 2015b).

In recent years, several commercial enterprises were interested in whether the biostimulant material could be applied as a component of a seed coating blend. In a recent study, Amirkhani et al. (2016) and co-workers showed that broccoli (*Brassica oleracea* L.) seed coating formulations (soy flour/cellulose fiber/diatomaceous earth, termed as SCD) using soy flour at a concentration of 10% had greater seedling shoot and root growth compared to uncoated seeds. In contrast, germination was negatively affected by seed coating with SCD likely due to the fact that the treatment binder may have acted as a barrier for water uptake and gas exchange (Mucke, 1988; Hill, 1999). However, after 1 month in the greenhouse, the fresh and dry biomass, plant height, leaf area, Soil Plant Analyses Development (SPAD) index, as well as total nitrogen of broccoli plantlets were always higher in plants with seed coatings of 30, 40, and 50% soy flour in comparison to the uncoated control. The authors concluded that using soy flour as seed coating materials improved several growth characteristics by triggering nitrogen uptake, assimilation and translocation, by enhancing some key enzymes involved in nitrogen metabolism. Colla et al. (2014) conducted laboratory bioassays using the PH 'Trainer®' containing 31% of soluble peptides and free amino acids. In their study, treatment of detached corn (*Zea mays* L.) coleoptiles with the plant-derived PH having elicited an accelerated coleoptile elongation in comparison to the non-treated control (i.e.,

deionized water). Moreover, no significant effects were recorded on coleoptile elongation rate among the four PH concentrations studied (0.375, 0.75, 1.5, and 3 ml/L) and IAA treatment. The authors concluded that a significant auxin-like activity occurred using the plant-derived PH 'Trainer®,' likely due to the presence of tryptophan, a major precursor for IAA biosynthesis and bioactive peptides. Like auxins, gibberellins are known to improve cell elongation and function as chemical signals promoting the biosynthesis of α-amylase, which is important during germination (Parrado et al., 2008). The application of PH 'Trainer®' at four doses (0.375, 0.75, 1.5, and 3 ml/L) enhanced the shoot length of gibberellin-dwarf pea (*Pisum sativum* L.) plants by 33% compared with the control treatment, with no significant differences between the four dose rates, providing clear evidence of gibberellin-like activity (Colla et al., 2014). The results of Colla et al. (2014) confirm a previous report by Ghosh et al. (2010), who provided evidence that wheat peptides mimic hormonal activity like that of gibberellins.

In addition to the beneficial role of plant-derived PHs on plant growth, the positive effects of animal-derived protein application also have been demonstrated. Gelatin, an animal-derived protein, applied as capsules placed near the seeds, has been shown to act as a biostimulant on greenhouse-grown cucumber (*Cucumis sativus* L.). Application of these gelatin capsules increased fresh and dry weight biomass, leaf area and nitrogen content of 2-week old plants compared with seeds sown without gelatin capsules (Wilson et al., 2015). Changes in plant biomass and nitrogen in response to the gelatin capsules were correlated with an up-regulation of both amino acids and N transporter genes and the xenobiotic detoxification system. The authors concluded that these genes, and their possible transcriptional regulation through the two transcription factors, could be an important mechanism regulating improved plant growth following gelatin seed treatment. The use of collagen hydrolysate in wheat seed treatment showed a stimulation of seed metabolism by increasing endogenous gibberellic acid, and an enhancement of emergence and seedling biomass, and a reduction of abnormal seedlings (Gaidau et al., 2015). Furthermore, Gaidau et al. (2013) showed that cereal seed treatments with collagen-based hydrolysate mixes with fungicides and insecticides reduced pesticide needs, with diminished environmental impact and reduced cost of seed treatment.

Similarly, a vegetal-PH based product (BioST VPH, Albaugh, LLC, Valdosta, GA, United States) containing a root hair promoting peptide has been successfully used as a seed treatment to stimulate early root growth, crop stress tolerance and promote adhesion of fungicides/micronutrients on seed surface in corn (Zea mays L.) and soybean [*Glycine max* (L.) Merr.] (Bonini et al., 2017).

Moreover, the use of these compounds as seed treatments can provide additional benefits, such as the reduction of dust formation and prevention of microbial inoculant detachment from the seed surface during handling. The adhesive properties of PHs are primarily related to the 'sticky' small cationic peptides. A recent patent (n. 201531523/3 presented on October 22, 2015) proposed by Agrotecnologías Naturales SL (Tarragona, Spain) showed that a soybean-derived PH was able to more than double the number of polyethylene microspheres (having 75 – 90 μm of diameter, and used as substitute for arbuscular mycorrhizal fungi spores) that stuck to the seed surface of wheat (*Triticum aestivum* L.), corn (Zea mays L.) and soybean [*Glycine max* (L.) Merr.] in comparison with water. Moreover, mechanical vibration of coated seeds, showed that adding the soybean-derived PH to the microsphere/water suspension increased the adhesion strength of the microspheres by about 96, 36, and 21% in wheat, corn and soybean seeds, respectively.

Plant Growth and Productivity

Several experimental studies testing the action of PHs under both open-field and controlled conditions, have demonstrated that they stimulate shoot and root biomass, resulting in increased productivity of several crops such as corn, kiwifruit, lettuce, lily, papaya, passiofruit, pepper and tomato (Schiavon et al., 2008; Ertani et al., 2009; Colla et al., 2014, 2015a, 2017; Halpern et al., 2015; Nardi et al., 2016). Foliar application of animal and plant-derived PHs has also been shown to promote the vegetative growth and yield of several fruit trees (Colla et al., 2015a). For instance, papaya (*Carica papaya* L.) plants sprayed at a 30-day interval with animal-derived PH 'Siapton' (i.e., increased crop productivity by 22% in comparison to the untreated control treatment (Morales-Pajan and Stall, 2004). Similarly, in banana (*Musa* spp.), foliar spray of banana plants with hydrolyzed poultry feather processing waste, condensed the time to harvest by 4 weeks and enhanced several yield components such as the number of hands per bunch and the mean bunch weight in comparison to untreated plants. Stimulation of banana crop performance and growth in these experiments was correlated with greater reducing sugars and chlorophyll concentrations in the PH-treated plants.

Greenhouse applications of an animal-derived PH 'Siapton,' and carob germ hydrolysate enhanced both plant height as well as number of flowers per plant in tomato (*Solanum lycopersicum* L.) compared with untreated plants, though only those sprayed with carob germ hydrolysate improved the number of fruit per plant after 18 weeks (Parrado et al., 2008). In greenhouse tomatoes, Koukounararas et al. (2013) showed that root or foliar spray of a PH commercial product, Amino 16®, containing 11.3% L-amino acids, enhanced yield by increasing both fruit number and mean weight, irrespective of fertilization rate. Similarly, foliar applications of the legume-derived PH 'Trainer®' at 5.0 ml L^{-1} increased marketable yield of two fresh-market tomato cultivars by modulating yield components differently depending on the cultivar, with higher number of fruits in Akyra and greater fruit mean weight in Sir Elyan (Rouphael et al., 2017b). Increasing the dose of plant-derived PH 'Trainer®' from 0 to 10 ml/L caused significant increase in shoots, root and total dry biomass, greenness readings as well as leaf N content (Colla et al., 2014) by 19.5, 27.5, 20.5, 15.1, and 21.5%, respectively, but there were no differences observed between the biostimulant at concentrations (5 and 10 ml/L). In the same study, enhancement in growth and nitrogen metabolism in PH-treated tomato plants was attributed to stimulation of nitrogen uptake and assimilation, which may improve net CO_2 assimilation and enhance the translocation of photosynthates (i.e., soluble sugars) via the phloem to potential

sinks (Ertani et al., 2009). A presumed mechanism involved in the stimulation of nitrogen assimilation in response to PHs, is the increase in the activity of two key enzymes, nitrate reductase and glutamine synthetase (Ertani et al., 2009). Another possible mechanism involved in the biostimulant effect of PH-treated plants could be related to stimulation of a more vigorous root system, which may enhance the efficiency of water and nutrient uptake, thus boosting crop yield. In a recent rooting experiment of tomato cuttings, Colla et al. (2014) observed that root dry weight, root length and surface area were greater in PH-treated plants in comparison to an untreated control, by 35, 24, and 26%. Increase in nitrogen assimilation and pigment synthesis in response to PH treatments has been also attributed to auxin as well as gibberellin-like activities (Ertani et al., 2009; Nardi et al., 2009; Colla et al., 2014). The biostimulant effects of low molecular size peptides and free amino acids have been also demonstrated by Matsumiya and Kubo (2011), who reported an increase of 25% in fresh weight of *Brassica rapa* with the addition of 12 mg-peptides/kg soil of degraded soybean meal products. The growth of eggplant, tomato and Indian mustard were also promoted by the addition of plant growth promoting peptides derived from soybean (Matsumiya and Kubo, 2011). In addition to stimulation of fresh weight, application of degraded soybean meal products increased the root hairs characteristics (number and length) of *Brassica oleracea* L., *Lactuca sativa* L., *Trifolium incarnatum* L., and *Gypsophila elegans* M. Bieb., thus favoring the uptake of water and nutrients via an increase in root surface. Similarly, Ugolini et al. (2015), reported that a sunflower meal hydrolysate containing free amino acids, with auxin-like but not gibberellins-like activity, stimulated root elongation, and increased transplanting success and crop productivity, indicating that this product could be an effective biostimulant in the agricultural field.

Protein hydrolysates have also been demonstrated to improve the productivity of ornamental plants. Application of two PHs derived from animal epithelia and alfalfa increased the diameter of flower buds, leaf area, stem quality and root biomass of lily (*Lilium longiflorum* Thunb. × *Lilium elegans* Thunb.) in comparison to untreated plants (De Lucia and Vecchietti, 2012). In contrast to studies demonstrating positive effects of PHs on plants, other experimental studies have found that foliar or root application of PHs has been minimal or non-significant (Kirn et al., 2010; Kunicki et al., 2010; Gajc-Wolska et al., 2012; Grabowska et al., 2012). For example, in these studies application of the animal-derived PH product 'Siapton,' had no effect on yield of endive, spinach, carrot and okra grown under open field conditions. The contrasting results may be due to the different origin of PHs (animal or vegetal origin), PH production process (chemical or enzymatic hydrolysis), plant species, rates of application and environmental conditions.

The amelioration of abiotic stress effects is the most commonly referred to benefit in relation to the use of biostimulants, since 60–70% of the yield losses in agriculture are estimated to be attributable to abiotic stresses (Rouphael et al., 2017c; Yakhin et al., 2017). Application of 'Stressal,' a commercial formulation of animal-derived PH, alleviated salt stress on persimmon (*Diospyros kaki* L.f.) by lowering chloride uptake and translocation to aerial parts, thus reducing leaf necrosis symptoms (Visconti et al., 2015). Greater tolerance to salt stress was associated with the composition of the PH, particularly compatible solutes such as proline and glycine betaine. When hydrolysate-based biostimulants from alfalfa containing triacontanol as well as inodole-3-acetic acid, were applied to maize under high salinity conditions, plants were better able to withstand salinity stress (Ertani et al., 2011). Under saline stress conditions, biostimulant-treated plants exhibited higher potassium and proline concentrations than untreated controls. In a similar experiment by the same authors, an alfalfa hydrolysate applied to maize grown in soilless culture under saline conditions also improved plant biomass and increased leaf proline, phenylalanine ammonia-lyase activity as well as gene expression relative to salt-stressed controls (Ertani et al., 2013). Lucini et al. (2015) showed that substrate drench, and to a higher extent foliar spray plus substrate drench applications of a plant-derived PH biostimulant product 'Trainer®,' helped plants maintain higher photochemical activity of the photosystem II, and obtain better nutritional status in lettuce (*Lactuca sativa* L.) shoot tissues under 25 mM NaCl, resulting in greater crop performance. The authors concluded that the potential for plants to withstand salinity stress in response to PH treatment, involved processes related to oxidative stress mitigation, change of hormonal balance, as well as production of secondary metabolites including glucosinolate, sterols and terpenes. In a similar experiment, Rouphael et al. (2017a) reported that the combination of a microbial biostimulant product 'Click Horto' (containing endophytic fungi such as *Rhizophagus intraradices* and *Trichoderma atroviride*) in combination with PH 'Trainer®,' induced a significant increase in crop productivity. Positive effects were associated with an increase in antioxidant enzymes activities (CAT and GPX), chlorophyll biosynthesis and improved mineral composition, likely through a stimulation of root morphology traits like total root length and root density. Cerdán et al. (2013) also demonstrated that the application of PH containing amino acids, particularly those derived from plant origin, enhanced tomato seedling growth under alkaline conditions due to an increase in leaf and root Fe reductase activities when applied to roots.

The potential for PH application to minimize the negative effects of thermal stress in several vegetable crops and perennial ryegrass have also been highlighted by several authors (Marfà et al., 2009; Botta, 2013). In the first experiment to investigate this potential benefit, the application of hydrolysates coming from animal hemoglobin did not improve strawberry (*Fragaria* × *ananassa* Duch.) plant survival following cold stress, through some growth promotion was recorded under non-cold stress conditions (Marfà et al., 2009). In contrast, enhancing plant tolerance to sub- and supra-optimal temperature conditions was observed in lettuce and ryegrass (*Lolium perenne* L.) when the commercial biostimulant 'Terra-Sorb foliar' containing amino acids was applied (Botta, 2013). In these experiments, PH-treated lettuce plants subjected to three cold stress treatments exhibited higher fresh weight compared to untreated plants, along with higher stomatal conductance, thus improving productivity. Moreover, PH-treat ryegrass plants subjected

to high temperatures (36°C) had improved photosynthetic efficiency, levels of chlorophylls and carotenoids over control plants.

Protein hydrolysates can also help plants to perform better under low nutrient availability through an increase of nutrient use efficiency. In fact, Colla et al. (2013) demonstrated that weekly foliar applications of 'Trainer®,' at a dose of 2.5 ml/L increased the yield, greenness readings (i.e., SPAD index) and N uptake of baby lettuce plants by 50%, 11% and 11%, respectively, under reduced nutrient solution concentration (10% of standard solution). Thus, application of PH-biostimulants could be considered an effective tool for obtaining high productivity with lower impact on the environment. Finally, according to scientific literature, some key amino acids (e.g., asparagine, cysteine and glutamine) and peptides (e.g., glutathione and phytochelatins), could play an important role in the tolerance of plants to a range of toxic and heavy metals (Cu, Zn, As, Cd, and Ni) through metal chelation and binding (Sharma and Dietz, 2009; Sytar et al., 2013).

Quality of Fruits and Vegetables

Over the past 20 years demand for high quality fruits and vegetables has been on the rise, in response to growing interest of consumers in healthy eating (Kyriacou et al., 2016, 2017). As reported in the previous sections, PHs have been shown to trigger several physiological mechanisms under optimal and sub-optimal conditions, stimulating the production and accumulation of specific molecules and secondary metabolites (i.e., ascorbate, tocopherols, carotenoids, glucosinolates). These metabolites perform a crucial role in supporting plant growth under suboptimal soil and ambient conditions, moreover such molecules confer an added value in promoting human well-being and longevity (Erba et al., 2013).

A significant improvement in protein, total phenolics, flavonoids, as well as antioxidant activity was observed when banana plants where fertilized with feather degradation products containing both amino acids and peptides (Gurav and Jadhav, 2013). This data confirmed results of earlier studies, which found an increase in phenols in various plant species, with the addition of organic wastes (McGrath et al., 1994). In red grape (*Vitis vinifera* L.), application of enzymatically treated vegetable extract coming from agricultural wastes increased the total phenolic and anthocyanin concentration by 22 and 70% respectively, over control plants (Parrado et al., 2007).

Ertani et al. (2014) conducted a greenhouse pot experiment with the goal of assessing the effects of two rates of biostimulants, one derived from alfalfa plants (25 and 50 ml/L) and another from red grapes (50 and 100 ml/L), on nutraceutical properties of *Capsicum chinensis* L. Results of these studies indicated that green pepper fruits of PH-treated plants had high concentrations of chlorogenic acid, and antioxidant activity, whereas both alfalfa and red grape PH-treated red pepper fruits were highly enriched with capsaicin. High-resolution magic-angle spinning-nuclear magnetic resonance spectra of red pepper fruits indicated that there were high amounts of $NADP^+$ in treated plants from both PH sources, while red grape-PH treatment improved glucose, ascorbate, thymidine and other high molecular weight species (Ertani et al., 2014).

In a greenhouse tomato trial, foliar applications of a legume-derived PH 'Trainer®' enhanced antioxidant activities, soluble solids, mineral composition (K and Mg) as well as bioactive molecules such as lycopene and ascorbic acid, thereby increasing the nutritional and functional quality of the tomato fruits (Rouphael et al., 2017b). Similar findings were also reported by Colla et al. (2017) in another greenhouse tomato trial using the same PH.

The beneficial effects of PH-biostimulant was also observed on the phytochemical profile of lemon balm (*Melissa officinalis* L.), an important aromatic plant (Mehrafarin et al., 2015). The authors reported that a foliar application of commercial formulations of Aminolforte and Fosnutren at 2 l/ha, increased contents of citronellal, neral, deltacadinene, germacrene, and geranial compared to untreated plants.

PH application has also been shown to reduce nitrates in leafy vegetables, which are noted for their high nitrate accumulation and potential to harm human health when provided excessive consumption of vegetable greens (Amr and Hadidi, 2001). For instance, Liu and Lee (2012) demonstrated that the application of mixed amino acids could substantially reduce nitrate accumulation in several leafy vegetables such as lettuce, rocket (*Eruca sativa* Mill.), Swiss chard (*Beta vulgaris* var. *cicla* L.) and spinach (*Spinacea oleracea* L.). A negative correlation was observed between the accumulation of nitrates in lettuce leaves and the application of Amino 16 (Tsouvaltzis et al., 2014). A reduction in nitrate accumulation with the use of single amino acids was also observed on hydroponically grown pack choi (*Brassica rapa* subsp. *chinensis* L.) (Wang et al., 2007). The potential of PHs in preventing the high concentration of nitrates could be attributed to the up-regulation of several metabolic pathways involved in nitrogen metabolism, in particular nitrite and nitrate reductase as well as glutamate synthase and glutamine synthetase activities (Calvo et al., 2014; Colla et al., 2015a).

EFFECTS ON MICROBIOME

Plant-associated microbes are increasingly being recognized for their potential to improve plant fitness by altering physiological and development processes (Philippot et al., 2013). Many of these interactions occur in the rhizosphere, a narrow zone of soil that surrounds and is influenced by plant roots, or in the phyllosphere, which covers plant leaf surfaces. The organic molecules in PHs could be used as a source of carbon, nitrogen and/or energy by the microbes residing in these unique habitats. Consequently, alteration of the composition and activity of plant microbiomes by PH's could be yet another mechanism responsible for the improvement in crop productivity by these products.

The Plant Microbiome

Plant-associated microorganisms have successfully coevolved with their host and are now known to play a crucial role in both crop growth and ecosystem functioning (Turner et al., 2013; Marin et al., 2017). To highlight the dependence of a plant on its microbiota at all stages of development, the concept of what constitutes an individual plant was redefined and plants are

now perceived as a "metaorganism" or "holobiont" (Bordenstein and Theis, 2015; Vandenkoornhuyse et al., 2015; Rosenberg and Zilber-Rosenberg, 2016) or, considering also the interactions with the environment and other organisms, as a "phytobiome" (Baltrus, 2017; Leach et al., 2017).

In nature, healthy and asymptomatic plants are not axenic organisms but host a complex microbial consortium comprising bacteria, fungi, protists and viruses, many of which interact with plants in various (beneficial, neutral or harmful) ways. These microbial communities can affect plant health and productivity (Berendsen et al., 2012; Berg et al., 2016); help the plant to overcome biotic or abiotic stresses (Vorholt, 2012; Bulgarelli et al., 2013); and prevent pathogen attack (Mendes et al., 2013). Recently, the use of high-throughput sequencing and microbial-specific databases have provided deep insights into the composition of above- and belowground compartments of various host plants, including *Arabidopsis thaliana* (Lundberg et al., 2012; Horton et al., 2014; Bai et al., 2015), barley (*Hordeum vulgare* L.; Bulgarelli et al., 2015), corn (Peiffer et al., 2013), grapevine (Zarraonaindia et al., 2015), lettuce (Williams and Marco, 2014), potato (*Solanum tuberosum* L.; İnceoğlu et al., 2011, 2012), tomato (Ottesen et al., 2013), rice (*Oryza sativa* L.; Knief et al., 2012), sugarcane (*Saccharum officinarum* L.; Yeoh et al., 2016), and soybean (Mendes et al., 2014). These studies have demonstrated that plants harbor different microbial communities specific for each organ and that there are conserved taxa that inhabit a given plant organ across multiple host species and environments (Müller et al., 2016). These studies indicate that the root microbiome of phylogenetically unrelated plant species is composed of only a few dominant phyla, mainly belonging to Proteobacteria, Actinobacteria, Bacteroidetes, and to a lesser extent, Firmicutes (Lundberg et al., 2012; Berg et al., 2016), whereas fungal communities appear to be subjected to greater variation, are more dependent on biogeography, plant species and compartment, and stochastic variations (Shakya et al., 2013). Consistent with this, Coleman-Derr et al. (2016) observed that geographic origin of the host was the major driving factor in fungal but not bacterial communities associated with cultivated and native agaves.

Bacteria also tend to be the most abundant microorganisms in phyllosphere communities. In particular, Proteobacteria, Firmicutes, and Actinobacteria often dominate the plant phyllosphere, with *Methylobacterium*, *Pseudomonas*, and *Sphingomonas* being among the most abundant genera at the leaf level in *A. thaliana*, soybean and grapevine (Delmotte et al., 2009; Zarraonaindia et al., 2015), and *Pseudomonas* and *Erwinia* (*Pantoea*) are the predominant taxa at the flower level, at least in grapevines (Zarraonaindia et al., 2015).

While the beneficial or detrimental effects of root-associated microbes have received considerable attention in recent years (Berendsen et al., 2012; Bulgarelli et al., 2013; Mendes et al., 2013; Philippot et al., 2013; Berg et al., 2016) the effects of epiphytes on plant health and productivity are not as well known. Like root-associated microbes, some epiphytic microbes have been demonstrated to promote plant growth via production of hormones (Wu et al., 2009; Ruzzi and Aroca, 2015) or synthesis of volatile organic compounds (VOCs), by biotransformation (Marmulla and Harder, 2014) or *de novo* biosynthesis (Schulz and Dickschat, 2007), that can have antimicrobial effects or serve as carbon sources for some microorganisms (Farré-Armengol et al., 2016). Plant pathogens can colonize the phyllosphere in the absence of any apparent infection (Vorholt, 2012), while other microbes such as some *Pseudomonas* and *Sphingomonas* species protect plants from pathogens by competing for limited nutrients, producing antibiotic compounds (Lindow and Brandl, 2003; İnnerebner et al., 2011; Ritpitakphong et al., 2016), and inducing systemic resistance (Conrath et al., 2006; Pieterse et al., 2012). Finally, the importance of the phyllospheric microbiota on the metabolic function of aromatic plant species has been recently analyzed in both *Sambucus nigra* L. and *Mentha piperita* Huds. del Rosario Cappellari et al. (2017) demonstrated that co-inoculation with selected *Pseudomonas* and *Bacillus* plant-growth promoting strains induced greater emissions of VOCs emission and synthesis of phenolic compounds in *M. piperita* plants. In contrast, Gargallo-Garriga et al. (2016) demonstrated that in *Sambucus nigra* L., suppression of phyllospheric microbial communities led to a decrease in several metabolites, such as citraconic acid, acetyl-CoA, isoleucine, as well as secondary compounds including terpenes and phenols.

In summary, a stable increase in plant productivity can be achieved if beneficial plant–microbiome relationships are established and maintained in the rhizosphere and phyllsophere. However, before this becomes a reality, greater understanding of factors that regulate these key plant-associated habitats is needed.

Plant–Microbial Habitats

In 1904, Lorenz Hiltner first coined the term "rhizosphere" and theorized that microbes inhabiting this plant-soil interface likely play a role in plant nutrition, growth promotion and suppression of plant pathogens (Hartmann et al., 2008). One hundred years later, it is now commonly accepted that the rhizosphere is one of the most dynamic and biologically active environments on earth, and microbes residing in this habitat are crucial for maintaining plant health in natural and managed ecosystems (Berendsen et al., 2012). Study of the rhizosphere and ways to manipulate this critical plant–soil interface to benefit plants in agricultural and horticultural systems has now become a prominent area of research.

While recent studies indicate that the composition of rhizosphere microbial communities is highly diverse, microbial taxa inhabiting this environment clearly differ from bulk soil (Berendsen et al., 2012; Philippot et al., 2013). This is likely due to differences in physicochemical characteristics between these two habitats. For example, nutrient and water availability as well as soil pH are modified by the presence of plant roots, and these conditions are likely to be important factors in the type of microbial taxa that can thrive in this environment. Moreover, plants roots actively and passively release up to 40% of their photosynthetically derived carbon via root exudates, mucilage, and sloughed off root cells (Bais et al., 2006). Release of these compounds signal and provide support for rhizosphere microbial communities. Composition of these carbon compounds, as well as rhizosphere microbial community structure, vary given plant species and genotype, root morphology, plant development and

maturation, and even location on the root (Berg and Smalla, 2009). Thus, while soil type, management history and climatic conditions arguably play a key role in shaping rhizosphere microbial communities since they influence the composition of microbial taxa available for colonization, it is clear that plants recruit specific microbial taxa and selectively shape this community via composition of these carbon compounds (Berg and Smalla, 2009).

Carbon compounds released from plant roots are made up of a mixture of low molecular weight (amino acids, organic acids, sugars and phenolics) as well as high molecular weight compounds (polysaccharides and proteins) (Badri et al., 2009). In particular, amino acids are the second most abundant compound released from plant roots, and the potential for rhizospheric microbes to utilize these compounds is thought to be a key characteristic of microbes residing in this habitat. In fact, over 80% of rhizosphere microbes have been found to possess this capability (Moe, 2013). Soil microbes have also been shown to specifically chemotax toward amino acids in root exudates (Nelson, 2004), another essential trait for rhizopshere colonization (de Weert et al., 2002), and the half-life of these compounds in soil averages just 1–6 h (Jones and Kielland, 2012). Amino acids are thought to provide an important food and energy source for microbes, as well as a mechanism to help modify various stress responses (Moe, 2013). Because of their importance, it has even been theorized that microbes produce compounds that help them to compete for amino acids with plants. For example, production of 2,4-diacetylphloroglucinol (DAPG), a compound commonly produced by many *Pseudomonas fluorescens* strains and frequently cited for its antagonistic activity toward plant pathogens, appeared to block amino acid influx by plants (Phillips et al., 2004).

The presence of amino acids in the rhizosphere has been shown to affect many key processes that help microbes survive in the rhizosphere and could also be indirectly affecting plants. For example, amino acids are major determinants of the synthesis and activity of auxin phytohormones like IAA (Staswick, 2009). Many rhizosphere-dwelling bacteria have been shown to synthesize IAA, with estimates that up to 80% possess this trait (Patten and Glick, 1996). Another key feature of microbial taxa inhabiting the rhizosphere is the ability to produce and reside in biofilms (Danhorn and Fuqua, 2007). Biofilms help microbes withstand environmental stress caused by pH, salt or toxic compounds produced by plants and other microbes. They also protect microbes from grazing by protozoa and facilitate horizontal gene transfer. Most importantly, they help microbes maintain critical mass for periods sufficient to initiate consortial metabolism that single cells cannot accomplish effectively. For example, the products of consortial metabolism include metabolites and exoenzymes important in degradation of organic matter, biocontrol activity and pathogenesis. Amino acid composition has been shown to be a key factor in biofilm formation by some microbial taxa, as well as disassembly in others (Kolodkin-Gal et al., 2010).

Because amino acids make up a significant component of PHs, it may be possible to modify the composition of these products and thereby alter rhizosphere microbial community structure and activity. For example, using soil dilutions, Halvorson (1972) found dramatic difference in the potential for soil microbes to utilize individual amino acids. The highest colony counts were observed in selective media containing threonine (79.2%), aspartate (70.8%), and glutamate (66.7%), whereas cysteine (8.1%) and tryptophan (7.7%) showed the lowest colony counts. Differences in preference among individual microbial taxa for specific amino acids have also been noted (Moe, 2013), providing further support for the theory that PHs could be specifically formulated to support specific microbial taxa.

The phyllosphere has received much less attention than the rhizosphere, though recent studies have begun to shed light on this important plant-microbial habitat. Unlike the rhizosphere, which is thought to favor copiotrophic organisms that can rapidly utilize labile carbon compounds released from plant roots, the phyllosphere is thought to be an oligotrophic environment, with few available nutrients, especially carbon (Vorholt, 2012). The phyllosphere is also expected to be a more ephemeral or short-lived habitat in comparison to the rhizosphere, with the microbes in this habitat subjected to more stressful environmental conditions. For example, phyllosphere microbes may be exposed to ultraviolet (UV) radiation as well as extreme drought due to the waxy cuticle covering plant leaves, which prevents water loss (Vorholt, 2012). At the same time, phyllosphere microbes are also subjected to intense rainfall events. While overall species richness in the phyllosphere is high, diversity compared to the rhizosphere and bulk soil is much lower, with over 70% of phyla characterized as Alphaproteobacteria, and the rest assigned primarily to Gammaproteobacteria, Bacteriodes and Actinobacteria (Vorholt, 2012). Consequently, manipulating the phyllosphere microbiome using PHs could prove easier than trying to manipulate the rhizosphere microbiome, since there are likely to be fewer microbial taxa inhabiting this environment and nutrients are scare.

Like the rhizosphere, distribution of phyllosphere microbes have been found to be highly heterogeneous, with microbes often residing in aggregates located near sites of nutrient leakage from plants such as the stomata and base of trichomes (Lindow and Brandl, 2003; Vorholt, 2012). Microbial composition has also been found to vary given plant species and genotype, development stage, as well as location on the leaf, which could be due to differences in surface appendages and composition of plant leachates (Lindow and Brandl, 2003; Vorholt, 2012; Ortega et al., 2016). Key traits expected to be essential for supporting microbial life in the phyllosphere include chemotaxis, and the ability to produce biofilms as well as pigments that aid in UV tolerance (Lindow and Brandl, 2003; Vorholt, 2012). Like rhizosphere microbes, the ability to withstand and produce antagonistic compounds is also expected to be essential to the ability of individual taxa to compete for nutrients and space. In particular, a high proportion of microbes isolated from the phyllosphere (up to 58%) were able to inhibit pathogen growth by production of VOCs (Ortega et al., 2016). The potential for microbes to synthesize IAA appears to be widespread among phyllosphere microbes and could be an important factor in facilitating colonization and helping microbes withstand drought

stress (Lindow and Brandl, 2003). There is also evidence that microbial synthesis of IAA could increase the availability of nutrients by loosening cell walls, thereby releasing saccharides from plants (Fry, 1989; Lindow and Brandl, 2003; Vorholt, 2012). Finally, microbial production of surfactants also appears to be an essential trait for helping phyllosphere microbes to withstand drought stress (Lindow and Brandl, 2003; Vorholt, 2012).

Protein Hydrolysates Effects on the Plant Microbiome

In response to increasing awareness of the plant microbiome and identification of specific microbial taxa than can benefit plants, scientists have begun to investigate whether PHs are indirectly affecting plant growth by altering these communities. To our knowledge, the only study to date that has specifically tested this hypothesis in soil is the study by Tejada et al. (2011), which correlated application of biostimulants with alterations in soil microbial community structure and greater soil microbial activity with improved plant establishment on degraded soils. Soil microbes are well known for their potential to produce extracellular enzymes that aid in decomposition of organic matter, producing compounds that could directly affect plants. For example, as noted above, an alkaline protease produced by *Bacillus circulans* HA12 has been used to produce a bioactive peptide called 'root hair promoting peptide' in a PH derived from soybeans (Matsumiya and Kubo, 2011). Composition of PHs resulting from the type of hydrolysis treatment used as well as the original feedstock is likely to affect microbial activity and corresponding plant benefits. Of the four biostimulants evaluated in the study by Tejada et al. (2011), the amendment derived from rice bran extract had the greatest effect on soil microbes and vegetal cover. The authors concluded that this was likely due to the fact that the rice bran extract contained the highest amount of protein and percentage of peptides under 3kDa, and the low molecular weight of these compounds could easily be assimilated by microbes. Furthermore, the authors suggested that a lower fat content in biostimulants could also favor nutrient and peptide absorption by microbes. Additional studies investigating relationships between PHs, soil and root microbiomes, and plant productivity are needed.

A few scientists have begun to try and untangle the complex relationships between biostimulant formulations, phyllosphere microbial community structure and activity, and plant health. For example, using a culture-dependent approach, Luziatelli et al. (2016) determined that a PH-based biostimulant product derived from a legume ('Trainer®') and another product derived from tropical plant extracts ('Auxym®'), altered phyllosphere microbial community diversity and increased lettuce growth and leaf chlorophyll content. Many of the microbes isolated from lettuce leaves subjected to foliar applications of these products, most notably isolates from the genera *Pantoea*, *Micrococcus,* and *Acinetobacter,* had the potential to solubilize phosphorous and produce indole acetic acid (IAA). Moreover, all *Bacillus* strains isolated from lettuce leaves exhibited strong inhibitory activity against two key plant pathogens (*Fusarium oxysporum* and *Erwinia amylovora*), and isolates of *Pantoea*, *Micrococcus* and *Pseudomonas* were active against *E. amylovora*. Results of this study indicate that alteration of the phyllospere microbial community not only stimulates plant growth, but could also help plants withstand pathogen stress.

Hydrolysates derived from casein and soybeans have previously been demonstrated to elicit grapevine defense mechanisms and suppress downy mildew, caused by *Plasmopara viticola* (Lachhab et al., 2014), but it was unclear whether this was due to direct induction of plant defense responses or an indirect effect from modification of phyllosphere microbial communities. Cappelletti et al. (2016) sought to answer this question by studying the effects of a protein derivative on downy mildew in grape in the absence (axenic conditions) and presence of phyllopshere microorganisms (protected cultivation). Results of these studies confirmed that the protein derivative could stimulate plant defense responses and reduce downy mildew in grape, and the authors concluded that multiple mechanisms of action were likely involved in the suppressive effects observed. For example, while induction of some defense genes were observed under axenic conditions, others were expressed only in the presence of phyllosphere microbial communities indicating that biocontrol activity of these microbes likely played a role in downy mildew suppression. Many of the microbial genera isolated from grapevine leaves in this study, including *Exiguobacterium*, *Pseudomonas, Serratia,* and *Lysobacter* species have previously been found to contribute to biocontrol activity via multiple strategies including competition for space and production of antagonistic compounds.

These studies provide evidence that PHs can modify microbial community structure and activity, and such changes could contribute to some of the beneficial effects observed after applying these products. Moreover, they provide tantalizing support for the hypothesis that these products could someday be specifically formulated to support beneficial plant-microbial relationships and further enhance plant productivity. In support of this hypothesis, Rouphael et al. (2017a) recently demonstrated that tolerance to alkalinity and salinity of lettuce plants could be improved by combining a PH with a microbial-based biostimulant containing *Rhizophagus intraradices* (an arbuscular mycorrhizal fungus, AMF) and *Trichoderma atroviride* (a filamentous fungus that functions as biocontrol agent). The effect of the combined application of the PH and fungi on plant growth was attributed to several factors, including increase in root surface area, greater chlorophyll synthesis and proline accumulation.

CONCLUSION

Protein hydrolysates have great potential to improve crop performance, especially under environmental stress conditions. Root applications of PHs have been shown to be beneficial by improving nutrient use efficiency, enhancing nutrient availability, root growth, nutrient uptake and assimilation in several crops. Moreover, foliar and root (substrate drench) applications of PHs exhibit hormone-like activities (especially auxin-like and gibberellin-like activity) leading to stimulation of seed

germination, plant growth, fruit set and enlargement. PHs not only increase yield but also improve some quality parameters such as fruit size, skin color, soluble solids, and antioxidant contents. Moreover, PHs have also great potential to reduce nitrate accumulation in leafy vegetables such as lettuce, spinach and rocket. However, mechanisms regulating the beneficial effects of PHs on plants are not completely understood and only recently, thanks to use of 'omics' sciences, is it becoming possible to clarify specific modes of action.

Recent studies have provided evidence that PHs can affect plant microbiomes and some of the benefits derived from these products might be due in part to changes in the composition and activity of these plant-associated communities. Combining PHs with specific microbial taxa that are well known for their potential to help plants acquire nutrients and withstand biotic and abiotic stress has been demonstrated to further enhance plant benefits. Someday it might be possible to build on the results of these studies by specifically formulating PHs to enhance the abundance and activity of beneficial microbes naturally inhabiting plant compartments, and/or develop consortia of fungi and bacteria that can be applied in combination with PHs to improve plant performance. For example, it has been reported that plant productivity is directly related to evenness (relative abundance) of members of the microbiome (Wilsey and Potvin, 2000), and increasing microbial biomass and/or diversity can enhance pathogen- or disease-suppressiveness (Larkin and Honeycutt, 2006). If microbial taxa that are evolutionarily adapted to particular host plants can be identified and applied in concert with PHs to support their colonization and survival on plants, these consortia could reduce the time required for the microbiome to achieve niche saturation and competitively exclude pathogens. The strategy of using plants as selective agents to improve beneficial microbial functions has the major advantage of not requiring any change in infrastructure or management.

FUTURE DIRECTIONS

Further maximizing the beneficial effects of PHs will require a better mechanistic understanding of how the rate, timing of application and composition of individual products specifically alters plant physiological processes. Moreover, there is growing consensus that small size peptides play an important role in the biological activity of PHs. However, only few bioactive peptides have been characterized. Therefore, more studies are necessary to discover the signaling peptides, which are responsible for the biostimulant activity of PHs. These findings may also help to make the PH production process more efficient in producing bioactive peptides.

Effectively manipulating plant microbiomes with PHs will require additional research to answer questions such as: how do individual microbial taxa respond to specific amino acids and other compounds in PHs; what is the optimal dose, time and mode of application to support specific microbial taxa that improve plant fitness; how much will plant species, genotype and the environment affect these relationships; can PHs be formulated with specific compounds to better support colonization and survival of microbial inoculants; and, will PHs need to be combined with inoculants that contain a microbial consortia with synergistic traits, thus providing more consistent effects? Application of new 'omics' sciences and high-throughput phenotyping platforms will aid in these studies, though partnerships between academic researchers and private industry will be required due the high costs of these studies. At the same time, further research investigating the effects of growing conditions on the interactions between PH formulation, plant species, developmental stage, application rate, microbiomes, etc., are also needed.

While there seem to be more questions than answers at this point in time, results of the few studies that have attempted to start to tease apart the complex relationships between PHs, the plant microbiome and changes in plant physiological processes suggest that altering these relationships will be possible and will be well worth the effort.

AUTHOR CONTRIBUTIONS

GC coordinated the review and he wrote many parts of the article. LH wrote part of the review dealing the effects of protein hydrolystes on microbiome and she contributed to improve the article. MR wrote part of the review dealing with the microbiome and he contributed to improve the article. MC wrote part of the review dealing with germination and seedling growth and she contributed to improve the article. PB wrote part of the review dealing with protein hydrolysate effects on plant growth. RC wrote part of the review dealing with chemical characteristics of protein hydrolysates. YR wrote many parts of the article especially those dealing with the effects of protein hydrolysates on yield and quality and he contributed to improve the article.

REFERENCES

Amirkhani, M., Netravali, A. N., and Huang, W. (2016). Investigation of soy protein–based biostimulant seed coating for broccoli seedling and plant growth enhancement. *Hortscience* 51, 1121–1126. doi: 10.21273/HORTSCI10913-16

Amr, A., and Hadidi, N. (2001). Effect of cultivar and harvest data on nitrate (NO3) and nitrite (NO2) content of selected vegetables gown under open field and greenhouse conditions in Jordan. *J. Food Comp. Anal.* 14, 59–67. doi: 10.1006/jfca.2000.0950

Badri, D. V., Weir, T. L., van der Lelie, D., and Vivanco, J. M. (2009). Rhizosphere chemical dialogues: plant-microbe interactions. *Curr. Opin. Biotechnol.* 20, 642–650. doi: 10.1016/j.copbio.2009.09.014

Baglieri, A., Cadili, V., Mozzetti Monterumici, C., Gennari, M., Tabasso, S., Montoneri, E., et al. (2014). Fertilization of bean plants with tomato plants hydrolysates. Effect on biomass production, chlorophyll content and N assimilation. *Sci. Hortic.* 176, 194–199. doi: 10.1016/j.scienta.2014.07.002

Bai, Y., Müller, D. B., Srinivas, G., Garrido-Oter, R., Potthoff, E., Rott, M., et al. (2015). Functional overlap of the Arabidopsis leaf and root microbiota. *Nature* 528, 364–369. doi: 10.1038/nature16192

Bais, H. P., Weir, T. L., Perry, L. G., Gilroy, S., and Vivanco, J. M. (2006). The role of root exudates in rhizosphere interactions with plants and other organisms. *Annu. Rev. Plant Biol.* 57, 233–266. doi: 10.1146/annurev.arplant.57.032905.105159

Baltrus, D. A. (2017). Adaptation, specialization, and coevolution within phytobiomes. *Curr. Opin. Plant Biol.* 38, 109–116. doi: 10.1016/j.pbi.2017.04.023

Berendsen, R. L., Pieterse, C. M., and Bakker, P. A. (2012). The rhizosphere microbiome and plant health. *Trends Plant Sci.* 17, 478–486. doi: 10.1016/j.tplants.2012.04.001

Berg, G., Rybakova, D., Grube, M., and Köberl, M. (2016). The plant microbiome explored: implications for experimental botany. *J. Exp. Bot.* 67, 995–1002. doi: 10.1093/jxb/erv466

Berg, G., and Smalla, K. (2009). Plant species and soil type cooperatively shape the structure and function of microbial communities in the rhizosphere. *FEMS Microbiol. Ecol.* 68, 1–13. doi: 10.1111/j.1574-6941.2009.00654.x

Bonini, P., Long, D. H., Canaguier, R., Colla, G., and Leman, J. (2017). "Seed treatments with endophytic fungi and biostimulant compounds enhance yield of corn and soybean," in *Proceedings of the 3rd World Congress on the Use of Biostimulants in Agriculture*, Miami, FL, 27–30.

Bonner, C. A., and Jensen, R. A. (1997). Recognition of specific patterns of amino acid inhibition of growth in higher plants, uncomplicated by glutamine-reversible 'general amino acid inhibition'. *Plant Sci.* 130, 133–143. doi: 10.1016/S0168-9452(97)00213-6

Bordenstein, S. R., and Theis, K. R. (2015). Host biology in light of the microbiome: ten principles of holobionts and hologenomes. *PLOS Biol.* 13:e1002226. doi: 10.1371/journal.pbio.1002226

Botta, A. (2013). Enhancing plant tolerance to temperature stress with amino acids: an approach to their mode of action. *Acta Hortic.* 1009, 29–35. doi: 10.1007/s00299-016-1980-4

Bulgarelli, D., Garrido-Oter, R., Münch, P. C., Weiman, A., Dröge, J., Pan, Y., et al. (2015). Structure and function of the bacterial root microbiota in wild and domesticated barley. *Cell Host Microbe* 17, 392–403. doi: 10.1016/j.chom.2015.01.011

Bulgarelli, D., Schlaeppi, K., Spaepen, S., van Themaat, E. V. L., and Schulze-Lefert, P. (2013). Structure and functions of the bacterial microbiota of plants. *Annu. Rev. Plant Biol.* 64, 807–838. doi: 10.1146/annurev-arplant-050312-120106

Calvo, P., Nelson, L., and Kloepper, J. W. (2014). Agricultural uses of plant biostimulants. *Plant Soil* 383, 3–41. doi: 10.1007/s11104-014-2131-8

Cappelletti, M., Perazzolli, M., Antonielli, L., Nesler, A., Torbol, E., Bianchedi, P. L., et al. (2016). Leaf treatments with a protein-based resistance inducer partially modify phyllosphere microbial communities of grapevine. *Front. Plant Sci.* 7:1053. doi: 10.3389/fpls.2016.01053

Cerdán, M., Sánchez-Sánchez, A., Jordá, D. J., Juárez, M., and Andreu, J. S. (2013). Effect of commercial amino acids on iron nutrition of tomato plants grown under lime-induced iron deficiency. *J. Plant Nutr. Soil Sci.* 176, 1–8. doi: 10.1002/jpln.201200525

Cerdán, M., Sánchez-Sánchez, A., Oliver, M., Juárez, M., and Sánchez-Andreu, J. J. (2009). Effect of foliar and root applications of amino acids on iron uptake by tomato plants. *Acta Hortic.* 830, 481–488. doi: 10.17660/ActaHortic.2009.830.68

Chalamaiah, M., Dinesh Kumar, B., Hemalatha, R., and Jyothirmayi, T. (2012). Fish protein hydrolysates: proximate composition, amino acid composition, antioxidant activities and applications: a review. *Food Chem.* 135, 3020–3038. doi: 10.1016/j.foodchem.2012.06.100

Coleman-Derr, D., Desgarennes, D., Fonseca-Garcia, C., Gross, S., Clingenpeel, S., Woyke, T., et al. (2016). Plant compartment and biogeography affect microbiome composition in cultivated and native *Agave* species. *New Phytol.* 209, 798–811. doi: 10.1111/nph.13697

Colla, G., Cardarelli, M., Bonini, P., and Rouphael, Y. (2017). Foliar applications of protein hydrolysate, plant and seaweed extracts increase yield but differentially modulate fruit quality of greenhouse tomato. *HortScience* 52, 1214–1220. doi: 10.21273/hortsci12200-17

Colla, G., Nardi, S., Cardarelli, M., Ertani, A., Lucini, L., Canaguier, R., et al. (2015a). Protein hydrolysates as biostimulants in horticulture. *Sci. Hortic.* 96, 28–38. doi: 10.1016/j.scienta.2015.08.037

Colla, G., Rouphael, Y., Di Mattia, E., El-Nakhel, C., and Cardarelli, M. (2015b). Co-inoculation of *Glomus intraradices* and *Trichoderma atroviride* acts as a biostimulant to promote growth, yield and nutrient uptake of vegetable crops. *J. Sci. Food Agric.* 95, 1706–1715. doi: 10.1002/jsfa.6875 doi: 10.1002/jsfa.6875

Colla, G., Rouphael, Y., Canaguier, R., Svecova, E., and Cardarelli, M. (2014). Biostimulant action of a plant-derived protein hydrolysate produced through enzymatic hydrolysis. *Front. Plant Sci.* 5:448. doi: 10.3389/fpls.2014.00448

Colla, G., Svecova, E., Rouphael, Y., Cardarelli, M., Reynaud, H., Canaguier, R., et al. (2013). Effectiveness of a plant –derived protein hydrolysate to improve crop performances under different growing conditions. *Acta Hortic.* 1009, 175–179. doi: 10.17660/ActaHortic.2013.1009.21

Conrath, U., Beckers, G. J., Flors, V., García-Agustín, P., Jakab, G., et al. (2006). Priming: getting ready for battle. *Mol. Plant Microbe Interact.* 19, 1062–1071. doi: 10.1094/MPMI-19-1062

Danhorn, T., and Fuqua, C. (2007). Biofilm formation by plant- associated bacteria. *Ann. Rev. Microbiol.* 61, 401–422. doi: 10.1146/annurev.micro.61.080706.093316

De Lucia, B., and Vecchietti, L. (2012). Type of biostimulant and application method effects on stem quality and root system growth in LA Lily. *Eur. J. Hortic. Sci.* 77, 1–10.

de Weert, S., Vermeiren, H., Mulders, I. H., Kuiper, I., Hendrickx, N., Bloemberg, G. V., et al. (2002). Flagella-driven chemotaxis towards exudate components is an important trait for tomato root colonization by *Pseudomonas fluorescens*. *Mol. Plant Microbe Interact.* 15, 1173–1180. doi: 10.1094/MPMI.2002.15.11.1173

del Rosario Cappellari, L., Chiappero, J., Santoro, M. V., Giordano, W., and Banchio, E. (2017). Inducing phenolic production and volatile organic compounds emission by inoculating *Mentha piperita* with plant growth-promoting rhizobacteria. *Sci. Hortic.* 220, 149–158. doi: 10.1016/j.scienta.2017.04.002

Delmotte, N., Knief, C., Chaffron, S., Innerebner, G., Roschitzki, B., Schlapbach, R., et al. (2009). Community proteogenomics reveals insights into the physiology of phyllosphere bacteria. *Proc. Natl. Acad. Sci. U.S.A.* 106, 16428–16433. doi: 10.1073/pnas.0905240106

du Jardin, P. (2015). Plant biostimulants: definition, concept, main categories and regulation. *Sci. Hortic.* 196, 3–14. doi: 10.1016/j.scienta.2015.09.021

Erba, D., Casiraghi, M. C., Ribas-Agustí, A., Cáceres, R., Marfà, O., and Castellari, M. (2013). Nutritional value of tomatoes (*Solanum lycopersicum* L.) grown in greenhouse by different agronomic techniques. *J. Food Compost. Anal.* 31, 245–251. doi: 10.1016/j.jfca.2013.05.014

Ertani, A., Cavani, L., Pizzeghello, D., Brandellero, E., Altissimo, A., Ciavatta, C., et al. (2009). Biostimulant activities of two protein hydrolysates on the growth and nitrogen metabolism in maize seedlings. *J. Plant. Nutr. Soil Sci.* 172, 237–244. doi: 10.1002/jpln.200800174

Ertani, A., Francioso, O., Tugnoli, V., Righi, V., and Nardi, S. (2011). Effect of commercial Lignosulfonate-humate on *Zea mays* L. metabolism. *J. Agric. Food Chem.* 59, 11940–11948. doi: 10.1021/jf202473e

Ertani, A., Pizzeghello, D., Francioso, O., Sambo, P., Sanchez-Cortes, S., and Nardi, S. (2014). *Capsicum chinensis* L. growth and nutraceutical properties are enhanced by biostimulants in a long-term period: chemical and metabolomic approaches. *Front. Plant Sci.* 5, 375. doi: 10.3389/fpls.2014.00375 doi: 10.3389/fpls.2014.00375

Ertani, A., Schiavon, M., Muscolo, A., and Nardi, S. (2013). Alfalfa plant-derived biostimulant stimulate short-term growth of salt stressed *Zea mays* L. plants. *Plant Soil* 364, 145–158. doi: 10.1007/s11104-012-1335-z

Farooq, M., Wahid, A., and Kadambot, H. M. S. (2012). Micronutrient application through seed treatments—a review. *J. Soil Sci. Plant Nutr.* 12, 125–142. doi: 10.4067/S0718-95162012000100011

Farré-Armengol, G., Filella, I., Llusia, J., and Peñuelas, J. (2016). Bidirectional interaction between phyllospheric microbiotas and plant volatile emissions. *Trends Plant Sci.* 21, 854–860. doi: 10.1016/j.tplants.2016.06.005

Fry, S. C. (1989). Cellulases, hemicellulases and auxin-stimulated growth: a possible relationship. *Physiol. Plant.* 75, 532–536. doi: 10.1111/j.1399-3054.1989.tb05620.x

Gaidau, C., Epure, D. G., Niculescu, M., Stepan, E., Radu, E., and Gidea, M. (2015). *Application of Collagen Hydrolysate in Cereal Seed Treatment*. XXXIII IULTCS

Congress. Available at: http://www.aaqtic.org.ar/congresos/brasil2015/pdf/072.pdf

Gaidau, C., Niculescu, M., Stepan, E., Epure, D.-G., and Gidea, M. (2013). New mixes based on collagen extracts with bioactive properties, for treatment of seeds in sustainable agriculture. *Curr. Pharm. Biotechnol. J.* 14, 792–801. doi: 10.2174/1389201014666131227112020

Gajc-Wolska, J., Kowalczyk, K., Nowecka, M., Mazur, K., and Metera, A. (2012). Effect of organic-mineral fertilizers on the yield and quality of endive (*Cichorium endivia* L.). *Acta Sci. Pol.* 11, 189–200.

Gargallo-Garriga, A., Sardans, J., Pérez-Trujillo, M., Guenther, A., Llusià, J., Rico, L., et al. (2016). Shifts in plant foliar and floral metabolomes in response to the suppression of the associated microbiota. *BMC Plant Biol.* 16:78. doi: 10.1186/s12870-016-0767-7

Ghosh, A., Madal, P., and Kumar Sircar, P. (2010). Wheat (*Triticum aestivum*) peptide (s) mimic gibberellin action and regulate stomatal opening. *Ind. J. Exp. Biol.* 48, 77–82.

Gorim, L., and Asch, F. (2012). Effects of composition and share of seed coatings on the mobilization efficiency of cereal seeds during germination. *J. Agron. Crop Sci.* 198, 81–91. doi: 10.1111/j.1439-037X.2011.00490.x

Grabowska, A., Kunicki, E., Sekara, A., Kalisz, A., and Wojciechowska, R. (2012). The effect of cultivar and biostimulant treatment on the carrot yield and its quality. *Veg. Crops Res. Bull.* 77, 37–48. doi: 10.2478/v10032-012-0014-1

Gurav, R. G., and Jadhav, J. P. (2013). A novel source of biofertilizer from feather biomass for banana cultivation. *Environ. Sci. Pollut. Res. Int.* 20, 4532–4539. doi: 10.1007/s11356-012-1405-z

Halmer, P. (2004). "Methods to improve seed performance in the field," in *Handbook of Seed Physiology: Applications to Agriculture*, eds R. L. Benech Arnold and R. A. Sanchez (New York, NY: Haworth Press), 120–156.

Halpern, M., Bar-Tal, A., Ofek, M., Minz, D., Muller, T., and Yermiyahu, U. (2015). The use of biostimulants for enhancing nutrient uptake. *Adv. Agron.* 130, 141–174. doi: 10.3389/fpls.2017.00597

Halvorson, H. (1972). Utilization of L-amino acids as sole source of carbon and nitrogen by bacteria. *Can. J. Biol.* 18, 1647–1650. doi: 10.1139/m72-255

Hartmann, A., Rothballer, M., and Schmid, M. (2008). Lorenz Hiltner, a pioneer in rhizosphere microbial ecology and soil bacteriology research. *Plant Soil* 312, 7–14. doi: 10.1007/s11104-007-9514-z

Hill, H. J. (1999). Recent developments in seed-technology. *J. New Seeds* 1, 105–112. doi: 10.1300/J153v01n01_09

Horton, M. W., Bodenhausen, N., Beilsmith, K., Meng, D., Muegge, B. D., Subramanian, S., et al. (2014). Genome-wide association study of *Arabidopsis thaliana* leaf microbial community. *Nat. Commun.* 5:5320. doi: 10.1038/ncomms6320

İnceoğlu, Ö., Al-Soud, W. A., Salles, J. F., Semenov, A. V., and van Elsas, J. D. (2011). Comparative analysis of bacterial communities in a potato field as determined by pyrosequencing. *PLOS ONE* 6:e23321. doi: 10.1371/journal.pone.0023321

İnceoğlu, Ö., Falcão Salles, J., and van Elsas, J. D. (2012). Soil and cultivar type shape the bacterial community in the potato rhizosphere. *Microb. Ecol.* 63, 460–470. doi: 10.1007/s00248-011-9930-8

İnnerebner, G., Knief, C., and Vorholt, J. A. (2011). Protection of *Arabidopsis thaliana* against leaf-pathogenic *Pseudomonas syringae* by *Sphingomonas* strains in a controlled model system. *Appl. Environ. Microbiol.* 77, 3202–3210. doi: 10.1128/AEM.00133-11

Jones, D. L., and Kielland, K. (2012). Amino acid, peptide and protein mineralization dynamics in a taiga forest soil. *Soil Biol. Biochem.* 55, 60–69. doi: 10.1016/j.soilbio.2012.06.005

Kirn, A., Kashif, S. R., and Yaseen, M. (2010). Using indigenous humic acid from lignite to increase growth and yield of okra (*Abelmoschus esculentus* L.). *Soil Environ.* 29, 187–191.

Knief, C., Delmotte, N., Chaffron, S., Stark, M., Innerebner, G., Wassmann, R., et al. (2012). Metaproteogenomic analysis of microbial communities in the phyllosphere and rhizosphere of rice. *ISME J.* 6, 1378–1390. doi: 10.1038/ismej.2011.192

Kolodkin-Gal, I., Romero, D., Cao, S., Clardy, J., Kolter, R., and Losick, R. (2010). D-amino acids trigger biofilm disassembly. *Science* 328, 627–629. doi: 10.1126/science.1188628

Koukounararas, A., Tsouvaltzis, P., and Siomos, A. S. (2013). Effect of root and foliar application of amino acids on the growth and yield of greenhouse tomato in different fertilization levels. *J. Food Agric. Environ.* 11, 644–648.

Kunicki, E., Grabowska, A., Sękara, A., and Wojciechowska, R. (2010). The effect of cultivar type, time of cultivation, and biostimulant treatment on the yield of spinach (*Spinacia oleracea* L). *Folia Hortic.* 22, 9–13. doi: 10.2478/fhort-2013-0153

Kyriacou, M. C., Rouphael, Y., Colla, G., Zrenner, R. M., and Schwarz, D. (2017). Vegetable grafting: the implications of a growing agronomic imperative for vegetable fruit quality and nutritive value. *Front. Plant Sci.* 8:741. doi: 10.3389/fpls.2017.00741

Kyriacou, M. C., Rouphael, Y., Di Gioia, F., Kyratzis, A., Serio, F., Renna, M., et al. (2016). Micro-scale food production and the rise of microgreens. *Trends Food Sci. Technol.* 57, 103–115. doi: 10.1016/j.tifs.2016.09.005

Lachhab, N., Sanzani, S. M., Adrian, M., Chilitz, A., Balacey, S., Boselli, M., et al. (2014). Soybean and casein hydrolysates induce grapevine immune responses and resistant against *Plasmopara viticola*. *Front. Plant Sci.* 5:716. doi: 10.3389/fpls.2014.00716

Larkin, R. P., and Honeycutt, C. W. (2006). Effects of different 3-year cropping systems on soil microbial communities and rhizoctonia diseases of potato. *Phytopathology* 96, 68–79. doi: 10.1094/PHYTO-96-0068

Leach, J. E., Triplett, L. R., Argueso, C. T., and Trivedi, P. (2017). Communication in the phytobiome. *Cell* 169, 587–596. doi: 10.1016/j.cell.2017.04.025

Lindow, S. E., and Brandl, M. T. (2003). Microbiology of the phyllosphere. *Appl. Environ. Microbiol.* 69, 1875–1883. doi: 10.1128/AEM.69.4.1875-1883.2003

Lisiecka, J., Knaflewski, M., Spizewski, T., Fraszczak, B., Kaluzewicz, A., and Krzesinski, W. (2011). The effect of animal protein hydrolysate on quantity and quality of strawberry daughter plants cv. *'Elsanta'*. *Acta Sci. Pol. Hortic.* 10, 31–40.

Liu, X. Q., and Lee, K. S. (2012). "Effect of mixed amino acids on crop growth," in *Agricultural Science*, ed. G. Aflakpui (Rijeka: InTech Europe Publisher), 119–158. doi: 10.5772/37461

Lucini, L., Rouphael, Y., Cardarelli, M., Canguier, R., Kumar, P., and Colla, G. (2015). The effect of a plant-derived biostimulant on metabolic profiling and crop performance of lettuce grown under saline conditions. *Sci. Hortic.* 182, 124–133. doi: 10.1016/j.scienta.2014.11.022

Lundberg, D. S., Lebeis, S. L., Paredes, S. H., Yourstone, S., Gehring, J., Malfatti, S., et al. (2012). Defining the core *Arabidopsis thaliana* root microbiome. *Nature* 488, 86–90. doi: 10.1038/nature11237

Luziatelli, F., Ficca, A. G., Colla, G., Svecova, E., and Ruzzi, M. (2016). Effects of a protein hydrolysate-based biostimulant and two micronutrient based fertilizers on plant growth and epiphytic bacterial populations of lettuce. *Acta Hortic.* 1148, 43–48. doi: 10.17660/ActaHortic.2016.1148.5

Marfà, O., Cáceres, R., Polo, J., and Ródenas, J. (2009). Animal protein hydrolysate as a biostimulant for transplanted strawberry plants subjected to cold stress. *Acta Hortic.* 842, 315–318. doi: 10.17660/ActaHortic.2009.842.57

Marin, I. A., Goertz, J. E., Ren, T., Rich, S. S., Onengut-Gumuscu, S., Farber, E., et al. (2017). Microbiota alteration is associated with the development of stress-induced despair behavior. *Sci. Rep.* 7:43859. doi: 10.1038/srep43859

Marmulla, R., and Harder, J. (2014). Microbial monoterpene trans-formations – a review. *Front. Microbiol.* 5:346. doi: 10.3389/fmicb.2014.00346

Matsumiya, Y., and Kubo, M. (2011). "Soybean peptide: novel plant growth promoting peptide from soybean," in *Soybean and Nutrition*, ed. H. El-Shemy (Rijeka: In Tech Europe Publisher), 215–230.

McGrath, S. P., Chang, A. C., Page, A. L., and Witter, E. (1994). Land application of sewage sludge: scientific perspectives of heavy metal loading limits in Europe and the United States. *Environ. Rev.* 2, 108–118. doi: 10.1139/a94-006

Mehrafarin, A., Qavami, N., Tahmasebi, Z., Naghdi Badi, H., Abdossi, V., and Seif Sahandi, M. (2015). Phytochemical and morpho-physiological responses of lemon balm (*Melissa officinalis* L.) to biostimulants Application. *J. Med. Plants* 14, 29–42.

Mendes, L. W., Kuramae, E. E., Navarrete, A. A., van Veen, J. A., and Tsai, S. M. (2014). Taxonomical and functional microbial community selection in soybean rhizosphere. *ISME J.* 8, 1577–1587. doi: 10.1038/ismej.2014.17

Mendes, R., Garbeva, P., and Raaijmakers, J. M. (2013). The rhizosphere microbiome: significance of plant beneficial, plant pathogenic, and human

pathogenic microorganisms. *FEMS Microbiol. Rev.* 37, 634–663. doi: 10.1111/1574-6976.12028

Moe, L. A. (2013). Amino acids in the rhizosphere: from plants to microbes. *Am. J. Bot.* 100, 1692–1705. doi: 10.3732/ajb.1300033

Morales-Pajan, J. P., and Stall, W. (2004). Passion fruit (*Passiflora edulis*) transplant production i affected by selected biostimulants. *Proc. Fla. State Hortic. Soc.* 117, 224–227.

Mucke, J. D. (1988). The regulation of water transport in pelleted sugar beet seed. *J. Agron. Crop Sci.* 161, 79–83.

Müller, D. B., Vogel, C., Bai, Y., and Vorholt, J. A. (2016). The plant microbiota: systems-level insights and perspectives. *Annu. Rev. Genet.* 50, 211–234. doi: 10.1146/annurev-genet-120215-034952

Nardi, S., Carletti, P., Pizzeghello, D., and Muscolo, A. (2009). "Biological activities of humic substances," in *Biophysico-Chemical Processes*, eds N. Senesi, B. Xing, and P. M. Huang (Hoboken, NJ: John Wiley & Sons).

Nardi, S., Pizzeghello, D., Schiavon, M., and Ertani, A. (2016). Plant biostimulants: physiological responses induced by protein hydrolyzed-based products and humic substances in plant metabolism. *Sci. Agric.* 73, 18–23. doi: 10.1590/0103-9016-2015-0006

Nelson, E. B. (2004). Microbial dynamics and interactions in the spermosphere. *Annu. Rev. Phytopathol.* 42, 271–309. doi: 10.1146/annurev.phyto.42.121603.131041

Ortega, R. A., Mahnert, A., Berg, C., Muller, H., and Berg, G. (2016). The plant is crucial: specific composition and function of the phyllosphere microbiome of indoor ornamentals. *FEMS Microbiol. Ecol.* 92:fiw173. doi: 10.1093/femsec/fiw173

Ottesen, A. R., González Peña, A., White, J. R., Pettengill, J. B., Li, C., Allard, S., et al. (2013). Baseline survey of the anatomical microbial ecology of an important food plant: *Solanum lycopersicum* (tomato). *BMC Microbiol.* 13:114. doi: 10.1186/1471-2180-13-114

Parrado, J., Bautista, J., Romero, E. J., Garcia-Martinez, A. M., Friaza, V., and Tejada, M. (2008). Production of a carob enzymatic extract: potential use as a biofertilizer. *Bioresour. Technol.* 99, 2312–2318. doi: 10.1016/j.biortech.2007.05.029

Parrado, J., Escudero-Gilete, M. L., Friaza, V., Garcia-Martinez, A., González-Miret, M. L., Bautista, J. D., et al. (2007). Enzymatic vegetable extract with bioactive components: influence of fertilizer on the colour and anthocyanins of red grapes. *J. Sci. Food Agric.* 87, 2310–2318. doi: 10.1002/jsfa.2989

Patten, C. L., and Glick, B. R. (1996). Bacterial biosynthesis of indole-3-acetic acid. *Can. J. Microbiol.* 42, 207–220. doi: 10.1139/m96-032

Pecha, J., Fürst, T., Kolomaznik, K., Friebrova, V., and Svoboda, P. (2012). Protein biostimulant foliar uptake modeling: the impact of climatic conditions. *AIChE J.* 58, 2010–2019. doi: 10.1002/aic.12739

Peiffer, J. A., Spor, A., Koren, O., Jin, Z., Tringe, S. G., Dangl, J. L., et al. (2013). Diversity and heritability of the maize rhizosphere microbiome under field conditions. *Proc. Natl. Acad. Sci. U.S.A.* 110, 6548–6553. doi: 10.1073/pnas.1302837110

Philippot, L., Raaijimakers, J. M., Lemanceau, P., and van der Putten, W. J. (2013). Going back to the roots: the microbial ecology of the rhizosphere. *Nat. Rev. Microbiol.* 11, 789–799. doi: 10.1038/nrmicro3109

Phillips, D. A., Fox, T. C., King, M. D., Bhuvaneswari, T. V., and Teuber, L. R. (2004). Microbial products trigger amino acid exudation from plant roots. *Plant Physiol.* 136, 2887–2894. doi: 10.1104/pp.104.044222

Pieterse, C. M., Van der Does, D., Zamioudis, C., Leon-Reyes, A., and Van Wees, S. C. (2012). Hormonal modulation of plant immunity. *Annu. Rev. Cell Dev. Biol.* 28, 489–521. doi: 10.1146/annurev-cellbio-092910-154055

Quartieri, M., Lucchi, A., Marangoni, B., Tagliavini, M., and Cavani, L. (2002). Effects of the rate of protein hydrolysis and spray concentration on growth of potted kiwifruit (*Actinidia deliciosa*) plants. *Acta Hortic.* 594, 341–347. doi: 10.17660/ActaHortic.2002.594.42

Ritpitakphong, U., Falquet, L., Vimoltust, A., Berger, A., Métraux, J.-P., and L'Haridon, F. (2016). The microbiome of the leaf surface of *Arabidopsis* protects against a fungal pathogen. *New Phytol.* 210, 1033–1043. doi: 10.1111/nph.13808

Rosenberg, E., and Zilber-Rosenberg, I. (2016). Microbes drive evolution of animals and plants: the hologenome concept. *MBio* 7:e01395. doi: 10.1128/mBio.01395-15

Rouphael, Y., Cardarelli, M., Bonini, P., and Colla, G. (2017a). Synergistic action of a microbial-based biostimulant and a plant derived-protein hydrolysate enhances lettuce tolerance to alkalinity and salinity. *Front. Plant Sci.* 8:131. doi: 10.3389/fpls.2017.00131

Rouphael, Y., Colla, G., Giordano, M., El-Nakhel, C., Kyriacou, M. C., and De Pascale, S. (2017b). Foliar applications of a legume-derived protein hydrolysate elicit dose dependent increases of growth, leaf mineral composition, yield and fruit quality in two greenhouse tomato cultivars. *Sci. Hortic.* 22, 353–360. doi: 10.1016/j.scienta.2017.09.007

Rouphael, Y., De Micco, V., Arena, C., Raimondi, G., Colla, G., and De Pascale, S. (2017c). Effect of Ecklonia maxima seaweed extract on yield, mineral composition, gas exchange and leaf anatomy of zucchini squash grown under saline conditions. *J. Appl. Phycol.* 29, 459–470. doi: 10.1007/s10811-016-0937-x

Ruiz, J. M., Castilla, N., and Romero, L. (2000). Nitrogen metabolism in pepper plants applied with different bioregulators. *J. Agric. Food Chem.* 48, 2925–2929. doi: 10.1021/jf990394h

Rushing, J. B., Baldwin, B. S., Taylor, A. G., Owens, V. N., Fike, H. J., and Moore, K. J. (2013). Seed safening from herbicidal injury in switch grass establishment. *Crop Sci.* 53, 1650–1657. doi: 10.2135/cropsci2013.01.0050

Ruzzi, M., and Aroca, R. (2015). Plant growth-promoting rhizobacteria act as biostimulants in horticulture. *Sci. Hortic.* 196, 124–134. doi: 10.1016/j.scienta.2015.08.042

Ryan, C. A., Pearce, G., Scheer, J., and Moura, D. S. (2002). Polypeptide hormones. *Plant Cell* 14, S251–S264. doi: 10.1105/tpc.010484

Schaafsma, G. (2009). Safety of protein hydrolysates, fractions thereof and bioactive peptides in human nutrition. *Eur. J. Clin. Nutr.* 63, 1161–1168. doi: 10.1038/ejcn.2009.56

Schiavon, M., Ertani, A., and Nardi, S. (2008). Effects of an alfaalfa protein hydrolysate on the gene expression and activity of enzymes of TCA cycle and N metabolism in *Zea mays* L. *J. Agric. Food Chem.* 56, 11800–11808. doi: 10.1021/jf802362g

Schulz, S., and Dickschat, J. S. (2007). Bacterial volatiles: the smell of small organisms. *Nat. Prod. Rep.* 24, 814–842. doi: 10.1039/B507392H

Searchinger, T. (2013). *The Great Balancing Act: Installment 1 of Creating a Sustainable Food Future*. Washington, DC: World Resources Institute.

Shakya, M., Gottel, N., Castro, H., Yang, Z. K., Gunter, L., Labbé, J., et al. (2013). A multifactor analysis of fungal and bacterial community structure in the root microbiome of mature *Populus deltoides* trees. *PLOS ONE* 8:e76382. doi: 10.1371/journal.pone.0076382

Sharma, S. S., and Dietz, K. J. (2009). The relationship between metal toxicity and cellular redox imbalance. *Trends Plant Sci.* 14, 43–50. doi: 10.1016/j.tplants.2008.10.007

Staswick, P. E. (2009). The tryptophan conjugates of jasmonic and indole 3-acetic acids are endogenous auxin inhibitors. *Plant Physiol.* 150, 1310–1321. doi: 10.1104/pp.109.138529

Sytar, O., Kumar, A., Latowski, D., and Kuczynska, P. (2013). Heavy metal-induced oxidative damage, defense reactions, and detoxification mechanisms in plants. *Acta Physiol. Plant.* 35, 985–999. doi: 10.1007/978-3-319-06746-9_1

Tejada, M., Benitez, C., Gomez, I., and Parrado, J. (2011). Use of biostimulants on soil restoration: effects on soil biochemical properties and microbial community. *Appl. Soil Ecol.* 49, 11–17. doi: 10.1016/j.apsoil.2011.07.009

Tsouvaltzis, P., Koukounaras, A., and Siomos, S. A. (2014). Application of amino acids improves lettuce crop uniformity and inhibits nitrate accumulation induced by the supplemental inorganic nitrogen fertilization. *Int. J. Agric. Biol.* 16, 951–955.

Turner, T. R., James, E. K., and Poole, P. S. (2013). The plant microbiome. *Genome Biol.* 14:209. doi: 10.1186/gb-2013-14-6-209

Ugolini, L., Cinti, S., Righetti, L., Stefan, A., Matteo, R., D'Avino, L., et al. (2015). Production of an enzymatic protein hydrolyzate from defatted sunflower seed meal for potential application as a plant biostimulant. *Ind. Crops Prod.* 75, 15–23. doi: 10.1016/j.indcrop.2014.11.026

Vandenkoornhuyse, P., Quaiser, A., Duhamel, M., Le Van, A., and Dufresne, A. (2015). The importance of the microbiome of the plant holobiont. *New Phytol.* 206, 196–206. doi: 10.1111/nph.13312

Visconti, F., de Paz, J. M., Bonet, L., Jordà, M., Quinones, A., and Intrigliolo, D. S. (2015). Effects of a commercial calcium protein hydrolysate on the salt tolerance of Diospyros kaki L. cv. "Rojo Brillante" grafted on *Diospyros lotus* L. *Sci. Hortic.* 185, 129–138. doi: 10.1016/j.scienta.2015.01.028

Vorholt, J. A. (2012). Microbial life in the phyllosphere. *Nat. Rev. Microbiol.* 10, 828–840. doi: 10.1038/nrmicro2910

Wang, H. J., Wu, L. H., Wang, M. Y., Zhu, Y. H., Tao, Q. N., and Zhang, F. S. (2007). Effects of amino acids replacing nitrate on growth, nitrate accumulation, and macroelement concentrations in Pak-choi (*Brassica chinensis* L.). *Pedosphere* 17, 595–600. doi: 10.1016/S1002-0160(07)60070-8

Williams, T. R., and Marco, M. L. (2014). Phyllosphere microbiota composition and microbial community transplantation on lettuce plants grown indoors. *mBio* 5:e01564-14. doi: 10.1128/mBio.01564-14

Wilsey, B. J., and Potvin, C. (2000). Biodiversity and ecosystem functioning: importance of species evenness in an old field. *Ecology* 81, 887–892. doi: 10.1890/0012-9658

Wilson, H. T., Xu, K., and Taylor, A. G. (2015). Transcriptome analysis of gelatin seed treatment as a biostimulant of cucumber plant growth. *Sci. World J.* 2015:391234. doi: 10.1155/2015/391234

Wu, C. H., Bernard, S. M., Andersen, G. L., and Chen, W. (2009). Developing microbe–plant interactions for applications in plant-growth promotion and disease control, production of useful compounds, remediation and carbon sequestration. *Microbiol. Biotechnol.* 2, 428–440. doi: 10.1111/j.1751-7915.2009.00109.x

Yakhin, O. I., Lubyanov, A. A., Yakhin, I. A., and Brown, P. H. (2017). Biostimulants in plant science: a global perspective. *Front. Plant Sci.* 7:2049. doi: 10.3389/fpls.2016.02049

Yeoh, Y. K., Paungfoo-Lonhienne, C., Dennis, P. G., Robinson, N., Ragan, M. A., Schmidt, S., et al. (2016). The core root microbiome of sugarcanes cultivated under varying nitrogen fertilizer application. *Environ. Microbiol.* 18, 1338–1351. doi: 10.1111/1462-2920.12925

Zarraonaindia, I., Owens, S. M., Weisenhorn, P., West, K., Hampton-Marcell, J., Lax, S., et al. (2015). The soil microbiome influences grapevine-associated microbiota. *mBio* 6, e02527-14. doi: 10.1128/mBio.02527-14

Conflict of Interest Statement: The authors declare that the research was conducted in the absence of any commercial or financial relationships that could be construed as a potential conflict of interest.

The reviewer AQ and handling Editor declared their shared affiliation.

frontiers in Plant Science

ORIGINAL RESEARCH
published: 16 February 2018
doi: 10.3389/fpls.2018.00154

Arbuscular Mycorrhiza Alleviates Restrictions to Substrate Water Flow and Delays Transpiration Limitation to Stronger Drought in Tomato

Michael Bitterlich, Martin Sandmann and Jan Graefe*

Leibniz-Institute of Vegetable and Ornamental Crops, Großbeeren, Germany

Arbuscular mycorrhizal fungi (AMF) proliferate in soil pores, on the surface of soil particles and affect soil structure. Although modifications in substrate moisture retention depend on structure and could influence plant water extraction, mycorrhizal impacts on water retention and hydraulic conductivity were rarely quantified. Hence, we asked whether inoculation with AMF affects substrate water retention, water transport properties and at which drought intensity those factors become limiting for plant transpiration. *Solanum lycopersicum* plants were set up in the glasshouse, inoculated or not with *Funneliformis mosseae*, and grown for 35 days under ample water supply. After mycorrhizal establishment, we harvested three sets of plants, one before (36 days after inoculation) and the second (day 42) and third (day 47) within a sequential drying episode. Sampling cores were introduced into pots before planting. After harvest, moisture retention and substrate conductivity properties were assessed and water retention and hydraulic conductivity models were fitted. A root water uptake model was adopted in order to identify the critical substrate moisture that induces soil derived transpiration limitation. Neither substrate porosity nor saturated water contents were affected by inoculation, but both declined after substrates dried. Drying also caused a decline in pot water capacity and hydraulic conductivity. Plant available water contents under wet (pF 1.8–4.2) and dry (pF 2.5–4.2) conditions increased in mycorrhizal substrates and were conserved after drying. Substrate hydraulic conductivity was higher in mycorrhizal pots before and during drought exposure. After withholding water from pots, higher substrate drying rates and lower substrate water potentials were found in mycorrhizal substrates. Mycorrhiza neither affected leaf area nor root weight or length. Consistently with higher substrate drying rates, AMF restored the plant hydraulic status, and increased plant transpiration when soil moisture declined. The water potential at the root surface and the resistance to water flow in the rhizosphere were restored in mycorrhizal pots although the bulk substrate dried more. Finally, substrates colonized by AMF can be more desiccated before substrate water flux quantitatively limits transpiration. This is most pronounced under high transpiration demands and complies with a difference of over 1,000 hPa in substrate water potential.

Keywords: arbuscular mycorrhiza, water retention, drought, tomato, transpiration, soil properties, hydraulic conductivity, root water uptake

OPEN ACCESS

Edited by:
Youssef Rouphael,
University of Naples Federico II, Italy

Reviewed by:
Alessandra Salvioli,
Università degli Studi di Torino, Italy
Jan Jansa,
Institute of Microbiology, Academy of Sciences of the Czech Republic, Czechia

****Correspondence:***
Michael Bitterlich
bitterlich@igzev.de

Specialty section:
This article was submitted to Plant Microbe Interactions, a section of the journal Frontiers in Plant Science

Received: *24 November 2017*
Accepted: *29 January 2018*
Published: *16 February 2018*

Citation:
Bitterlich M, Sandmann M and Graefe J (2018) Arbuscular Mycorrhiza Alleviates Restrictions to Substrate Water Flow and Delays Transpiration Limitation to Stronger Drought in Tomato. Front. Plant Sci. 9:154. doi: 10.3389/fpls.2018.00154

INTRODUCTION

Biostimulants in agri- and horticulture are defined as substances or microorganisms applied to plants in minute quantities aiming to improve crop quality traits, stress tolerance and nutrient efficiency, without being mineral nutrients, soil improvers or pesticides, which are applied in high quantities (du Jardin, 2015). Those involve humic acids, protein hydrolysates, seaweed extracts, biopolymers (Colla et al., 2015; du Jardin, 2015) and beneficial microbes such as plant growth promoting rhizobacteria (Ruzzi and Aroca, 2015) and arbuscular mycorrhizal fungi (AMF) (Rouphael et al., 2015). AMF in particular are considered as potential biostimulants, because they are able to colonize many important crop species and contribute to the abiotic stress tolerance, disease resistance and nutrient acquisition of their hosts (Rouphael et al., 2015). The provision of phosphorus (P) from soil to plants by AMF is well documented and is based on the increased surface area for P absorption provided by hyphae that proliferate in substrates beyond relatively short ranged root P depletion zones (Smith and Read, 2008). When extraradical hyphae spread in soils or substrates, they will penetrate areas beyond the ambit of roots and compete for resources and space with roots and other microbes. Soils are self-organizing systems, their structure builds up hierarchically and AMF are important contributors (Tisdall and Oades, 1982; Milleret et al., 2009; Daynes et al., 2013). Filamentous AMF hyphae are seen as sticky-string bags that influence physical and chemical properties of the substrate area around them by exudation, enmeshment and entanglement of particles and, the release of intrahyphal components during turnover (Miller and Jastrow, 2000). They have physical contact with roots, have direct access to plant derived carbon and constitute a network, which redistributes organic carbon in the soil (Miller and Jastrow, 2000). In those ways, AMF can contribute positively to the formation and stabilization of aggregation in soils (Augé et al., 2001; Rillig et al., 2002; Piotrowski et al., 2004; Rillig and Mummey, 2006), can affect soil water repellency (Rillig et al., 2010) and logically would affect pore volume, pore distribution and wettability. Similar to roots (Bodner et al., 2014), AMF influence soil structure, which has been investigated extensively, but quantification of the impact of mycorrhizal effects on soil hydraulic properties remains less clear and has gained surprisingly little attention (Querejeta, 2017). Vice versa, an understanding of soil properties is crucial for the efficient use of AMF in biological systems (Frey and Ellis, 1997).

Substrate water retention characteristics and hydraulic conductivity are specific for every soil or substrate and depend on texture (particle size distribution) and structure (particle arrangement) (Querejeta, 2017). The latter could be affected by AMF and in turn, AMF could affect substrate water retention (Augé et al., 2001) and hydraulic conductivity. If influenced by AMF, changes in water retention and hydraulic conductivity could have significant impacts on the ability of the host to extract water and the stress plants would experience at particular levels of substrate moisture. Under moisture stress, AMF may confer drought tolerance to hosts (Ruiz-Lozano et al., 1995; Augé, 2001; Augé et al., 2015). Under defined drought conditions, colonized plants often grow better than their non-mycorrhizal counterparts (e.g., Ruiz-Lozano et al., 1995), stomatal conductance is often enhanced under drought (Khalvati et al., 2005; Augé et al., 2015) and higher drying rates were observed in substrates that contain such symbiotic plant-fungus associations (Khalvati et al., 2005; Ruth et al., 2011). Several hypotheses of underlying mechanisms do exist, but it was also shown that non-host mutant plants growing in a mycorrhizal soil can maintain stomata opening, although there is no functional symbiosis (Augé, 2004). This indicates a substrate originated effect and might be related to changes in substrate water retention and transport properties, which could be caused by e.g., formation of soil structure, a higher degree of particle-particle contact and increases and conservation of pore connectivity by proliferation into additional pores. However, up to date, mycorrhizal effects on water retention have been scarcely examined (Augé et al., 2001, 2004; Bearden, 2001; Daynes et al., 2013) and, to our best knowledge, AMF effects on substrate conductivity as a strict physical property are not yet reported.

Roots do affect soil structure (Bodner et al., 2014) and induction of aggregate formation by roots may be dominant over mycorrhizal effects (Hallett et al., 2009), but roots and AMF together can result in largest changes in substrate hydraulic characteristics (Daynes et al., 2013). In the past, scientist have used root free compartments to study mycorrhizal functioning (George et al., 1992; Ruth et al., 2011), but the realistic scenario for the host is a substrate that contains roots and AMF. Especially, when host physiological responses to substrate properties are examined, roots should not be excluded. Thus we chose an experimental design that uses tomato as the host, which is one of the most important vegetable crops worldwide, compatible with many AMF species and, which allows avoidance of large confounding effects by biomass development. To our experience and to that of others, healthy wild type tomato plants frequently lack strong growth responses, although a functional symbiosis was verified by root colonization, nutritional, physiological and/or metabolic reactions (Pozo et al., 1999; Smith et al., 2004; Neumann and George, 2005; Boldt et al., 2011; Rivero et al., 2015). Tomato plants of similar size were grown under unlimited water supply and exposed to a subsequent drying episode. We asked whether AMF can affect plant water availability (plant available water content), extractability (substrate water potential) and water transport through the substrate (hydraulic conductivity) in equally rooted substrates. To elucidate the physiological relevance of substrate property alterations, we integrated root morphology and substrate hydraulic properties by adopting a root water uptake model. We investigated whether AMF induce changes in the critical substrate water potential that limits plant transpiration under different atmospheric demands.

MATERIALS AND METHODS

Plant Growth, Inoculation, and Experimental Design

Two weeks after germination in wet sand in the greenhouse, 100 tomato plants (*Solanum lycopersicum* cv. Moneymaker)

were transplanted at the three-leaf-stage in 4 L open pots with 3.5 L of a sand/vermiculite mixture (sand: grain size 0.2–1 mm; Euroquarz, Ottendorf-Okrilla, Germany, vermiculite: agra-vermiculite, Pullrhenen, Rhenen, The Netherlands; 1:1 v:v) and set up in the greenhouse in a randomized block design with four blocks. Temperature regulation was set to 22: 17°C (day: night), relative humidity was between 50 and 75% during the day and intensity of photosynthetic active radiation (PAR) at canopy height ranged from 150 to 660 μmol $m^{-2}s^{-1}$. Half of the plants were inoculated with *Funneliformis mosseae* BEG12 (MycAgro Laboratory, Breteniere, France) with 10% of the substrate volume. The inoculum carrier material was a mixture of clay and zeolite. Non-mycorrhizal (NM) counterparts were inoculated with a filtrate of the inoculum and the same amount of autoclaved inoculum (2 h, 121°C). The filtrate was produced for every pot by filtration of 200 mL deionized water through Whatman filter (particle retention 4–7 μm; GE Healthcare Europe GmbH, Freiburg, Germany) containing approx. 200 mL of inoculum. The same amount of deionized water (200 mL) was added to mycorrhizal pots. The pots were fertilized approx. every other day the first 3 weeks and subsequently every day with 400 mL of nutrient solution (De Kreij et al., 1997; 40% of full strength) with 10% of the standard phosphate to guarantee good colonization (N: 10.32 mM; P: 0.07 mM, K: 5.5 mM, Mg: 1.2 mM, S: 1.65 mM, Ca: 2.75 mM, Fe: 0.02 mM, pH: 6.2, EC: 1.6 mS). Until the start of the drying cycle 35 days after inoculation and transplanting, ample water conditions were maintained by irrigating with deionized water until pot water capacity and additional water was applied to plates under the pots to guarantee water accessibility throughout whole daytimes. At day 36, all plants were irrigated in the morning to a total of 1,500 mL (≈ pot water capacity), which was sufficient to maintain ample water conditions (WW) during day 36. Afterwards, water was subsequently withheld from pots and two additional harvests were done under water-deficient conditions at day 42 (WD1) and day 47 (WD2).

Leaf Area, Root Morphology, and Fungal Colonization

Leaf area (LA) was measured with the LI-3100 Area Meter (LICOR, Lincoln, USA). For leaves that required immediate sampling, leaf length was measured from the first pinnate to the distal end and, area was estimated after Schwarz and Klaering (2001).

Root systems were carefully washed and analyzed with image processing software WinRHIZO Arabidopsis 2012b (Regent instruments, Québec, Canada). Before (further) analysis, washed roots were centrifuged in a common salad spinner to discard adhesive water. The whole root system was divided into three parts (0–5, 5–20, and >20 cm of depth from top), individual parts were weighed and a representative subsample of 25% of fresh matter was analyzed. Weight based upscaling to the bulk root was performed to assess total root length, surface, volume, the mean root diameter and root length density (L_v) in the substrate volume.

Fungal staining was done with Trypan blue modified after Koske and Gemma (1989). A fine root subsample of 2 g was stored in 15% ethanol, incubated for 20 min at 60°C in 10% KOH, subsequently acidified for 2 min in 2 N HCl and then stained in 0.05% trypan blue in lactic acid for 20 min at 60°C. The percent of mycorrhizal colonization was assessed on 100 root pieces by the grid line intersection method (Giovannetti and Mosse, 1980).

Substrate Hydraulic Conductivity and Substrate Water Potential

We used the simplified evaporation method (Schindler, 1980), which is a continuous dry out of a substrate sample under laboratory conditions. Before planting, standard soil sampling cores ($V = 250$ mL, $h = 5$ cm) were introduced into a subset of pots ($n = 4$–6 per treatment and harvest date) in a way that the cylinder diameter covered the central section of the substrate filling level and the depth of the cylinder covered the radius from the center to the rim of the pot. The cylinders were covered with a 2 mm mesh that allowed root and fungal ingrowth and undisturbed harvesting of the incorporated substrate. Roots were cut with a sharp knife along the outside of the mesh while the cylinder remained in the pot. After harvest, sampling cores were weighed and water saturated for 24 h. All measurements were done with the HYPROP system (UMS GmbH, Munich, Germany) according to Peters and Durner (2008). Two tensiometers in different heights (1.25 and 3.75 cm) were introduced into the soil core. The tension was recorded every 10 min and water loss was determined by weighing at least two times a day, resulting in retention functions of the volumetric water content (Θ) at the average tension (h) of both tensiometers which equal the bulk substrate water potential (Ψ_S). During the measurement water evaporates from the sample surface and the measurement is terminated when air enters the tensiometer ceramic and the tension drops down to 0 hPa. After termination, substrate samples were dried (105°C, 24 h) to obtain the substrate dry mass. For very low water potentials subsamples of the substrate have been taken and measured with C-30 chambers (Wescor Inc., Logan, USA), containing a wet bulb depression psychrometer connected to a PSYRO water potential data logger (Wescor Inc., Logan, USA). After 15 min of temperature equilibration in a water bath (22°C), data were logged every 5 min until occurrence of a plateau. The obtained values were added to the water retention data.

Assuming half of the water flow for evaporation deriving from the upper cylinder height and a linear gradient of volumetric water content (Θ) from bottom to top, a function for the hydraulic conductivity $K(h)$ can be estimated as:

$$K(h_i) = \frac{0.5q}{\frac{\Delta h}{z_1 - z_2} - 1}, \quad (1)$$

where q is the water flow, Δh is the mean tension difference of the two tensiometers and z_i are the depths of the tensiometers (Peters and Durner, 2008).

Although made for soils, water retention models can also be used for substrate mixes (Fonteno, 1992). Thus, several water retention models were tested (van Genuchten unimodal, van

Genuchten bimodal, Brooks and Corey, Ross-Smettem, Fayer-Simmons, Kosugi). Their performance was evaluated based on the Akaike Information Criterion (AICc) for finite sample sizes (Akaike, 1974), which penalizes model complexity, so candidate models with minimum AICc are preferred. To the relationship of Θ vs. Ψ_S the bimodal van Genuchten model for water retention was fitted (Durner, 1994) and parameters were estimated with the HYPROP-DES software (UMS GmbH, Munich, Germany). The model allows a mixture of two pore size distributions, which is reasonable for a two component substrate:

$$S_e(h) = \sum_{i=1}^{2} \omega_i \left(\frac{1}{1+(\alpha_i |h|)^{n_i}} \right)^{1-\frac{1}{n_i}}, \quad (2)$$

where S_e is the effective saturation defined as $S_e = (\Theta - \Theta_r)/(\Theta_S - \Theta_r)$ (Mualem, 1976) with Θ_r and Θ_S are the residual and saturated water content, respectively. ω_i is a weighing factor, n_i is the pore size distribution parameter and α_i is the reciprocal potential at the air entry water tension of the substrate. The input is the geometric mean of h of both tensiometers (Peters and Durner, 2008). Based on the estimated retention model, the volumetric substrate water content at the time of harvest (Θ_H) was used to compute the substrate water potential at time of harvest (Ψ_{SH}).

The retention function was coupled to a model for hydraulic conductivities (K) in unsaturated porous media (Mualem, 1976):

$$K(h) = K_s S_e^{\tau} \left(\frac{\int_0^{S_e} h^{-1}\, dS_e(h)}{\int_0^1 h^{-1} dS_e(h)} \right)^2, \quad (3)$$

where K_S and τ are the saturated conductivity and a pore tortuosity parameter, respectively. Overall 9 parameters were identified simultaneously from combined retention and conductivity data: Θ_S, Θ_r, w_2, α_1, α_2, n_1, n_2, K_S, and τ. Parameter estimation was carried out as described in Peters and Durner (2008).

Computation of Derived Hydraulic Parameters

The matrix flux potential (M), which is defined as:

$$M(h) = \int_h^{h_{PWP}} K(h)\, dh \quad (4)$$

has been shown to be a useful parameter to describe direct soil water limitations as the instantaneous water influx ($T_{p,s}$) occurring at a maximum soil water potential gradient between the bulk soil (h) and the root surface ($\max(h_{rs}) = h_{\mathrm{PWP}} = 15{,}000$ cm, $M(h_{\mathrm{PWP}}) = 0$)

$$T_{p,s}(h) = \rho\left(M(h) - M(h_{rs})\right) \text{ and } \rho = \frac{4z}{r_0^2 - a^2 r_m^2 + 2(r_m^2 + r_0^2)\ln\left(\frac{a r_m}{r_0}\right)} \quad (5)$$

of a proxy rhizosphere model geometry (ρ) with the root radius r_0, the mean half inter-root distance r_m, the pot height z and the relative location of the bulk substrate water potential a (0.53) (de Jong van Lier et al., 2008, 2013). Using a prescribed atmospherically demanded potential transpiration ($T_{p,a}$), the extent of direct substrate originating water limitations can be expressed as relative transpiration:

$$rT(h) = min\left[\frac{T_{p,s}(h)}{T_{p,a}}, 1\right]. \quad (6)$$

The water potential at the root surface (h_{rs}) was also calculated from Equation (5), but using the measured plant transpiration rate (T_a) at the time of plant harvest in place of $T_{p,s}$. Adopting the root conductance relation from de Jong van Lier et al. (2013) the resistance of water flow toward roots (R_{SOIL}) present at harvest dates was calculated as:

$$R_{SOIL} = \frac{2z(h_r - h)}{T_a r_m^2 (a r_m / r_0)}, \quad (7)$$

where mean values have been used for bulk substrate water potential (h) and actual transpiration T_a to enable treatment comparison at the same soil water content and water flux. Apart from commonly used uniquely sized rhizospheres with a unique radius $r_m = (1/\pi L_V)^{0.5}$, we followed a recently proposed approach (Graefe and Bitterlich, submitted), which allows for different rhizosphere sizes $r_{m,i}$ following the distance distribution of a 2D Poisson point process (Moltchanov, 2012). So, Equation (5) is rewritten as:

$$T_{p,s}(h) = \overline{\rho}(M(h) - M(h_r), \quad (8)$$

where now a mean parameter function $\overline{\rho}$ is computed over i rhizosphere size classes (Graefe and Bitterlich, submitted).

The volumetric water content Θ in the range between field capacity (FC, pF = 1.8) and the permanent wilting point (PWP, pF = 4.2) is termed plant available water content (PAW), because a proportion of water residing in macro pores would be lost by draining at degrees of saturation that correspond to pF values lower than FC. And, at water contents corresponding to a pF higher than PWP, water resides in micro pores that cannot be extracted by plants (Blume et al., 2009)

Plant Water Potentials and Evapotranspiration

Water potentials of the compound leaf xylem (Ψ_L) and the root system (Ψ_R) were determined using a pressure chamber (Scholander et al., 1965). The first fully expanded leaf was cut at the main stem Both, the leaf and the root system including a short stem base (~5 cm) were covered with plastic foil and immediately inserted into a SKPM 1400 pressure chamber (UP GmbH, Cottbus, Germany). Roots were obtained carefully from pots, separated from adhering substrate while avoiding root tissue damage.

Pressure was increased at a rate of 0.1 bars s^{-1} until the meniscus of xylem sap appeared at the cut surface. For measurements of the leaf lamina potential, leaf discs (2 cm in diameter) have been put into a C-52 sample chamber

(Wescor Inc., Logan, USA) containing a wet bulb depression psychrometer connected to a PSYRO water potential data logger (Wescor Inc., Logan, USA). After 15 min of temperature equilibration in a polystyrol box, data were logged every 5 min until occurrence of a plateau. The reference temperature was 22°C. The same part of the lamina was then squashed to destroy cells to determine the osmotic potential accordingly. Turgor pressure (Ψ_T) was calculated as the difference between the leaf lamina potential and the osmotic potential of the leaf disc.

Plant evapotranspiration was analyzed by weighing of pots every hour during daytime on days of the respective harvests and additionally on day 39 between WW and WD1. For the respective harvest times, larger sets of plants ($n \geq 15$) were used to estimate actual transpiration rates by fitting a third order polynomial to the daytime time courses between 9 a.m. and 6 p.m.

Statistical Analysis

Statistical analysis ($\alpha = 0.05$; normal distribution, homogeneity of variances, ANOVA, *t*-test and regressions) were computed with STATISTICA 12 software (StatSoft, Tulsa, OK, USA). In case of violation of assumptions data sets have been log transformed. The goodness of fit (root mean squared error) and the AICc for model selection was computed with the HYPROP-DES software (UMS GmbH, Munich, Germany).

RESULTS

Plant Growth and Substrate Hydraulic Properties

In order to relate substrate hydraulic properties to plant and fungal growth in a pot specific manner, **Tables 1**, **2** display data obtained only from those pots that contained sampling cores for hydraulic assessments. Please refer also to the Supplementary Material, which is in the following referred to as S1 to S5.

NM roots did not contain fungal structures. The mean AMF root colonization intensity was 16.4, 25.9, and 30.5% at WW, WD1, and WD2, respectively (see also **Figure 4**). We found intraradical hyphae and arbuscules in all cases, indicating the establishment of a functional symbiosis (not shown).

Plants did not entirely stop growing during the drying episode as root and leaf biomass increased with harvest dates (**Table 1**). Leaf area did not change during that time, likely resulting from a shrinking lamina due to leaf desiccation, because leaves stayed vital during that period. Thus, root/shoot dry weight ratios were not different between harvest dates, but the root/leaf area ratio slightly increased ($P = 0.056$; **Table 1**). Roots were growing by approx. 2.5 cm per cm^3 substrate volume from WW to WD1, but nearly entirely stopped growing between WD1 and WD2 (**Table 2**). Root surface and volume densities developed accordingly. With exception of slight mycorrhizal effects on root diameters, none of the plant growth parameters was influenced by inoculation with *F. mosseae* and no interaction between the factors harvest date and inoculation was detected. Root volumes constituted 5.16, 6.45, and 6.49% of the substrate volume at WW, WD1 and WD2, respectively. The marginal differences between mycorrhizal and NM pots in root volume densities did not exceed 0.45% of the substrate volume. The coarsely textured substrate had a low bulk density and high porosity (**Table 3**). During the drying phase total dry porosity declined and a loss of water volume that can be withheld during saturating of the substrate sample, i.e., the saturated water content (Θ_{SAT}; **Table 3**) was observed. Importantly, those soil parameters were not affected by AMF.

Losses of total porosity from WW to WD2 may partly offset the water retention curves toward lower water contents (**Figure 1**; Table S1). The substrate lost a proportion of its water capacity when sampled during the drying phase (WD1 and WD2). We fitted the water retention model to every individual dataset, resulting in replicates of water content values at particular reference water potentials. The water content at the so called "field capacity" (FC, pF = 1.8) declined from 22 to 14.6% and 12.8% in average at WW, WD1, and WD2, respectively and was only marginally affected by the mycorrhizal treatment (see Table S1). In contrast, mycorrhizal inoculation caused a change in water contents that correspond to a particular level of water potential in the plant relevant range from FC to the PWP. When harvested after withholding water (WD1 and WD2), Θ comparatively starts to decline at pF = 2.5 in colonized substrates and was significantly lower from pF = 3.5 (see also Table S1). Consistently in all three harvests, the pF in colonized substrates declined less per unit of Θ (see Table S2) as soon as pF = 2 was approached.

Interestingly, mycorrhization enhanced plant available water contents under moist (pF = 1.8–4.2) and dry conditions (pF = 2.5–4.2) up to 75 and 56 mL, respectively (**Figure 2**). This was fairly conserved when corrected for the root length (**Figure 2**, Table S3).

Another important characteristic of substrates is the water transport capacity through the pore space, i.e., the hydraulic conductivity (K). We found K invariably increased in mycorrhizal substrates at all three harvest dates between pF of 1.8 and 4.2, but also under water saturation (**Figure 3**, Table S4). K is shown on a logarithmic scale. At WD2 the actual enhancement of average K in mycorrhizal substrates in the FC - PWP moisture range was up to 300% (**Figure 4**). Absolute values of Θ(pF) and K(pF) declined with harvest time, once substrates started to desiccate. Therefore, we calculated mycorrhizal response ratios with the mean observed in NM pots as the basis. And, AMF stimulation of K from FC to PWP and plant available water contents responded similarly to root colonization intensities observed at the respective harvest dates (**Figure 4**).

Plant Physiological Responses and Substrate Depletion during Drying

Substrates that contained *F. mosseae* were characterized by higher plant available water contents and a lower resistance to water flow (inverse of conductivity) in the plant relevant range of substrate moisture potentials, therefore enhancing the potential supply of water. The water flow toward the root system as driven by plant transpiration will depend on these substrate hydraulic properties. Vice versa, plants are able to sense substrate moisture stress

TABLE 1 | Plant development and biomass allocation of mycorrhizal (AM) and non-mycorrhizal (NM) substrates as present at the three harvests (WW, WD1, WD2) during the drying episode.

Variable	Inoculation	Harvest time			ANOVA		
		WW	WD1	WD2	Harvest	Inoculation	H × I
					$F_{(2, 23)}$ P	$F_{(1, 23)}$ P	$F_{(2, 23)}$ P
Plant fresh matter [g]	NM	275.0 ±10.9	312.2 ± 7.8	324.1 ± 4.5	**(15.48) < 0.001**	(0.08) 0.778	(0.18) 0.841
	AM	275.7 ± 6.3	313.1 ± 12.3	316.4 ± 8.1			
		A	B	B			
Leaf dry matter [g]	NM	16.7 ± 0.39	19.9 ± 0.63	20.5 ± 0.30	**(26.22) < 0.001**	(4.04) 0.056	(0.20) 0.820
	AM	15.2 ± 0.70	19.3 ± 0.80	19.5 ± 0.63			
		A	B	B			
Root dry matter [g]	NM	1.54 ± 0.27	1.91 ± 0.13	1.78 ± 0.07	**(5.732) < 0.001**	(0.66) 0.425	(0.65) 0.532
	AM	1.29 ± 0.10	1.82 ± 0.18	1.85 ± 0.09			
		A	B	B			
Leaf area [dm^2]	NM	40.4 ± 1.90	40.4 ± 2.06	40.8 ± 2.41	(0.572) 0.572	(2.78) 0.109	(0.73) 0.491
	AM	42.1 ± 0.97	45.9 ± 1.89	41.7 ± 1.70			
		A	A	A			
Root area [dm^2]	NM	43.5 ± 4.03	53.2 ± 1.27	51.1 ± 1.61	(10.03) < 0.001	(0.29) 0.597	(0.20) 0.823
	AM	43.0 ± 1.57	54.7 ± 3.71	53.4 ± 1.35			
		A	B	B			
Root/shoot ratio	NM	0.063 ± 0.01	0.060 ± 0.01	0.051 ± 0.01	(2.010) 0.157	(0.13) 0.724	(1.35) 0.280
	AM	0.057 ± 0.01	0.063 ± 0.01	0.058 ± 0.01			
		A	A	A			
Root/leaf area ratio	NM	1.10 ± 0.15	1.32 ± 0.10	1.28 ± 0.10	(3.327) 0.054	(0.61) 0.443	(0.30) 0.742
	AM	1.02 ± 0.02	1.20 ± 0.10	1.30 ± 0.08			
		A	A	A			

The three harvests occurred 36 days after inoculation under ample water conditions (WW) and 42 and 47 days under water deficient conditions (WD1 and WD2, respectively) after withholding water. The data (mean ± SE, n = 4–6) was analyzed by two way ANOVA (α = 0.05) with significant P-values highlighted in bold. Different capital letters indicate significant differences between harvest dates and asterisks (second column) indicate whether inoculation caused a significant effect (Tukey HSD).

and can quickly adjust transpiration to avoid or delay wilting without direct or relaxed hydraulic feedbacks. To study that, we determined substrate water contents and water potentials present at the time of harvest (Θ_H, Ψ_{SH}).

From WW to WD2 a stronger decline in substrate water contents at harvest was observed in mycorrhizal pots. Both NM and mycorrhizal pots approached plant unavailable water contents (Θ_{PWP}) at the last harvest (**Table 4**). Consistently, substrate water potentials were higher (in means of pF) in mycorrhizal pots at WD1, concurring with higher cumulative evapotranspiration rates. Since the amount of water residing in plants was not altered by AMF inoculation (**Table 4**), more water was flowing through the mycorrhizal substrate-plant-air continuum between the first two harvests. Cumulative evapotranspiration was about 85 mL higher in colonized pots in the end, which is in good agreement with the improvement in PAW in the substrate observed at WD2 (≈72 mL, see **Figure 2**). Whole plant transpiration rates were calculated as the average weight loss from harvest to harvest subtracted by the average biomass increment. In mycorrhizal pots, whole plant transpiration rates were declining less between WW and WD1 and stronger between WD1 and WD2. Based on the findings in water retention and the observation made directly at harvest date, the significant interaction in evapotranspiration rates is consistent. From WW to WD1 transpiration depleted the substrate to a degree of saturation where the stress (pF) in mycorrhizal pots declined less per unit water content and substrate conductivity was higher. Remarkably, leaf turgidity, the leaf xylem water potential and the root water potential were equal in NM and mycorrhizal pots, although mycorrhizal plants grew in stronger water depleted substrates (**Table 4**). At WD2 leaves of two NM and two mycorrhizal plants lost turgidity. Hence, NM and mycorrhizal plants were equally on the brink of wilting at WD2 and the mean negative turgidity of colonized plants at WD2 is not indicating a general turgor loss. Within the rhizosphere, the resistance of water flow toward roots (R_{SOIL}) and the water potential estimated at the root surface (Ψ_{RS}) was conserved in colonized pots, although mycorrhizal substrates were more desiccated (**Table 4**).

TABLE 2 | Root development of mycorrhizal (AM) and non-mycorrhizal (NM) substrates as present at the three harvests (WW, WD1, WD2) during the drying episode.

Variable	Inoculation	Harvest time			ANOVA		
		WW	WD1	WD2	Harvest	Inoculation	H × I
					$F_{(2, 23)}$ P	$F_{(1, 23)}$ P	$F_{(2, 23)}$ P
Root length density [cm cm^{-3}]	NM	9.11 ± 0.94	12.01 ± 0.38	12.26 ± 0.34	**(16.15) < 0.001**	(1.51) 0.232	(0.08) 0.920
	AM	9.77 ± 0.36	12.37 ± 0.91	13.12 ± 0.50			
		A	B	B			
Root surface density [cm^2 cm^{-3}]	NM	1.24 ± 0.12	1.54 ± 0.04	1.57 ± 0.05	**(15.82) < 0.001**	(0.31) 0.583	(0.21) 0.812
	AM	1.22 ± 0.05	1.59 ± 0.11	1.64 ± 0.04			
		A	B	B			
Root volume density [mm^3 cm^{-3}]	NM	53.9 ± 4.15	63.9 ± 0.99	63.7 ± 2.53	**(14.14) < 0.001**	(0.01) 0.917	(0.91) 0.415
	AM	49.4 ± 1.79	65.2 ± 4.23	66.3 ± 1.06			
		A	B	B			
Root diameter [mm]	NM	0.44 ± 0.01 b	0.41 ± 0.01 ab	0.41 ± 0.01 b	**(3.720) 0.040**	**(9.11) 0.006**	**(5.21) 0.014**
	AM	0.40 ± 0.01 a	0.41 ± 0.01 b	0.40 ± 0.01 b			

The three harvests occurred 36 days after inoculation under ample water conditions (WW) and 42 and 47 days under water deficient conditions (WD1 and WD2, respectively) after withholding water. The data (mean ± SE, n = 4–6) was analyzed by two way ANOVA (α = 0.05) with significant P-values highlighted in bold. In case of significant interaction, values followed by the same small letter are not significantly different (Tukey HSD). Different capital letters indicate significant differences between harvest dates and asterisks (second column) indicate whether inoculation caused a significant effect (Tukey HSD).

TABLE 3 | Substrate properties of mycorrhizal (AM) and non-mycorrhizal (NM) substrates as present at the three harvests (WW, WD1, WD2) during the drying episode.

Variable	Inoculation	Harvest time			ANOVA		
		WW	WD1	WD2	Harvest	Inoculation	H × I
					$F_{(2, 23)}$ P	$F_{(1, 23)}$ P	$F_{(2, 23)}$ P
Bulk density [g cm^{-3}]	NM	0.88 ± 0.02	0.92 ± 0.05	0.98 ± 0.01	**(7.01) 0.004**	(0.28) 0.604	(0.64) 0.537
	AM	0.91 ± 0.02	0.92 ± 0.01	0.97 ± 0.02			
		A	AB	B			
Total porosity [–]	NM	0.67 ± 0.01	0.65 ± 0.02	0.63 ± 0.01	**(5.87) 0.009**	(0.40) 0.534	(0.47) 0.632
	AM	0.65 ± 0.01	0.65 ± 0.01	0.64 ± 0.01			
		A	AB	B			
Θ_{SAT} [cm^3 cm^{-3}]	NM	0.56 ± 0.12	0.51 ± 0.05	0.51 ± 0.08	**(16.1) < 0.001**	(1.24) 0.277	(0.35) 0.710
	AM	0.57 ± 0.07	0.53 ± 0.09	0.51 ± 0.09			
		A	B	B			

Θ_{SAT} denotes the saturated water content. The three harvests occurred 36 days after inoculation under ample water conditions (WW) and 42 and 47 days under water deficient conditions (WD1 and WD2, respectively) after withholding water. The data (mean ± SE, N = 4–6) was analyzed by two way ANOVA (α = 0.05) with significant P-values highlighted in bold. Different capital letters indicate significant differences between harvest dates and asterisks (second column) indicate whether inoculation caused a significant effect (Tukey HSD).

Limitation of Transpiration by Restriction of Substrate Water Flow

Measured transpiration rates under ample water conditions (WW) have been highest at noon (1.6 cm d^{-1}) and lowest in the morning and evening (0.4 cm d^{-1}). Mean daytime transpiration rates have been 1.3, 0.9, and 0.6 cm d^{-1} at WW, WD1 and WD2, respectively. The atmospheric conditions during the experiment constituted low to moderate atmospheric demands (T = 22: 17°C, day: night; rH = 50–75%; PAR = 150–660 μmol m^{-2}s^{-1}). Based on our measurements, we chose actual transpiration rates of 2 cm d^{-1} for high atmospheric demands, which would be realistic at higher temperatures, higher light intensities and/or lower rH. For low and moderate atmospheric demands, 0.5 and 1 cm d^{-1} were chosen as scenarios that would apply for growing conditions present in the early and late morning/afternoon, respectively, or under drought.

Figure 5 illustrates the limitation of transpiration by substrate water flux expressed as relative transpiration rates (potential

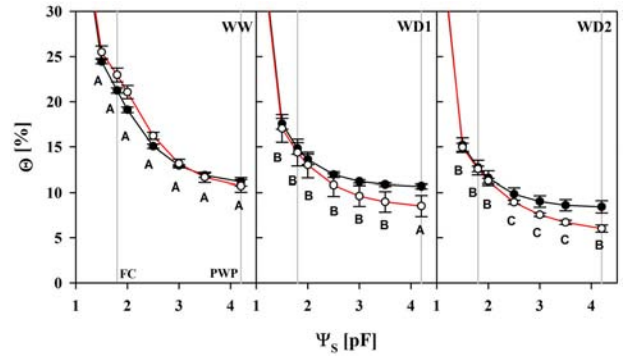


FIGURE 1 | The substrate volumetric water content (Θ) as a function of the substrate water potential (Ψ_S) of mycorrhizal (white) and non-mycorrhizal (black) substrates sampled at the three harvests. The three harvests occurred 36 days after inoculation under ample water conditions (WW) and 42 and 47 days under water deficient conditions (WD1 and WD2, respectively) after withholding water during the drying episode. Significant differences between harvest dates ($p < 0.05$) were detected from $\Psi_S = 1.5$–4.2. Different capital letters indicate significant differences between harvest dates at particular levels of Ψ_S. Signifcant differences between mycorrhizal and non-mycorrhizal plants ($p < 0.05$) were detected at $\Psi_S = 3.5$ and 4.2. No significant interaction was detected (mean ± *SE*, $N = 4$–6, two way ANOVA, Tukey HSD). For absolute values and statistical analyses see also Tables S1, S2.

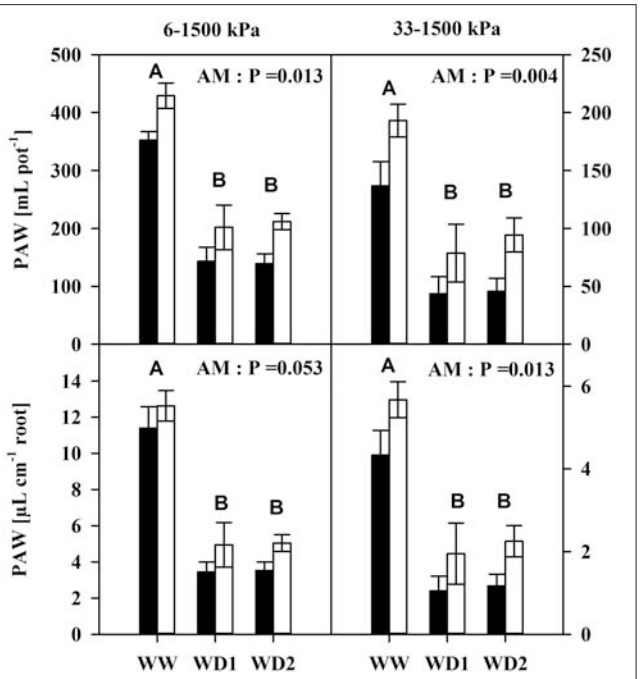


FIGURE 2 | The Plant available water content (PAW) of mycorrhizal (white bars) and non-mycorrhizal (black bars) in substrates **(Top)** and per unit root length **(Bottom)** as present during the three harvests. PAW was quantified as the difference in water content between 6 kPa (pF = 1.8) and 1500 kPa (pF = 4.2) for moist conditions (left), and between 33 kPa (pF = 2.5) and 1,500 kPa for dry conditions (right). The three harvests occurred 36 days after inoculation under ample water conditions (WW) and 42 and 47 days under water deficient conditions (WD1 and WD2, respectively) after withholding water during the drying episode. Significance of factors harvest date (H), inoculation with *F. mosseae* (AM) and their interaction (H × I) was analyzed by two way ANOVA (mean ± *SE*, $N = 4$–6; $\alpha = 0.05$). No significant interaction was detected. Different capital letters indicate significant differences between harvest dates (Tukey HSD) and the *P*-value for main factor inoculation (AM) is shown. For complete ANOVA results please refer to the Table S3.

transpiration allowed by substrates/actual transpiration demands). As long as relative transpiration is equal to 1, the potential substrate water flux is higher than the actual plant transpiration demand and not limiting. Relative transpiration lower than 1, indicates transpiration limitation by substrate water flux.

Relative transpiration already decreased at a lower pF in NM substrates than in colonized substrates (**Figure 5**). The AMF effect at WW (**Figures 5A,C,E**) is not yet significant, but becomes clearly pronounced at the last harvest where AMF root colonization was highest (WD2; **Figures 5B,D,F**). Similar to WW, no significant differences were observed at WD1 (not shown). The critical drought intensity (pF) at which transpiration becomes limited is highest under low transpirative demands (**Figures 5A,B**) and gradually shifts to lower drought intensities at moderate (**Figures 5C,D**) and high transpirative demands (**Figures 5E,F**). When colonized by AMF, substrates harvested at WD2 can provide water at sufficient rates to match high transpirative demands (**Figure 5F**) until a pF of 3.24 is reached. In NM pots, substrate limitation already set in at a pF of 2.83 (**Figure 5F**). Expressed on an absolute basis, under those conditions, mycorrhizal pots are able to fulfill high transpiration demands for an extra substrate water potential depletion of 1,079 hPa. The critical water content (Θ_{CRIT}) where transpiration becomes limited by substrate drought was reduced in mycorrhizal substrates at all three harvest dates and the mycorrhizal effect becomes more pronounced with experiment duration (**Figure 6**; Table S5). The critical soil water potential (Ψ_{CRIT}) was also reduced except at WD1 (**Figure 6**; Table S5), which is coinciding with the smallest differences in hydraulic conductivity between pF 3 and pF 4.2 (see Table S4).

DISCUSSION

As anticipated, we did not observe a growth response to AMF inoculation, but intraradical hyphae, arbuscules and the maintenance of the plant hydraulic state in stronger water depleted substrates indicate a symbiotic relationship, because the latter is a common observation in mycorrhizal experiments. An extensive review (including more than 200 studies, 90 host species and at least 22 AMF species) revealed that in 75% of the cases where soil moisture was measured, mycorrhizal plants were observed to deplete the soil water more thoroughly, before achieving a similar shoot response, which is often not associated with better growth (Augé, 2001). This was verified here. We used a substrate for our study that is reproducible and characterized by a high porosity and a low bulk density. Similar to other horticultural substrates this allows easy extraction of water with a low risk of inducing hypoxia under high saturation (Fonteno, 1992). We are aware that the applied water retention model was originally developed for soils. However, such models can be used also for horticultural substrates (Fonteno, 1992),

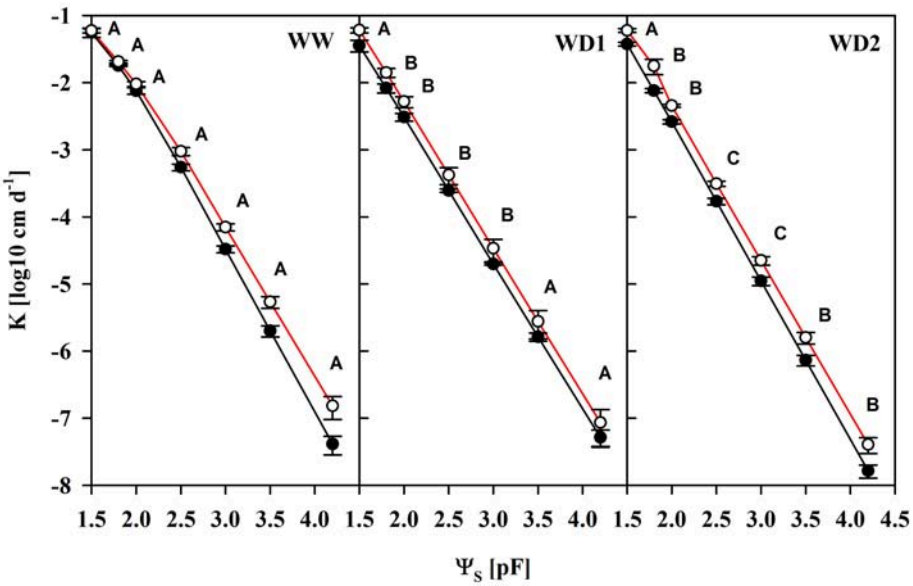


FIGURE 3 | The unsaturated substrate hydraulic conductivity (K) as a function of the substrate water potential (Ψ_S) of mycorrhizal (white) and non-mycorrhizal (black) substrates sampled at three harvests. The three harvests occurred 36 days after inoculation under ample water conditions (WW) and 42 and 47 days under water deficient conditions (WD1 and WD2, respectively) after withholding water during the drying episode. Significant differences between harvesrfddds a t dates ($p < 0.05$) were detected from $\Psi_S = 1.8$–4.2. Different capital letters indicate significant differences between harvest dates at particular levels of Ψ_S. Signifcant differences between mycorrhizal and non-mycorrhizal plants ($p < 0.05$) were detected at $\Psi_S = 1.5$–4.2. No significant interaction was detected (mean ± *SE*, $N = 4$–6, two way ANOVA, Tukey HSD). For absolute values and statistical analyses see also Table S4.

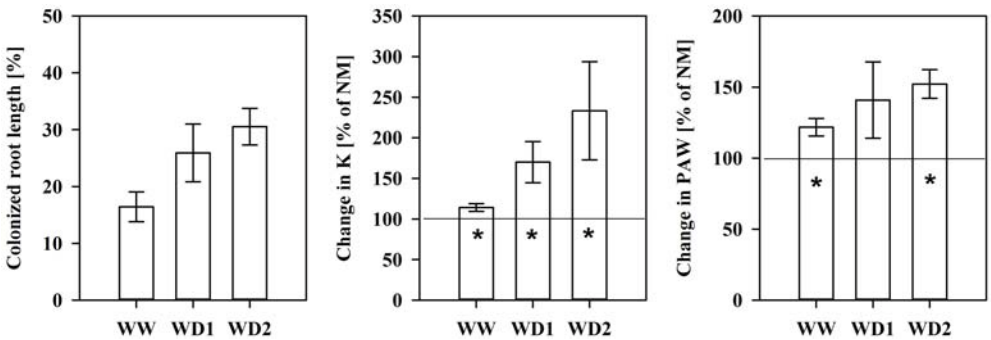


FIGURE 4 | The colonized root length **(Left)**, the relative mycorrhizal improvement in the mean substrate hydraulic conductivity **(Middle)** and plant available water content (PAW, **Right)** between field capacity and the permanent wilting point calculated using the mean of non-mycorrhizal plants as the reference (baseline at 100%) for the harvests during the drying episode. The three harvests occurred 36 days after inoculation under ample water conditions (WW) and 42 and 47 days under water deficient conditions (WD1 and WD2, respectively) after withholding water during the drying episode (mean ± *SE*, $N = 4$–6). Asterisks indicate whether the mean of mycorrhizal plants is higher than that that of non-mycorrhizal plant (*t*-test, $\alpha = 0.05$).

especially when they fit well to measured data as judged by the AICc.

The loss of substrate water capacity with experiment duration could be related to several factors, but the root volume increment alone is not large enough to explain the changes in Θ_{SAT}. There was no substrate shrinkage detected, when substrates desiccated during the water retention measurements. The time of re-saturation could have been too short (24 h), because substrates that contain vermiculite may require longer times to fully saturate than soils (Fonteno, 1992). Increased water repellency could be a factor. Substrate water repellency can increase with root and fungal colonization, and with drought, but longer water contact times can recover wettability (Doerr et al., 2000). Although not predicted, this also constitutes the realistic scenario for the plant during the drying episode and would also apply when pots would be re-irrigated. We found the smallest differences in hydraulic properties at WD1, which requires further research. Substrates containing AMF have been stronger depleted of water in the course of the experiment, which putatively increases repellency and may have partly offset positive

TABLE 4 | Substrate and plant hydraulic properties observed at harvest of mycorrhizal (AM) and non-mycorrhizal (NM) substrates as present at the three harvests (WW, WD1, WD2) during the drying episode.

Variable	Inoculation	Harvest time			ANOVA		
		WW	WD1	WD2	Harvest	Inoculation	H × I
					$F_{(2, 23)}$ P	$F_{(1, 23)}$ P	$F_{(2, 23)}$ P
Θ_H [%]	NM	47.7 ± 0.5	12.7 ± 1.4	8.5 ± 0.9	**(795) <0.001**	**(4.77) 0.039**	(0.20) 0.824
	AM*	46.5 ± 0.9	10.8 ± 1.9	6.3 ± 0.3			
		A	B	C			
Θ_{PWP} [%]	NM	11.2 ± 0.43	10.9 ± 0.3	8.4 ± 0.7	**(14.8) <0.001**	**(7.62) 0.011**	(1.06) 0.364
	AM*	10.7 ± 0.69	9.0 ± 1.1	6.0 ± 0.4			
		A	A	B			
Ψ_{SH} [pF]	NM	1.00 ± 0.02	2.60 ± 0.24	3.48 ± 0.12	**(88.5) <0.001**	**(4.72) 0.041**	(1.15) 0.332
	AM*	1.01 ± 0.01	3.19 ± 0.45	3.81 ± 0.03			
		A	B	C			
Ψ_{RS} [pF]	NM	1.01 ± 0.01	3.56 ± 0.01	3.74 ± 0.02	**(3,409) <0.001**	(1.20) 0.283	(1.50) 0.250
	AM	1.01 ± 0.01	3.57 ± 0.01	3.71 ± 0.01			
		A	B	C			
K_H [log10 cm d^{-1}]	NM	0.28 ± 0.21	−3.79 ± 0.56	−6.12 ± 0.31	**(94.0) <0.001**	(2.84) 0.106	(1.03) 0.372
	AM	0.37 ± 0.06	−5.03 ± 1.08	−6.56 ± 0.13			
		A	B	C			
R_{SOIL} [d cm^{-1}]	NM	$4*10^{-1}$	$2.2*10^{4}$	$2.7*10^{5}$	**(1070) <0.001**	(1.15) 0.294	(2.45) 0.109
	AM	$4*10^{-1}$	$2.7*10^{4}$	$7.7*10^{4}$			
		A	B	C			
Cumulative evapotranspiration [mL]	NM	222.8 ± 6.2 a	1187 ± 25.0 b	1704 ± 32.6 d	**(4,438) <0.001**	**(6.93) 0.015**	**(7.25) 0.002**
	AM*	222.1 ± 4.2 a	1277 ± 19.6 c	1790 ± 30.1 d			
Evapotranspiration rate [mL d^{-1}]	NM	247.8 ± 6.0 d	205.5 ± 4.3 b	122.9 ± 3.3 a	**(420) <0.001**	(1.10) 0.305	**(2.36) 0.045**
	AM	246.2 ± 4.4 d	220.7 ± 3.4 c	122.4 ± 3.8 a			
Water in the plant [g]	NM	249.1 ± 9.9	278.7 ± 8.30	287.2 ± 4.9	**(10.8) <0.001**	(0.10) 0.922	(0.18) 0.838
	AM	252.0 ± 5.7	282.3 ± 10.8	282.6 ± 7.5			
		A	B	B			
Turgor pressure [MPa]	NM	0.38 ± 0.05	0.35 ± 0.08	0.14 ± 0.19	(3.28) 0.056	(0.16) 0.692	(0.19) 0.828
	AM	0.35 ± 0.03	0.38 ± 0.09	−0.01 ± 0.15			
		A	A	A			
Leaf water potential [MPa]	NM	−0.69 ± 0.05	−0.80 ± 0.09	−0.98 ± 0.03	**(13.2) <0.001**	(0.01) 0.967	(0.44) 0.648
	AM	−0.51 ± 0.05	−0.84 ± 0.12	−1.01 ± 0.05			
		A	AB	B			
Root water potential [MPa]	NM	−0.06 ± 0.01	−0.55 ± 0.07	−0.65 ± 0.04	**(68.9) <0.001**	(2.21) 0.151	(1.19) 0.321
	AM	−0.06 ± 0.01	−0.51 ± 0.06	−0.51 ± 0.06			
		A	B	B			

Θ_H, Ψ_{SH}, and K_H denote the substrate water content, water potential and substrate hydraulic conductivity present at harvest, respectively. $\Theta_{4.2}$ denotes the water content at the permanent wilting point. Ψ_{RS} and R_{SOIL} are the mean water potential estimated at the root surface and the resistance of water flow to roots, respectively. The three harvests occurred 36 days after inoculation under ample water conditions (WW) and 42 and 47 days under water deficient conditions (WD1 and WD2, respectively) after withholding water. The data (mean ± SE, n = 4–6, transpiration data: n ≥15) was analyzed by two way ANOVA (α = 0.05) with significant P-values highlighted in bold. In case of significant interaction, values followed by the same small letter are not significantly different (Tukey HSD). Different capital letters indicate significant differences between harvest dates and asterisks (second column) indicate whether inoculation caused a significant effect (Tukey HSD).

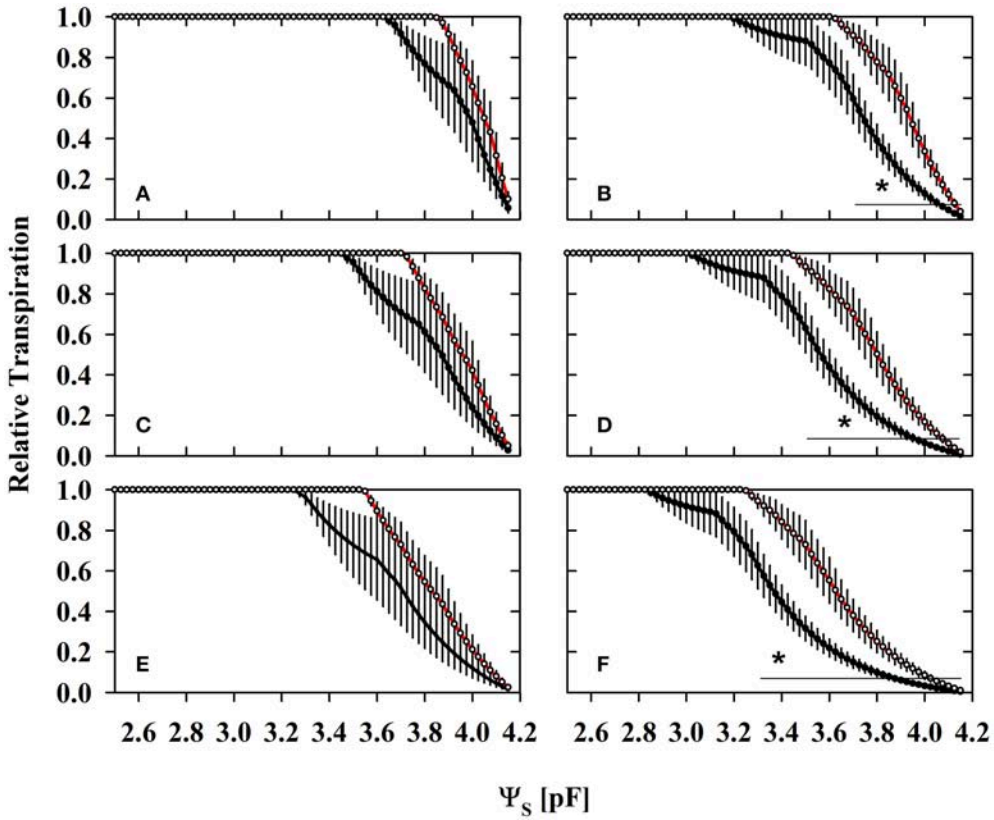


FIGURE 5 | The substrate related water limitation expressed as relative transpiration (potential root water influx/transpiration demand) under different levels of atmospheric demands **(A,B)** 0.5 cm d^{-1}; **(C,D)** 1 cm d^{-1}; **(E,F)** 2 cm d^{-1} in mycorrhizal (red lines) and non-mycorrhizal (black lines) substrates harvested under ample water conditions (WW; **A,C,E)** and under water deficient conditions (WD2; **B,D,F)** (mean ± SE, $N = 4$–6). The highest measured actual transpiration rates during the experiment have been 1.6 cm d^{-1} at noon and lowest in the morning and evening (0.4 cm d^{-1}) at WW. Average daytime transpiration rates have been 1.3, 0.9, and 0.6 cm d^{-1} at WW, WD1, and WD2, respectively under growing conditions with moderate atmospheric demands ($T = 22{:}17°C$, day: night; rH = 50–75%; PAR = 150–660 μmol $m^{-2}s^{-1}$). A value of 1 denotes that water flux allowed by substrates is higher than the level of assumed actual transpiration rates (0.5, 1, and 2 cm d^{-1}). Values lower than one indicate substrate water flux limitations, i.e., substrate water flow rates are smaller than actual transpiration rates. Lines with an asterisk denote the ra006Ege where relative transpiration was different between NM and AM pots (Students *t*-test: $\alpha = 0.05$).

mycorrhizal effects caused by changes in pore distribution or connectivity.

The influence of AMF on water retention is not abundantly reported and especially scarce under experimental conditions, where rooting is equal like here or in Augé et al. (2001). Our finding of a stronger decline in water contents per unit pF in mycorrhizal substrates is consistent with observations made Augé et al. (2001) in an equally rooted mix of loamy soil and quartz sand. Both studies have in common that water retention curves are characteristic for a coarsely textured substrate with a significant proportion of sand. On such coarsely textured substrates, differences in substrate structure and water retention induced by AMF would be expected to be strongest (Leifheit et al., 2014; Querejeta, 2017).

Soil water retention is largely determined by texture and soil structure while roots and AMF can affect structure. Although roots may have the largest influence on structure (Hallett et al., 2009; Daynes et al., 2013), hyphae are sufficient to influence substrate properties like water repellency (Rillig et al., 2010) and can promote the formation of aggregates within rooted substrates (Augé et al., 2001; Leifheit et al., 2014). A hierarchical development of aggregates from micro-aggregates (<20 μm) is probably not very pronounced in our case, because the sand and the vermiculite grain size was larger than 200 μm and the experiment duration was rather short. Although vermiculite particles may also partly disintegrate with rooting or drying and the carrier material also provided some clay particles, substrate water retention characteristics were typical for a coarse texture. On dune sand or sandy soils, sand particles are enmeshed and entangled to aggregates by hyphae and adhere to the hyphal surface (Clough and Sutton, 1978; Forster, 1979) that is covered by mucilage and polysaccharides among other "sticky" substances (Miller and Jastrow, 2000; Rillig and Mummey, 2006). Such a direct effect of hyphae is likely to occur on the used substrate, because its coarse texture requires hyphae or roots bridging large pores (Miller and Jastrow, 2000) and probably does need shorter colonization times than a hierarchical formation of aggregates from e.g., microbial and plant debris. Those processes would influence the size, shape and connectivity of the pore space where the water flow through substrates occurs.

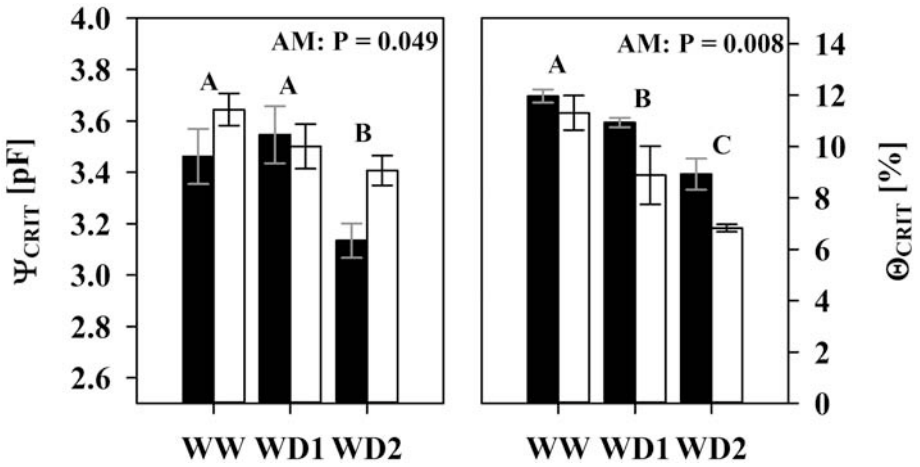


FIGURE 6 | The critical substrate water potential (Ψ_{CRIT}) and water content (Θ_{CRIT}) that start to limit root water influx under high atmospheric demands (2 cm d^{-1}) of mycorrhizal (white bars), and non-mycorrhizal (black bars) plants as sampled during the three harvests. The three harvests occurred 36 days after inoculation under ample water conditions (WW) and 42 and 47 days under water deficient conditions (WD1 and WD2, respectively) after withholding water during the drying episode. Significance of factors harvest date (H), inoculation with *F. mosseae* (I) and their interaction (H × I) was analyzed by two way ANOVA (mean ± *SE*, $N = 4$–6, $\alpha = 0.05$). No significant interaction was detected. Different captial letters indicate significant differences between harvest dates (Tukey HSD) and the *P*-value for main factor inoculation (AM) is shown. For complete ANOVA results please refer to the Table S5.

We did not find any changes in total porosity upon AMF colonization. The effects of AMF on total dry porosity might just be marginal, either because the volume of hyphae and spores is too small to detect a change in pore volume and/or AMF induces processes that create and reduce pore volume simultaneously and thus compensate each other. Significant changes in substrate water retention indicate reorganization in discrete pore size distributions (Daynes et al., 2013). Indeed, the AMF induced increases in PAW without changes in total porosity found here is consistent with another study using a substrate deriving from coarse spoil (Daynes et al., 2013) and may be indicative for a gain of partial porosity of the pore space. Because total porosity and the saturated water content were not influenced by AMF, the suggested increase of porosity related to plant extractable pore volume has come by the expense of other pores or pore property transformations. Although porosity inside aggregates might not be strongly altered by AMF (Hallett et al., 2009), aggregate formation might alter inter- and intra-aggregate proportions of total porosity or the interconnectivity of the inter-aggregate pore space. The degree of aggregation increases with hyphal length (Miller and Jastrow, 2000) and we found PAW to relatively increase with time and root colonization as a surrogate for fungal development. Many other processes occur in biologically active substrates that could change the water potential at a particular degree of saturation. Hydrophobicity influences the contact angle of the liquid-solid phase (Letey et al., 1962) and hydrophobicity of hyphae or exudates could change the effective wettable pore space. AMF may also influence the microbial community that alters porosity, water repellency or change the properties of particle surfaces. Future studies could use sampling cores covered by meshes only allowing fungal access on substrates that facilitate harvest of hyphae to further elucidate these mechanisms (Querejeta, 2017).

Hydraulic substrate conductivity measures how water can be transmitted through the pore space and depends on pore connectivity, pore geometry and tortuosity (Durner, 1994). Some studies showed a significant water transport across compartments only connected by extraradical hyphae under ongoing plant transpiration (Khalvati et al., 2005; Ruth et al., 2011). Those observations may be plant driven, because intrahyphal transport of P seems to be accelerated by the plant transpiration stream along intrahyphal water potential gradients (Bitterlich and Franken, 2016; Kikuchi et al., 2016). Hydraulic conductivity as a strict physical substrate property, which does not require plant or fungal activity, was improved upon AMF colonization. We did not find any other study to compare our mycorrhizal effects. By theory, unsaturated hydraulic conductivity is mainly determined by the largest water filled pores and is thus related to pore size distribution (Mualem, 1976; Durner, 1994). However, varying pore geometry as induced by aggregate formation can alter hydraulic conductivity although substrate water retention remains the same (Durner, 1994). We cannot clarify the mechanistic background of our observations in water retention and conductivity apart from fungal presence, but the decreased resistance of water flow may indicate altered pore space geometry. In addition to aggregation processes, hyphae may bridge air filled pores and restore root-substrate contact, fill voids of particular pore sizes or retain pore connectivity by smoothing the surface profile of particles. Non-septate (dead, disrupted) AMF hyphae of about 5 μm in diameter (Staddon et al., 2003) could also constitute less tortuous bio-pores that would be emptied at substrate water potentials considered as low moisture conditions. If the quantity of hyphae or pores left behind after hyphae degradation could explain our observation cannot be answered here, but is at least debatable, because unrealistic high flow rates within hyphae would be required to

significantly affect plant water uptake (George et al., 1992). But, roots and AMF hyphae are able to enhance the structural pore volume by several magnitudes higher than can be explained by the biomass volume alone (Milleret et al., 2009).

Changes in hydraulic properties upon AMF inoculation can have consequences for plants to take up water, their stress response and solute transport into the vicinity of roots. To study the impact of AMF on plant activity we performed the quantitative limitation analysis. From water retention and hydraulic conductivity functions alone, it remains elusive, which impact the observed AMF effects have on the plant's ability to acquire water from the substrate. Whole plant transpiration depends on atmospheric conditions and plant size. Plant transpiration will only become restricted by substrate water flux when the water flow to the root surface provided by the whole pot cannot compensate aerial transpirative demands. The substrate water flux will depend on the total amount of water ($\Theta \times$ substrate volume), water extractability (Ψ_S), water mobility (K) and the rhizosphere size (rooting density, root diameter). We integrated those factors with the approach described in section 2.4 in order to assess substrate derived limitation to transpiration quantitatively under different atmospheric demands.

For the first time we show here that substrate water flux limitation to plant transpiration is delayed to stronger drought intensities in mycorrhizal substrates. This is caused by effects that relate to the water flow within the rhizosphere outside of roots. Our analysis illustrates that the mass flow of substrate water, and with that, solute transport, can be maintained in mycorrhizal substrates during higher drought intensities under distinct transpirative demands. This may be of high ecological relevance and could contribute to the frequently observed AMF growth promotion and nutrient acquisition in drought stress experiments (Augé, 2001). Moreover, the limiting drought intensity decreased with the transpirative demand induced by the atmosphere. We are convinced that this important to understand how growing conditions, which vary largely between studies, are decisive for intermittent mycorrhizal effects and the choice and effectiveness of distinct drought treatments.

Plants have developed mechanisms to sense substrate drought. The mechanism has a hydraulic and a biochemical component (ABA) and the message serves to inform the plant about the soil water status (Tardieu and Davies, 1993). This enables plants to avoid exhaustive behavior and regulate transpiration via stomatal movement (Tardieu and Simonneau, 1998). From an ecological point of view this is mandatory to sustain viability and prolong survival times under such conditions. Indeed, we found indication for such reactions. Under our experimental conditions, water flux limitations to transpiration would only occur at drought intensities that were achieved at the end of the drying episode (WD2), but plant transpiration (calculated as the daily average between harvests) already decreased in between the first two harvests. This requires a plant feed-forward response to substrate drought. Mycorrhizal plants did transpire more water in the early phase of drying, which suggests a higher leaf conductance on the plant level. Mycorrhizal plants may not show altered sensitivities of stomatal conductance to xylem [ABA] (Duan et al., 1996), but the authors suggested that mycorrhizal plants are able to better scavenge water in drier soils, which alleviates ABA production in roots. Within the early drying phase (WW to WD1) plants already passed the phase where the substrate pF in mycorrhizal pots declined less with a reduction of Θ caused by plant activity (see Table S3). In combination with the improvements in substrate conductivity this could have caused an alleviation of the stress response in mycorrhizal roots and in turn, to higher transpiration.

Actually, for pot cultures, mycorrhizal plants exerted a more exhaustive behavior. However, such behavior of mycorrhizal plants is potentially advantageous for water and nutrient acquisition in scenarios of alternate irrigation as applied in many reductionist pot experiments. Indeed, AM plants have grown better under pulsed irrigation treatments (Birhane et al., 2012). And, in an earlier study we found stomatal condcutance to be improved in mycorrhizal tomatoes, colonized by the same fungus on the same substrate (Boldt et al., 2011). In field scenarios, where water contents decline less quickly due to subsequent water delivery from the periphery (Tardieu and Simonneau, 1998), a longer duration of phases, where stress responses are alleviated in mycorrhizal plants, is possible when those effects also occur in soils.

CONCLUSIONS

Our study revealed that inoculation with arbuscular mycorrhiza can result in an improvement in water availability and water transport within colonized substrates. Physiologically, this indicates that plants may experience or sense less stress at the root surface at equal substrate moisture, when substrate moisture declines. For experimental systems that investigate the drought tolerance of mycorrhizal plants, it would be important to consider those effects when particular irrigation treatments are used. The fact that mycorrhiza delays the critical substrate water potential for transpiration inhibition to stronger stress levels cannot be seen as a general transference of drought tolerance in every scenario. In pots, the latter can lead to higher resource acquisition, because the mass flow in substrates declines later, but would also cause higher resource depletion rates. To benefit from that, a timely irrigation in e.g., hydroponic pot systems would be required. In systems where water flow from the periphery is possible, e.g., in field scenarios, the observed mycorrhizal effect putatively increases plant resource use efficiency, when total availability of water and solutes increases by subsequent delivery from areas outside the ambit of roots. This is either driven by higher transpiration or allowed by higher substrate conductivity. Finally, the suggested increment in substrate mass flow by AMF under severe drought may contribute to the acquisition of mobile nutrients such as NO_3 and K under drought conditions.

Further studies are required to elucidate whether easier water extractability and improved hydraulic conductivity at equal drought intensity in mycorrhizal substrates would lead to alleviation of the physiological stress response in the plant. But if this is the case, mycorrhizal plants could be able to invest more resources in biomass development instead of e.g., osmotic adjustments when substrate moisture declines before

transpiration is limited by substrate moisture. And, they sustain water and nutrient acquisition under severe drought, because of delayed substrate born limitations to transpiration. Such scenarios would have a strong ecological significance and would reason targeting the use of AMF in crop production systems. Therefore, mycorrhizal effects on substrate hydraulic properties are worth investigating on different crops and substrates with different textures.

In the future, the underlying mechanisms responsible for alterations of substrate hydraulic properties by mycorrhizal colonization could be investigated by applying water retention measurements on substrate proportions that exclude root ingrowth and/or by the use of mutants resistant to mycorrhizal colonization. Subsequently, with such experimental systems, changes in hydraulic properties could then be associated directly to hyphal length, induction of aggregation, water repellency or pore clogging. Standard methods like sieving techniques, measurements of water contact angle or water drop penetration tests, only to name a few, are well established, but have only scarcely been used in combination with water retention assessments to quantify mycorrhizal effects. This however, is crucial to understand the relevance of direct extraradical mycorrhizal effects for plant physiology and for the induction of plant drought stress response. Furthermore, since water and solute transport are closely linked, our proposed approach can help distinguishing between direct hyphal delivery of N and P and indirect mycorrhizal effects that alleviate substrate mass flow restrictions, by using isotopic labeling techniques.

AUTHOR CONTRIBUTIONS

MB: conducted the experiments, analyzed the data and wrote the manuscript; MS: set up the HYPROP method, revisedthe manuscript and contributed to writing; JG: conducted the experiments, developed and conducted root water uptake modeling and revised the manuscript.

ACKNOWLEDGMENTS

We would like to thank Birgit Löffelbein for excellent technical assistance. MB was supported by an Elsa Neumann grant of Berlin. The project has received funding from the Ministry of Consumer Protection, Food and Agriculture of the Federal Republic of Germany, from the Ministry for Science, Research and Culture of the State of Brandenburg, and from the Thuringian Ministry of Infrastructure and Agriculture.

SUPPLEMENTARY MATERIAL

The Supplementary Material for this article can be found online at: https://www.frontiersin.org/articles/10.3389/fpls.2018.00154/full#supplementary-material

REFERENCES

Akaike, H. (1974). A new look at the statistical model identification. *Auto. Control IEEE Trans.* 19, 716–723. doi: 10.1109/TAC.1974.1100705

Augé, R. M. (2001). Water relations, drought and vesicular-arbuscular mycorrhizal symbiosis. *Mycorrhiza* 11, 3–42. doi: 10.1007/s005720100097

Augé, R. M. (2004). Arbuscular mycorrhizae and soil/plant water relations. *Can. J. Soil Sci.* 84, 373–381. doi: 10.4141/S04-002

Augé, R. M., Stodola, A. J. W., Tims, J. E., and Saxton, A. M. (2001). Moisture retention properties of a mycorrhizal soil. *Plant Soil* 230, 87–97. doi: 10.1023/A:1004891210871

Augé, R. M., Sylvia, D. M., Park, S., Buttery, B. R., Saxton, A. M., Moore, J. L., et al. (2004). Partitioning mycorrhizal influence on water relations of *Phaseolus vulgaris* into soil and plant components. *Can. J. Bot.* 82, 503–514. doi: 10.1139/b04-020

Augé, R. M., Toler, H. D., and Saxton, A. M. (2015). Arbuscular mycorrhizal symbiosis alters stomatal conductance of host plants more under drought than under amply watered conditions: a meta-analysis. *Mycorrhiza* 25, 13–24. doi: 10.1007/s00572-014-0585-4

Bearden, B. N. (2001). Influence of arbuscular mycorrhizal fungi on soil structure and soil water characteristics of vertisols. *Plant Soil* 229, 245–258. doi: 10.1023/A:1004835328943

Birhane, E., Sterck, F. J., Fetene, M., Bongers, F., and Kuyper, T. W. (2012). Arbuscular mycorrhizal fungi enhance photosynthesis, water use efficiency, and growth of frankincense seedlings under pulsed water availability conditions. *Oecologia* 169, 895–904. doi: 10.1007/s00442-012-2258-3

Bitterlich, M., and Franken, P. (2016). Connecting polyphosphate translocation and hyphal water transport points to a key of mycorrhizal functioning. *New Phytol.* 211, 1147–1149. doi: 10.1111/nph.14104

Blume, H. P., Brümmer, G. W., Horn, R., Kandeler, E., Kögel-Knabner, I., Kretzschmar, R., et al. (2009). *Scheffer/Schachtschabel: Lehrbuch der Bodenkunde*. Wiesbaden: Springer-Verlag.

Bodner, G., Leitner, D., and Kaul, H. P. (2014). Coarse and fine root plants affect pore size distributions differently. *Plant Soil* 380, 133–151. doi: 10.1007/s11104-014-2079-8

Boldt, K., Pörs, Y., Haupt, B., Bitterlich, M., Kühn, C., Grimm, B., et al. (2011). Photochemical processes, carbon assimilation and RNA accumulation of sucrose transporter genes in tomato arbuscular mycorrhiza. *J. Plant Physiol.* 168, 1256–1263. doi: 10.1016/j.jplph.2011.01.026

Clough, K. S., and Sutton, J. C. (1978). Direct observation of fungal aggregates in sand dune soil. *Can. J. Microbiol.* 24, 333–335. doi: 10.1139/m78-056

Colla, G., Nardi, S., Cardarelli, M., Ertani, A., Lucini, L., Canaguier, R., et al. (2015). Protein hydrolysates as biostimulants in horticulture. *Sci. Hortic.* 196, 28–38. doi: 10.1016/j.scienta.2015.08.037

Daynes, C. N., Field, D. J., Saleeba, J. A., Cole, M. A., and McGee, P. A. (2013). Development and stabilisation of soil structure via interactions between organic matter, arbuscular mycorrhizal fungi and plant roots. *Soil Biol. Biochem.* 57, 683–694. doi: 10.1016/j.soilbio.2012.09.020

de Jong van Lier, Q., van Dam, J. C., Durigon, A., dos Santos, M. A., and Metselaar, K. (2013). Modeling water potentials and flows in the soil–plant system comparing hydraulic resistances and transpiration reduction functions. *Vadose Zone J.* 12:vzj2013.02.0039. doi: 10.2136/vzj2013.02.0039

de Jong van Lier, Q., van Dam, J. C., Metselaar, K., de Jong, R., and Duijnisveld, W. H. M. (2008). Macroscopic root water uptake distribution using a matric flux potential approach. *Vadose Zone J.* 7, 1065–1078. doi: 10.2136/vzj2007.0083

De Kreij, C., Voogt, W., van den Bos, A. L., and Baas, R. (1997). *Voedingsoplossingen voor de teelt van tomaat in gesloten teeltsystemen*. Brochure VG Tomaat, PBG Naaldwijk.

Doerr, S. H., Shakesby, R. A., and Walsh, R. (2000). Soil water repellency: its causes, characteristics and hydro-geomorphological significance. *Earth Sci. Rev.* 51, 33–65. doi: 10.1016/S0012-8252(00)00011-8

Duan, X. G., Neuman, D. S., Reiber, J. M., Green, C. D., Saxton, A. M., and Augé, R. M. (1996). Mycorrhizal influence on hydraulic and hormonal factors

implicated in the control of stomatal conductance during drought. *J. Exp. Bot.* 47, 1541–1550. doi: 10.1093/jxb/47.10.1541

du Jardin, P. (2015). Plant biostimulants: definition, concept, main categories and regulation. *Sci. Hortic.* 196, 3–14. doi: 10.1016/j.scienta.2015.09.021

Durner, W. (1994). Hydraulic conductivity estimation for soils with heterogeneous pore structure. *Water Resour. Res.* 30, 211–223. doi: 10.1029/93WR02676

Fonteno, W. C. (1992). Problems & considerations in determining physical properties of horticultural substrates. *Acta Hortic.* 342, 197–204.

Forster, S. M. (1979). Microbial aggregation of sand in an embryo dune system. *Soil Biol. Biochem.* 11, 537–543. doi: 10.1016/0038-0717(79)90014-2

Frey, J. E., and Ellis, J. R. (1997). Relationship of soil properties and soil amendments to response of *Glomus intraradices* and soybeans. *Can. J. Bot.* 75, 483–491. doi: 10.1139/b97-052

George, E., Haussler, K. U., Vetterlein, D., Gorgus, E., and Marschner, H. (1992). Water and nutrient translocation by hyphae of glomus-mosseae. *Can. J. Bot. Revue Can. Bot.* 70, 2130–2137. doi: 10.1139/b92-265

Giovannetti, M., and Mosse, B. (1980). Evaluation of techniques for measuring vesicular arbuscular mycorrhizal infection in roots. *New Phytol.* 84, 489–500. doi: 10.1111/j.1469-8137.1980.tb04556.x

Hallett, P. D., Feeney, D. S., Bengough, A. G., Rillig, M. C., Scrimgeour, C. M., and Young, I. M. (2009). Disentangling the impact of AM fungi versus roots on soil structure and water transport. *Plant Soil* 314, 183–196. doi: 10.1007/s11104-008-9717-y

Khalvati, M. A., Hu, Y., Mozafar, A., and Schmidhalter, U. (2005). Quantification of water uptake by arbuscular mycorrhizal hyphae and its significance for leaf growth, water relations, and gas exchange of barley subjected to drought stress. *Plant Biol.* 7, 706–712. doi: 10.1055/s-2005-872893

Kikuchi, Y., Hijikata, N., Ohtomo, R., Handa, Y., Kawaguchi, M., Saito, K., et al. (2016). Aquaporin mediated long distance polyphosphate translocation directed towards the host in arbuscular mycorrhizal symbiosis: application of virus induced gene silencing. *New Phytol.* 211, 1202–1208. doi: 10.1111/nph.14016

Koske, R. E., and Gemma, J. N. (1989). A modified procedure for staining roots to detect VA mycorrhizas. *Mycol. Res.* 92, 486–488. doi: 10.1016/S0953-7562(89)80195-9

Leifheit, E. F., Veresoglou, S. D., Lehmann, A., Morris, E. K., and Rillig, M. C. (2014). Multiple factors influence the role of arbuscular mycorrhizal fungi in soil aggregation: a meta-analysis. *Plant Soil* 374, 523–537. doi: 10.1007/s11104-013-1899-2

Letey, J., Osborn, J., and Pelishek, R. E. (1962). The influence of the water-solid contact angle on water movement in soil. *Hydrol. Sci. J.* 7, 75–81. doi: 10.1080/02626666209493272

Miller, R. M., and Jastrow, J. D. (2000). "Mycorrhizal fungi influence soil structure," in *Arbuscular Mycorrhizas: Physiology and Function*, eds Y. Kapulnik and D. Douds (Dordrecht: Springer), 3–18.

Milleret, R., Le Bayon, R. C., Lamy, F., Gobat, J. M., and Boivin, P. (2009). Impact of roots, mycorrhizas and earthworms on soil physical properties as assessed by shrinkage analysis. *J. Hydrol.* 373, 499–507. doi: 10.1016/j.jhydrol.2009.05.013

Moltchanov, D. (2012). Distance distributions in random networks. *Ad Hoc Networks* 10, 1146–1166. doi: 10.1016/j.adhoc.2012.02.005

Mualem, Y. (1976). A new model for predicting the hydraulic conductivity of unsaturated porous media. *Water Resour. Res.* 12, 513–522. doi: 10.1029/WR012i003p00513

Neumann, E., and George, E. (2005). Does the presence of arbuscular mycorrhizal fungi influence growth and nutrient uptake of a wild-type tomato cultivar and a mycorrhiza-defective mutant, cultivated with roots sharing the same soil volume? *New Phytol.* 166, 601–609. doi: 10.1111/j.1469-8137.2005.01351.x

Peters, A., and Durner, W. (2008). Simplified evaporation method for determining soil hydraulic properties. *J. Hydrol.* 356, 147–162. doi: 10.1016/j.jhydrol.2008.04.016

Piotrowski, J. S., Denich, T., Klironomos, J. N., Graham, J. M., and Rillig, M. C. (2004). The effects of arbuscular mycorrhizas on soil aggregation depend on the interaction between plant and fungal species. *New Phytol.* 365–373. doi: 10.1111/j.1469-8137.2004.01181.x

Pozo, M. J., Azcon-Aguilar, C., Dumas-Gaudot, E., and Barea, J. M. (1999). beta-1,3-glucanase activities in tomato roots inoculated with arbuscular mycorrhizal fungi and/or Phytophthora parasitica and their possible involvement in bioprotection. *Plant Sci.* 141, 149–157. doi: 10.1016/S0168-9452(98)00243-X

Querejeta, J. I. (2017). "Soil water retention and availability as mediated by mycorrhizal symbiosis: consequences for individual plants, communities, and ecosystems," in "*Mycorrhizal Mediation of Soil: Fertility, Structure, and Carbon Storage*, eds N. C. Johnson, C. A. Gehring, and J. Jansa (Amsterdam: Elsevier Inc.), 299–317.

Rillig, M. C., Mardatin, N. F., Leifheit, E. F., and Antunes, P. M. (2010). Mycelium of arbuscular mycorrhizal fungi increases soil water repellency and is sufficient to maintain water-stable soil aggregates. *Soil Biol. Biochem.* 42, 1189–1191. doi: 10.1016/j.soilbio.2010.03.027

Rillig, M. C., and Mummey, D. L. (2006). Mycorrhizas and soil structure. *New Phytol.* 171, 41–53. doi: 10.1111/j.1469-8137.2006.01750.x

Rillig, M. C., Wright, S. F., and Eviner, V. T. (2002). The role of arbuscular mycorrhizal fungi and glomalin in soil aggregation: comparing effects of five plant species. *Plant Soil* 238, 325–333. doi: 10.1023/A:1014483303813

Rivero, J., Gamir, J., Aroca, R., Pozo Ma, J., and Flors, V. (2015). Metabolic transition in mycorrhizal tomato roots. *Front. Microbiol.* 6:598. doi: 10.3389/fmicb.2015.00598

Rouphael, Y., Franken, P., Schneider, C., Schwarz, D., Giovannetti, M., Agnolucci, M., et al. (2015). Arbuscular mycorrhizal fungi act as biostimulants in horticultural crops. *Sci. Hortic.* 196, 91–108. doi: 10.1016/j.scienta.2015.09.002

Ruiz-Lozano, J. M., Azcon, R., and Gomez, M. C. (1995). Effects of Arbuscular-mycorrhizal glomus species on drought tolerance - physiological and nutritional plant-responses. *Appl. Environ. Microbiol.* 61, 456–460.

Ruth, B., Khalvati, M., and Schmidhalter, U. (2011). Quantification of mycorrhizal water uptake via high-resolution on-line water content sensors. *Plant Soil* 342, 459–468. doi: 10.1007/s11104-010-0709-3

Ruzzi, M., and Aroca, R. (2015). Plant growth-promoting rhizobacteria act as biostimulants in horticulture. *Sci. Hortic.* 196, 124–134. doi: 10.1016/j.scienta.2015.08.042

Schindler, U. (1980). Ein schnellverfahren zur messung der wasserleitfähigkeit im teilgesättigten boden an stechzylinderproben. *Arch. Acker Pfl. Boden.* 24, 1–7.

Scholander, P. F., Hammel, H. T., Bradstre, E. D., and Hemmings, E. A. (1965). Sap pressure in vascular plants - negative hydrostatic pressure can be measured in plants. *Science* 148, 339–346. doi: 10.1126/science.148.3668.339

Schwarz, D., and Klaering, H.-P. (2001). Allometry to estimate leaf area in tomato. *J. Plant Nutr.* 24, 1291–1309. doi: 10.1081/PLN-100106982

Smith, S. E., and Read, D. J. (2008). *Mycorrhizal Symbiosis*. London: Academic Press.

Smith, S. E., Smith, F. A., and Jakobsen, I. (2004). Functional diversity in arbuscular mycorrhizal (AM) symbioses: the contribution of the mycorrhizal P uptake pathway is not correlated with mycorrhizal responses in growth or total P uptake. *New Phytol.* 162, 511–524. doi: 10.1111/j.1469-8137.2004.01039.x

Staddon, P. L., Ramsey, C. B., Ostle, N., Ineson, P., and Fitter, A. H. (2003). Rapid turnover of hyphae of mycorrhizal fungi determined by AMS microanalysis of C-14. *Science* 300, 1138–1140. doi: 10.1126/science.1084269

Tardieu, F., and Davies, W. J. (1993). Integration of hydraulic and chemical signalling in the control of stomatal conductance and water status of droughted plants. *Plant Cell Environ.* 16, 341–349. doi: 10.1111/j.1365-3040.1993.tb00880.x

Tardieu, F., and Simonneau, T. (1998). Variability among species of stomatal control under fluctuating soil water status and evaporative demand: modelling isohydric and anisohydric behaviours. *J. Exp. Bot.* 49, 419–432. doi: 10.1093/jxb/49.Special_Issue.419

Tisdall, J. M., and Oades, J. (1982). Organic matter and water stable aggregates in soils. *Eur. J. Soil Sci.* 33, 141–163. doi: 10.1111/j.1365-2389.1982.tb01755.x

Conflict of Interest Statement: The authors declare that the research was conducted in the absence of any commercial or financial relationships that could be construed as a potential conflict of interest.

frontiers in Plant Science

ORIGINAL RESEARCH
published: 27 February 2018
doi: 10.3389/fpls.2018.00249

Physiological and Metabolic Responses Triggered by Omeprazole Improve Tomato Plant Tolerance to NaCl Stress

Youssef Rouphael[1†], Giampaolo Raimondi[1†], Luigi Lucini[2], Petronia Carillo[3], Marios C. Kyriacou[4], Giuseppe Colla[5], Valerio Cirillo[1], Antonio Pannico[1], Christophe El-Nakhel[1] and Stefania De Pascale[1]*

[1] Department of Agricultural Sciences, University of Naples Federico II, Portici, Italy, [2] Department for Sustainable Food Process, Università Cattolica del Sacro Cuore, Piacenza, Italy, [3] Department of Environmental, Biological and Pharmaceutical Sciences and Technologies, University of Campania "Luigi Vanvitelli", Caserta, Italy, [4] Department of Vegetable Crops, Agricultural Research Institute, Nicosia, Cyprus, [5] Department of Agricultural and Forestry Sciences, University of Tuscia, Viterbo, Italy

OPEN ACCESS

Edited by:
Antonio Ferrante, Università degli Studi di Milano, Italy

Reviewed by:
Vincenzo Candido, University of Basilicata, Italy
Spyridon Alexandros Petropoulos, University of Thessaly, Greece
Georgia Ntatsi, Agricultural University of Athens, Greece

***Correspondence:**
Stefania De Pascale
depascal@unina.it

[†] *These authors have contributed equally to this work and Co-first authors.*

Specialty section:
This article was submitted to Crop and Product Physiology, a section of the journal Frontiers in Plant Science

Received: *25 January 2018*
Accepted: *12 February 2018*
Published: *27 February 2018*

Citation:
Rouphael Y, Raimondi G, Lucini L, Carillo P, Kyriacou MC, Colla G, Cirillo V, Pannico A, El-Nakhel C and De Pascale S (2018) Physiological and Metabolic Responses Triggered by Omeprazole Improve Tomato Plant Tolerance to NaCl Stress. Front. Plant Sci. 9:249. doi: 10.3389/fpls.2018.00249

Interest in the role of small bioactive molecules (< 500 Da) in plants is on the rise, compelled by plant scientists' attempt to unravel their mode of action implicated in stimulating growth and enhancing tolerance to environmental stressors. The current study aimed at elucidating the morphological, physiological and metabolomic changes occurring in greenhouse tomato (cv. Seny) treated with omeprazole (OMP), a benzimidazole inhibitor of animal proton pumps. The OMP was applied at three rates (0, 10, or 100 μM) as substrate drench for tomato plants grown under nonsaline (control) or saline conditions sustained by nutrient solutions of 1 or 75 mM NaCl, respectively. Increasing NaCl concentration from 1 to 75 mM decreased the tomato shoot dry weight by 49% in the 0 μM OMP treatment, whereas the reduction was not significant at 10 or 100 μM of OMP. Treatment of salinized (75 mM NaCl) tomato plants with 10 and especially 100 μM OMP decreased Na^+ and Cl^- while it increased Ca^{2+} concentration in the leaves. However, OMP was not strictly involved in ion homeostasis since the K^+ to Na^+ ratio did not increase under combined salinity and OMP treatment. OMP increased root dry weight, root morphological characteristics (total length and surface), transpiration, and net photosynthetic rate independently of salinity. Metabolic profiling of leaves through UHPLC liquid chromatography coupled to quadrupole-time-of-flight mass spectrometry facilitated identification of the reprogramming of a wide range of metabolites in response to OMP treatment. Hormonal changes involved an increase in ABA, decrease in auxins and cytokinin, and a tendency for GA down accumulation. Cutin biosynthesis, alteration of membrane lipids and heightened radical scavenging ability related to the accumulation of phenolics and carotenoids were observed. Several other stress-related compounds, such as polyamine conjugates, alkaloids and sesquiterpene

lactones, were altered in response to OMP. Although a specific and well-defined mechanism could not be posited, the metabolic processes involved in OMP action suggest that this small bioactive molecule might have a hormone-like activity that ultimately elicits an improved tolerance to NaCl salinity stress.

Keywords: benzimidazole, gas exchange, hormone-like activity, ion homeostasis, metabolomics, salt stress, *Solanum lycopersicum* L

INTRODUCTION

Salinity affects more than 45 million hectares (20%) of irrigated soils accounting for one-third of worldwide food production (Machado and Serralheiro, 2017). In Europe, about 4 million hectares have been impoverished by human activities, in particular along the Mediterranean coast (Daliakopoulos et al., 2016). Climate change, rise in evapotranspiration, intensive farming, excessive over-pumping of groundwater for irrigation (especially in coastal areas with consequent sea-water infiltration into fresh aquifers) and use of low quality water (brackish water or treated wastewater) in irrigation contribute synergically to soil salinization (Rana and Katerji, 2000; Costantini and Lorenzetti, 2013; Daliakopoulos et al., 2016). Under these circumstances, continuous exposure to hyperosmotic stress and seasonal effects linked to salt accumulation in the roots highly affect crop yield (Rana and Katerji, 2000).

Osmotic stress and ion toxicity are the main problems that affect salt stressed plants (Munns and Tester, 2008; Gorham et al., 2010). Under high salinity, roots are unable to uptake water from the soil and toxic concentrations of sodium and chloride build up in the cytosol and organelles, resulting in plant nutritional disorders and oxidative stress (Hasegawa et al., 2000; Munns, 2002; Tavakkoli et al., 2010). Sodium interferes with potassium and calcium uptake, negatively affecting stomatal control; moreover, it can replace potassium in key enzymatic reactions. Therefore, the salt stress status of a crop depends mainly on the potassium-to-sodium ratio than on the absolute amount of sodium in the cytosol (Shabala and Cuin, 2008; Asins et al., 2013; Annunziata et al., 2017). Instead, chloride competes with nitrate for uptake and translocation within the plants by nitrate transporter proteins, exerting direct and indirect effects mediated by nitrate decrease on chlorophyll degradation as well as on the PSII quantum yield and photochemical quenching (Carillo et al., 2005; Tavakkoli et al., 2011). This double effect reduces plant growth and causes irreversible cell damage. However, plants try to adapt to salinity by osmo-regulating cellular compartments and controlling ion and water homeostasis to reduce stress damage and resume growth (Hasegawa et al., 2000; Woodrow et al., 2017). In particular, a ubiquitous mechanism of plant cells involves compartmentalization of toxic ions in the vacuoles as inexpensive osmotica and synthesis and/or accumulation of organic osmolytes in the cytosol for osmotic adjustment and protection against oxidative stress (Carillo et al., 2008; Hasegawa, 2013; Shabala, 2013). In this important process, plasma membrane and vacuolar H^+-ATPases have a key role in cytosol detoxification by creating an electrochemical H^+ gradient across the membranes used to drive a secondary active transport for Na^+ compartmentalization within the vacuole or its extrusion from the cell (Blumwald et al., 2000; Pardo et al., 2006; Ji et al., 2013). In fact, it is generally accepted that salt stress induces H^+-pumping capacity in plant tissues, mainly to energize Na^+/H^+ exchanger activity (Cuin et al., 2011; Bose et al., 2015). Moreover, the electrochemical gradient built up can be channeled for driving the active co-transport with H^+ of nitrate, phosphate, sulfate, sucrose, hexoses, and amino acids against their gradient (Batelli et al., 2007; Silva and Gerós, 2009; Conde et al., 2011).

Proton pump activity is continuously modulated by all the important factors controlling the plant physiology, subject to activation/deactivation foremost in response to abiotic stresses (Chelysheva et al., 1999; Hasegawa, 2013). Salt tolerance in *Arabidopsis* is enhanced as a result of increased ion compartmentalization facilitated by overexpressing vacuolar H^+-PPase AVP1 (Fuglsang et al., 2011) and, furthermore, by co-overexpressing vacuolar H^+-PPase*AVP1* and Na^+/H^+ antiporter *AtNHX1* genes simultaneously (Shen et al., 2015). Conversely, inhibition of plasma membrane H^+-ATPase by vanadate decreases the K^+/Na^+ ratio rendering the plant more susceptible to salinity (Li et al., 2014).

Homologues of plant proton pumps are the gastric H^+/K^+-ATPases, members of the P2-type ATPase family, responsible for gastric acid secretion, which include also membrane Ca^{2+} pumps and Na^+/K^+-transporters (Shin et al., 2009). The introduction and use of substituted benzimidazoles as proton pump inhibitors (PPI) targeted to the gastric H^+/K^+-ATPases has been essential for the treatment of peptic ulcers and gastroesophageal reflux disease (Fellenius et al., 1981). Omeprazole (OMP) has been the first PPI pharmaceutical introduced in the market, which specifically and irreversibly inhibits the P2-type ATPases (Shin and Kim, 2013). It is thus used for the treatment of dyspepsia, peptic ulcer, gastroesophageal reflux disease or *Helicobacter pylori* infection (Seoane et al., 2017).

Over the past few decades, plant scientists have started to identify the targets and mode of action in plants of signaling small molecules (< 500 Da) derived from human/animal research (Kaschani and van der Hoorn, 2007; Lace and Prandi, 2016). These small bioactive molecules created on the basis of natural or synthetic low-molecular weight compounds could be considered an efficient and safe approach to stimulate plant growth and elicit tolerance to environmental stressors (Kaschani and van der Hoorn, 2007; Lace and Prandi, 2016; Tsygankova et al., 2016).

Notwithstanding P2-type ATPases are not present in plants, Van Oosten et al. (2017) have demonstrated OMP (345.4 Da) as being effective at micromolar (μM) concentrations in stimulating tomato plant growth and enhancing tolerance to salinity. However, the experiments discussed by Van Oosten et al. (2017)

pertained to a short term trial, while in horticultural context plants growing on saline soils usually encounter long-term sodium chloride salinization. Moreover, though the effectiveness of OMP in inducing stress tolerance has been partially clarified, conclusive evidences regarding its molecular targets have not become available yet. Nonetheless, it is important to unravel the molecular basis of the improved stress tolerance imparted by OMP treatments, in order to elucidate the physiological and biochemical mechanisms involved, thus supporting a rationale for their application in agriculture. In this context, an untargeted approach facilitated by metabolomics has proved a powerful strategy for shedding light onto the role of secondary metabolites in mediating plant response to abiotic stressors (Nakabayashi and Saito, 2015).

Indisputably, the elucidation of fundamental plant molecular responses to OMP can be instrumental to unraveling adaptive strategies against salinity stress; hence the aim of this study was to investigate morphological, physiological, and metabolic changes in response to OMP application onto greenhouse tomato subjected to salt stress conditions. Untreated and treated tomato plants were characterized and compared in terms of growth, root morphology, ion content, gas exchange parameters, water relations and metabolic profiling.

MATERIALS AND METHODS

Plant Material, Greenhouse Conditions, and Crop Management

The experimental trial was carried out in the 2016 summer season in an unheated glasshouse at the experimental station of the University of Naples Federico II, located in Bellizzi, Salerno province (43° 31′ N, 14° 58′ E; 60 m asl), Italy. The tested vegetable species for the current experiment was tomato (*Solanum lycopersicum* L.) cv. Seny (Seminis Monsanto, Milano, Italy). Tomato plant were grown under natural light conditions and the daily air temperature inside the glasshouse was maintained between 18 and 30°C.

Cultivar Seny is a round-fruited, indeterminate tomato vine widely cultivated under greenhouse conditions in Italy due to its high productivity and resistance to cracking. Tomato seedlings were transplanted on May 2, at the three-true-leaf phenological stage into plastic pots (h 20 cm; d 20 cm) containing 5.3 L of a peat/perlite mixture in 2:1 volume ratio. The Lithuanian peat containing sphagnum peat moss (Agraria Di Vita, Pistoia, Italy) had the following physicochemical properties: 80% water holing capacity, pH 4.0, electrical conductivity 0.1 dS m^{-1}, 11 g kg^{-1} N, 0.1 g kg^{-1} P, 0.1 g kg^{-1} K, 1.8 g kg^{-1} Ca, 2.0 g kg^{-1} Mg, 70 mg kg^{-1} Fe, 15 mg kg^{-1} Mn, and 4 mg kg^{-1} Zn. Plastic pots were arranged in double rows. Plant rows were 0.9 m apart, and the space between plants within a row was 0.3 m. The distance between the centers of double rows was 2.22 m, resulting in a plant density of 3 plants m^{-2}, as normally practiced among fresh tomato greenhouse growers. Throughout the cultural cycle pathogens and pests were controlled based on standard phytoprotective practices used by commercial tomato growers in Italy.

Experimental Design, Omeprazole Application, and Nutrient Solution Management

The experiment was designed as a two-way factorial design encompassing combinations of two sodium chloride (NaCl) concentrations (1 mM nonsaline control and 75 mM NaCl) in the nutrient solution and three omeprazole (OMP) application levels (0 control, 10 and 100 μM OMP). The treatments were arranged in a randomized complete-block design with four replicates, amounting to a total of 24 experimental units with four plants each ($n = 96$ plants). The OMP was applied as substrate drench treatment five times during the growing cycle at weekly intervals starting on 10 May (9 days after transplanting; DAT). All OMP applications were delivered at a uniform rate of 100 mL per plant.

The basic (nonsaline) nutrient solution had the following composition: 13.6 mM N-NO_3, 2.0 mM S, 1.4 mM P, 6.0 mM K, 4.5 mM Ca, 2.0 mM Mg, 1 mM Na, 1 mM Cl, 20 μM Fe, 9 μM Mn, 1.5 μM Cu, 3 μM Zn, 20 μM B, and 0.3 μM Mo with an electrical conductivity (EC) of 2.0 dS m^{-1}. The saline nutrient solution treatment consisted of the same basic composition plus an additional 75 mM NaCl, yielding an EC value of 9.2 dS m^{-1}. The pH of the two nutrient solutions was 6.2 ± 0.3. The nonsaline and saline nutrient solutions were prepared using deionized water. Saline treatment was initiated on May 18 (17 DAT).

The nutrient solution was pumped from independent tanks and delivered through a drip irrigation system with one emitter per plant at a flow rate of 2 L h^{-1}. All plants received the same amount of solution with a leaching fraction of 20% to avoid build up of salinity into the substrate. A leaching fraction of 20% is needed to maintain the EC in the substrate to a similar level to the nutrient solution EC (Colla et al., 2012, 2013).

Yield, Growth Measurements, and Root Characteristics

The number of fully ripe fruits as well as the fresh weight of marketable fruit of the first two trusses were recorded on all plants. At the end of the experiment (July 5, 65 DAT), plants were separated into leaves, stems and roots. All plant tissues were dried at 80°C for 72 h until they reached a constant weight which corresponded to their dry biomasses. Shoot dry weight was equal to the sum of the aerial vegetative parts (leaves + stems), and the root-to-shoot ratio was also calculated. Dried plant tissues were sampled for ion analyses. The total leaf area per plant was measured using an electronic area meter (Li-Cor3000, Li-Cor, Lincoln, NE, USA).

The plant height as well as the number of leaves per plant were counted. Also, the root system architecture components were determined. Root system collection and sample preparation were performed following the protocol described previously by Lucini et al. (2015) and Rouphael et al. (2017a). The determination of root morphology characteristics was performed using WinRHIZO Pro (Regent Instruments Inc., Canada), connected to an image analysis scanner (STD 4800 Regent Instruments Inc., Canada). Three-dimensional images were captured and the following root characteristics were determined: root diameter, total root length and surface.

Leaf Water Potential, Relative Water Content, and Leaf Gas Exchange Measurements

On June 6 (36 DAT), leaf water potential (Ψ_l) measurements were performed on three replicates per treatment, using a dew-point psychrometer (WP4; Decagon Devices, Pullman, WA). The Relative Water Content (RWC) of basal and apical tomato leaves was calculated following the formula described by Jones and Turner (1978) (RWC = [FW−DW]/[TW−DW] × 100); where FW, DW and TW corresponded to fresh, dry and turgid weight, respectively.

At 58 DAT, the net CO_2 assimilation rate (A_{CO2}), stomatal resistance (r_s) and transpiration rate (E) were measured with a portable gas exchange analyzer (LCA-4; ADC BioScientific Ltd., Hoddesdon, UK) equipped with a broadleaf chamber (cuvette window area, 6.25 cm^2). This measurement was carried out within 2 h across solar noon (i.e., between 11.00 and 13.00) on the youngest fully expanded leaves, using six replicates for each treatment. Photosynthetically active radiation (PAR), Relative humidity (RH) and CO_2 concentration (593 ± 8 μmol m^{-2} s^{-1}, RH 50 ± 0.6% and 377 ± 0.6 mg kg^{-1}, respectively) were set at ambient value and the flow rate of air was 400 mL s^{-1}. The Water Use Efficiency (WUE) was calculated as A_{CO2}/E.

Ion Analyses

Dried plant tissues (leaf, fruit, and root) were ground separately in a Wiley mill (IKA, MF10.1, Staufen, Germany) to pass through 0.5 mm sieve, and then were used for ion analyses.

For the cations (K^+, Ca^{2+}, Mg^{2+}, and Na^+) and anions (NO_3^-, PO_4^{3-}, and Cl^-) analysis, 250 mg of dried material was extracted in 50 mL of ultrapure water (Milli-Q, Merck Millipore, Darmstadt, Germany) using a shaking water bath (ShakeTemp SW22, Julabo, Seelbach, Germany) at 80°C for 10 min as described previously by Rouphael et al. (2017b,d). The mixture was centrifuged at 6000 rpm for 10 min (R-10 M, Remi Elektrotechnik Limited, India), then filtered through a 0.20 μm filter paper (Whatman International Ltd., Maidstone, U.K.). The monovalent and bivalent cations were separated by ion chromatography (ICS-3000, Dionex, Sunnyvale, CA, USA) and quantified through an electrical conductivity detector. An IonPac CG12A (4 × 50 mm, Dionex, Corporation) guard column and IonPac CS12A (4 × 250 mm, Dionex, Corporation) analytical column were used for the separation of the four cations, whereas for anions an IonPac AG11-HC guard (4 × 50 mm) column and IonPac AS11-HC analytical column (4 × 250 mm) were used.

Collection of Samples and Metabolomic Analysis

Two terminal leaflets were sampled from the first fully expanded leaves of two plants per experimental plot at the end of the experiment, and immediately frozen in liquid nitrogen before stored at −80°C for metabolomic analysis. Tissue samples (1.0 g) of four replicates per treatment were extracted in 10+5 mL of 0.1% HCOOH in 80% methanol, using an Ultra-Turrax (Ika T-25, Staufen, Germany), then filtered through a 0.22 μm cellulose membrane disposable filter and finally transferred to an amber vial for analysis. The untargeted metabolite screening was carried out using a 1290 UHPLC liquid chromatography system coupled to a G6550 quadrupole-time-of-flight mass spectrometer, equipped with a JetStream dual Electrospray ionization source (UHPLC-ESI/QTOF-MS) (Agilent Technologies Santa Clara, CA, USA).

The parameters for metabolomic investigations in plant tissues were set out in previous experiments (Pretali et al., 2016). Briefly, chromatographic separation was achieved on an Agilent Zorbax Eclipse-plus column (75 × 2.1 mm i.d., 1.8 μm) using a mobile phase consisting of water (A) and methanol (B), flowing at 220 μL min^{-1} and 35°C. The gradient was initiated with 5% B and increased to 90% B within 35 min, whereas the mass spectrometer was run in positive scan mode (range of 100–1200 m/z) using a nominal mass resolution of 30,000 FWHM. Concerning electrospray conditions, nebulizer pressure was 60 psig, capillary voltage was 4 kV, sheath gas was nitrogen at 10 L min^{-1} (350°C), and drying gas was nitrogen at 10 L min^{-1} (280°C).

Raw data were processed using Profinder B.05 (from Agilent Technologies) for feature initial deconvolution. Compounds identification was carried out using the whole isotopic pattern (i.e., accurate mass, isotope accurate spacing and isotope ratio). Compounds were aligned for both mass and retention time, then annotated using the database PlantCyc 9.5 (Plant Metabolic Network, http://www.plantcyc.org; released November 2014). A filter-by-frequency post-processing was applied retaining only those compounds that were present in 100% of replications within at least one treatment. Therefore, identification was carried out as Level 2 (putatively annotated compounds), according to COSMOS Metabolomics Standards Initiative (http://cosmos-fp7.eu/msi).

Statistical Analysis of Experimental Data

Experimental data were subjected to two-way analysis of variance (ANOVA) using the SPSS 10 software package. Treatment means within each measured parameter were separated by Duncan's multiple range test performed at a significance level of $P \leq 0.05$. Principal component analysis (PCA) was also performed using Minitab 16.2.1 statistical software, aimed to extract trends by formulating new variables correlated to the original ones (Lawless and Heymann, 2010; Rouphael et al., 2017d). The PCA outputs included variable loading to each selected component and treatment component scores (Ciarmiello et al., 2015; Rouphael et al., 2017c).

Metabolomics data were formerly elaborated using Agilent Mass Profiler Professional B.12.06 (from Agilent Technologies). Compounds were filtered by abundance (area > 10,000 counts), normalized at the 75th percentile and baselined to the median of control. Unsupervised hierarchical cluster analysis was carried out setting similarity measure as "Euclidean" and "Wards" linkage rule. Fold-change analysis was also carried

out, using a cut-off value of 2. Thereafter, the dataset was exported onto SIMCA 13 (Umetrics, Malmo, Sweden), UV-scaled and elaborated for partial least square discriminant analysis (PLS-DA) and Orthogonal Projections to Latent Structures Discriminant Analysis (OPLS-DA) modeling together with unsupervised methods (Worley and Powers, 2013). Hierarchical cluster analysis can be applied in order to reveal differences between classes without supervision, whilst the utilization of class membership in OPLS-DA allows a better separation between classes in score plot hyperspace while effectively separating Y-predictive variation from Y-uncorrelated variation in X. In particular, OPLS-DA allowed separating variation between the groups into predictive and orthogonal (i.e., ascribable to technical and biological variation) components. Outliers were excluded using the distance from the origin in the OPLS-DA model, according to Hotelling's T2 and adopting 95 and 99% confidence limits for suspect and strong outliers respectively. Model overfitting was excluded through cross validation CV-ANOVA ($p < 0.01$) and permutation testing. Model parameters (goodness-of-fit R^2Y and goodness-of-prediction Q^2Y) were also produced. Regarding Q^2Y prediction ability, a value >0.5 was adopted as a threshold to identify acceptable models, according to software recommendation and as set out in literature (Rombouts et al., 2017). Variable importance in projection (VIP analysis) was used to evaluate the importance of metabolites and to select those having the highest discrimination potential (VIP score >1.3). To achieve information on the regulation of biochemical processes related to OMP treatment either under salinity or nonsaline control, a following fold-change analysis was performed for those metabolites highlighted by VIP analysis.

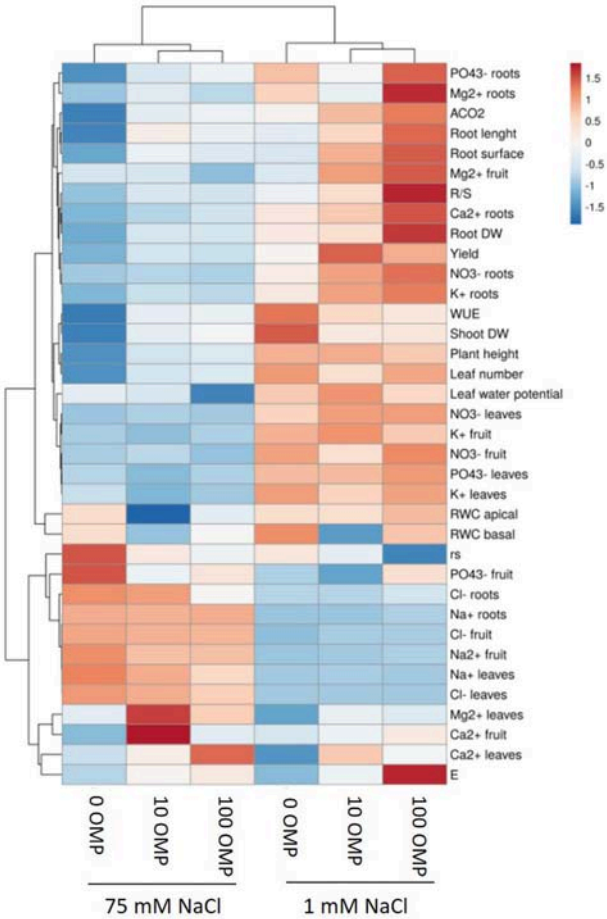


FIGURE 1 | Heat map analysis summarizing the plant responses to NaCl concentration in the nutrient solution and OMP treatments. Results were calculated as Logarithm base 2 (Log2) of untreated and OMP-treated plants under to salinity levels (1 or 75 mM NaCl) and were visualized using a false color scale with red indicating an increase and blue a decrease of plants values compared to values relative to those in control condition. No differences were visualized by white squares.

RESULTS

Morphological Parameters, Yield, and Root Characteristics

Plant height, number of leaves per plant, total leaf area as well as shoot biomass were influenced by salinity and omeprazole (OMP) treatments with significant salinity × OMP interaction. In treated and untreated tomato plants, the plant height number of leaves, leaf area and dry biomass decreased as the salinity level increased, with a more detrimental effect recorded in untreated plants (**Figures 1**, **2**). In fact, increasing NaCl concentration in the nutrient solution from 1 to 75 mM decreased the tomato shoot biomass by 49% in the control treatment, whereas the dry shoot reduction was not significant when 10 μM (−10%) and 100 μM (−7%) of OMP were used, with no significant difference between the two OMP concentrations.

Except from the root diameter, which was not affected by either salinity or OMP, root dry weight, total root length and surface as well as the root-to-shoot ratio (R/S) incurred significant salinity × OMP interaction (**Figure 1**). The root dry weight, total length and surface area were negatively influenced by salt stress treatment (**Figure 3**). Under nonsaline conditions, the drench application of OMP elicited dose-dependent increases in root dry weight, total length and surface, whereas under saline conditions significant differentiation was observed with respect to the 0 μM control but not between the 10 and 100 μM treatments (**Figures 1**, **3**).

Tomato yield and the mean fruit weight were significantly affected by salinity and OMP treatments with no salinity × OMP interaction. Neither salinity nor OMP treatment had a significant effect on tomato fruit number (data not shown). Irrespective of OMP treatment, fresh tomato yield decreased with increasing salinity in the nutrient solution (**Figure 4**). Moreover, when averaged over salt-treatment levels, the yield of OMP-treated plants was higher than those of untreated plants by 44.5% (**Figure 4**).

Physiological Parameters

The net CO_2 assimilation rate (A_{CO2}) and stomatal resistance (r_s) of tomato plants were significantly affected by salinity and OMP treatments, with no salinity × OMP application interaction;

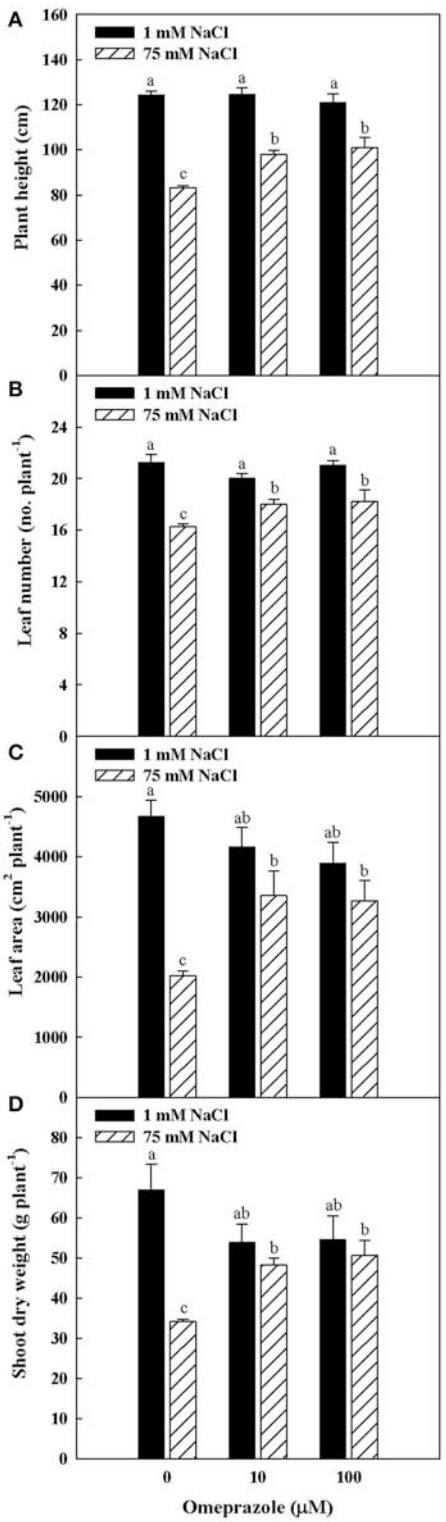


FIGURE 2 | Effects of NaCl concentration in the nutrient solution and omeprazole application on plant height **(A)**, number of leaves per plant **(B)**, total leaf area **(C)**, and shoot dry biomass **(D)** of greenhouse tomato plants. Different letters indicate significant differences according to Duncan's test ($P = 0.05$). The values are the means of four replicate samples. Vertical bars indicate ± SE of means.

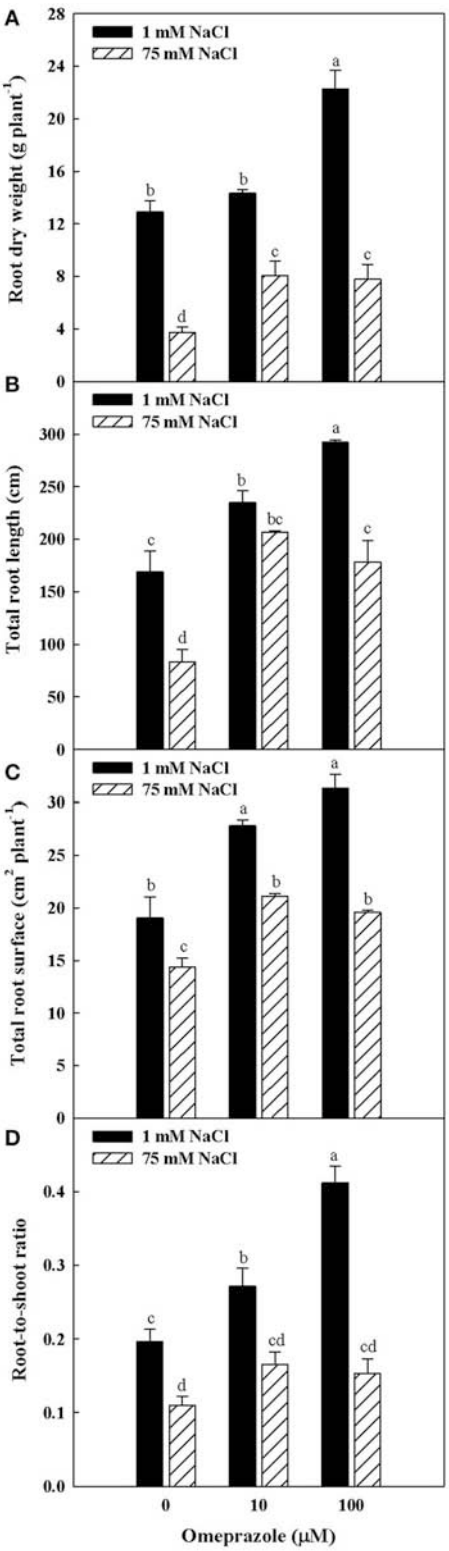


FIGURE 3 | Effects of NaCl concentration in the nutrient solution and omeprazole application on root dry weight **(A)**, total root length **(B)**, total root surface **(C)**, and root-to-shoot ratio **(D)** of greenhouse tomato plants. Different letters indicate significant differences according to Duncan's test ($P = 0.05$). Vertical bars indicate ± SE of means.

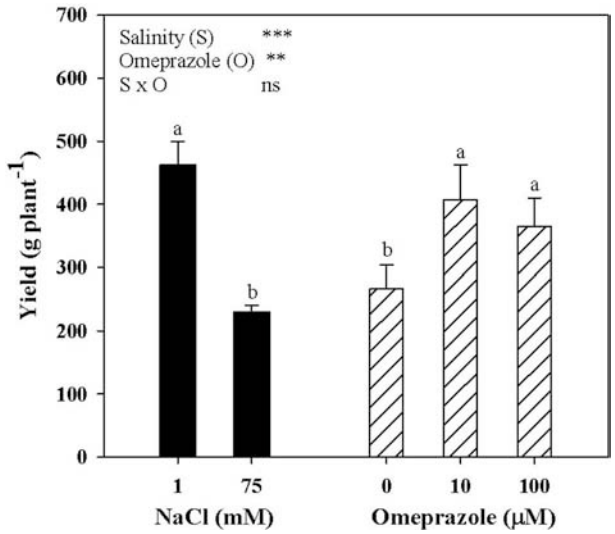


FIGURE 4 | Mean effects of NaCl concentration in the nutrient solution and omeprazole application on yield of greenhouse tomato plants. Different letters indicate significant differences according to Duncan's test ($P = 0.05$). The values are the means of four replicate samples. Vertical bars indicate ± SE of means. ns, **, *** Nonsignificant or significant at $P \leq 0.01$, and 0.001.

whereas the leaf water potential (Ψl) and WUE were only affected by the salinity treatment (**Table 1**). Increasing the sodium chloride concentration in the nutrient solution from 1 to 75 mM reduced Ψl, A_{CO2}, and WUE by 27, 37, and 34%, respectively, while it increased r_s values by 23% (**Table 1**). Substrate drench application of OMP induced significant increase of A_{CO2} (+48%), with no significant difference between the two OMP concentrations (**Table 1**). The higher A_{CO2} in OMP-treated tomato plants was accompanied by an increase in E values. Averaged over salinity treatments, OMP application induced lower values of r_s in comparison to untreated plants (**Table 1**). Conversely to the leaf gas exchange parameters, no significant differences between treatments were recorded in RWC of basal leaves (**Table 1**).

Ion Content and Partitioning

Except for the bivalent cations (Ca^{2+} and Mg^{2+}) in leaf tissue, the NO_3^-, PO_4^{3-}, K^+ in both leaves and roots as well as Ca^{2+} and Mg^{2+} in roots, were negatively affected by 75 mM NaCl in the nutrient solution (**Table 2**). Moreover, the concentrations of both toxic elements (Na^+ and Cl^-), which accumulated mainly in leaves and to a lesser extent in roots, were significantly influenced by salt stress treatment (**Table 2**). In OMP untreated plants, the concentrations of Na^+ and Cl^- were 50- and 23-fold higher as the salinity level in the nutrient solution increased (**Table 2**). The K^+/Na^+ ratio, initially equal to 21.4 in leaves and 11.9 in roots, was drastically reduced at 75 mM of NaCl to a value of 0.3 and 0.4, respectively.

The OMP treatment, averaged over salt stress levels, affected NO_3^- and Ca^{2+} concentrations in leaf tissue which were higher by about 23% than in OMP untreated tomato plants (**Table 2**). Interestingly, under nonsaline conditions the application of 10 and 100 μM of OMP as substrate drench induced a significant increase of NO_3^- in root tissue (**Table 2**). Significant OMP × salinity interaction was observed as the OMP treatment effectively reduced the Na+ and Cl− accumulation in leaf tissue under saline (75 mM NaCl) but not under nonsaline (1 mM NaCl) conditions. Under saline conditions the OMP application significantly reduced Na^+ and Cl^- accumulation in leaf tissue in a dose-dependent manner: −14 and −31% Na^+, and −7 and −20% Cl^- in response to 10 and 100 μM OMP, respectively. However, significant reductions in leaf Na^+ and Cl^- concentrations were attained in response to the 100 μM OMP level. Root Na^+ and Cl^- concentrations were lower by 31 and 20%, respectively, when 100 μM OMP was delivered to tomato plants (**Table 2**, **Figure 1**).

The fruit mineral composition was significantly affected by salinity and to a lesser extent by the OMP application. Increasing the NaCl concentration in the nutrient solution decreased the concentrations of NO_3^-, K^+ and Mg^{2+}, whereas an opposite trend was observed for Na^+ and Cl^- (**Supplementary Table 1**). Finally, the highest Mg^{2+} concentration in tomato fruit was observed at 1 mM NaCl combined with the application of 10 and 100 μM OMP (**Supplementary Table 1**).

Metabolic Profiling of Leaves

The salinity × OMP application interaction was also analyzed using an untargeted metabolomics approach based on UHPLC-ESI/QTOF-MS. Overall, this analytical approach allowed annotating 2,019 compounds. The entire list of compounds identified across the samples is provided as **Supplementary Table 2**, together with annotations and composite MS spectra.

Both the non-averaged unsupervised hierarchical cluster analysis and the supervised orthogonal projection to latent structures discriminant analysis (OPLS-DA) multivariate statistical approaches (see Materials and Methods section) allowed differentiating between treatments (**Figures 5** and **6**), suggesting that the metabolic profiles were affected by the treatments. In particular, when looking at the heat-map based on the fold-change analysis (**Figure 5**), two main clusters could be identified, representing 1 and 75 mM NaCl respectively. These findings indicated that salinity was the main clustering factor. Nonetheless, OMP treated plants could be discriminated from those without OMP, and a dose-dependent response was also observed under salt stress conditions (i.e., 75 mM NaCl). Indeed, three separated sub-clusters could be defined under salinity, whereas OMP treated plants clustered together under 1 mM NaCl conditions. The following OPLS-DA supervised multivariate analysis provided an output that was consistent with hierarchical clustering, suggesting that salinity was the principal factor followed by OMP concentration (**Figure 6**). To better point out the response related to OMP itself rather than its specific biochemical role in promoting salt stress tolerance, two different OPLS-DA models were built, under 1 and 75 mM NaCl, respectively (**Supplementary Figure 1**). Both models fitting parameters were more than adequate, being goodness-of-fit R2Y = 0.99 under both salinity conditions and

TABLE 1 | Analysis of variance and mean comparisons for leaf water potential (Ψ_l), relative water content (RWC) of apical and basal leaves, net CO_2 assimilation rate (A_{CO2}), stomatal resistance (rs), transpiration rate (E), and water use efficiency (WUE) of tomato plants grown under two salinity levels and treated with omeprazole (OMP) at three rates of application.

Source of variance	Ψ_l (MPa)	RWC apical (%)	RWC basal (%)	A_{CO2} (μmol CO_2 m^{-2} s^{-1})	r_s (m^2 s^1 mol^{-1})	E (mol H_2O m^{-2} s^{-1})	WUE (μmol CO_2 mol^{-1} H_2O)
Salinity (S)	***	ns	ns	***	*	ns	*
Omeprazole (OMP)	ns	*	ns	*	**	*	ns
S × O	ns	ns	ns	ns	ns	ns	ns
SALINITY (mM NaCl)							
1	−1.39a	83.88	91.51	6.22a	16.84b	1.78	3.58a
75	−1.90b	77.33	86.94	3.95b	20.64a	1.69	2.35b
OMEPRAZOLE (μM)							
0	−1.58	83.37a	95.57	3.87b	22.06a	1.41b	3.02
10	−1.54	76.36b	81.36	5.46a	18.65ab	1.70ab	2.95
100	−1.81	82.10a	91.81	5.92a	15.52b	2.03a	2.93
S ×OMP							
1 mM NaCl × 0 μM OMP	−1.42	83.29	96.66	5.22	19.86	1.38	4.13
1 mM NaCl × 10 μM OMP	−1.28	83.08	80.14	6.36	17.73	1.66	3.41
1 mM NaCl × 100 μM OMP	−1.46	85.28	93.93	7.09	12.91	2.27	3.16
75 mM NaCl × 0 μM OMP	−1.74	83.44	92.31	2.53	24.25	1.46	1.37
75 mM NaCl × 10 μM OMP	−1.80	69.64	82.57	4.56	19.56	1.73	2.64
75 mM NaCl × 100 μM OMP	−2.15	78.92	88.63	4.75	18.12	1.79	2.70

*ns, *, **, *** Nonsignificant or significant at $P \leq 0.05$, 0.01, and 0.001, respectively. Different letters within each column indicate significant differences according to Duncan's multiple-range test ($P = 0.05$).*

goodness-of-prediction Q2Y 0.59 and 0.73 for 1 and 75 mM NaCl, respectively. Both models provided 100% accuracy in class prediction (Fischer's probability: 0.0002), whereas cross validation CV-ANOVA ($p < 0.01$) and permutation testing excluded model overfitting. No outlier replicates could be identified using Hotelling's T2 under 95 and 99% confidence limits for suspect and strong outliers, respectively.

Given the adequate fitting of OPLS-DA models, a subsequent investigation was done aiming to identify the compounds differences could be attributed to. With this purpose, the investigation of the most discriminant compounds in the OPLS-DA model (i.e., variables of importance in projection—VIP analysis) was carried out. **Table 3** reports the metabolites identified (i.e., >1.3) by VIP analysis, together with individual scores and their standard error, as well as Log fold-change values and regulation. Overall, 84 compounds were identified as those variables mostly contributing to class discrimination in OPLS-DA. Discriminating compounds were grouped in functional classes; hormones, membrane lipids, terpenes, and alkaloids were the most represented classes. Among hormones, compounds related to almost all classes could be discerned. In more detail, the brassinosteroid brassinolide, auxin inactivation compounds (oxindole-3-acetyl-aspartate-N-beta-glucosyl-beta-1,4-glucose; 2-oxindole-3-acetyl-hexose; indole-3-acetyl-tryptophan), inactive forms of gibberellins (A34, A98, A51-catabolite), a precursor and a catabolite of abscisic acid (abscisic aldehyde and dihydroxyphaseic acid respectively), methyl jasmonate and a cytokinin (trans-zeatin riboside triphosphate) were identified. Among lipids, several membrane lipids (glyco- and phospholipids) were identified in VIP analysis, together with cutin biosynthetic intermediates [9,10-epoxystearate and (9R,10S)-dihydroxystearate]. Sesquiterpene lactones were also among discriminating compounds, including lubimin-related sesquiterpenoid phytoalexins (3-hydroxylubimin and 2-dehydrolubimin), parthenolide, two costunolide-related compounds [3-beta-hydroxycostunolide and germacra-1(10),4,11(13)-trien-12-oate] as well as zealexins A1 and A3. Furthermore, several alkaloids and phenolics were outlined as OPLS-DA discriminants; among the seconds, tetramethylquercetagetin, conjugated cyanidins, and hydroxycinnamics were the most represented. However, ajmaline and sarpagine, lupanine, and cinchona alkaloids were the most common. Carotenoids (mainly ascribable to xanthins), polyamines and their conjugates, pteridine as well as porphyrin biosynthetic precursors were also selected among discriminating compounds. Among amino acids, asparagine, lysine, saccharopine (a lysine degradant), and cystathionine (involved in cysteine/homocysteine interconversion) were pointed out. Finally, some other compounds could be recognized as differential, including L-dopachrome (intermediate in eumelanin biosynthesis), 6,7-dimethyl-8-(1-D-ribityl) lumazine (flavin biosynthesis), a plastoquinone, an acetyl-hexosamine and two glucosinolate-related compounds (9-methylthiononylhydroximoyl-glutathione and 7-methylthioheptyldesulfoglucosinolate).

Principal Component Analysis

To obtain a broad overview on the morphological and physiological changes of greenhouse tomato plants in response to OMP application under both saline and nonsaline conditions,

TABLE 2 | Analysis of variance and mean comparisons for nitrate, phosphate, potassium, calcium, magnesium, sodium and chloride ions in leaves and roots of tomato plants grown under two salinity levels and treated with omeprazole (OMP) at three rates of application.

Source of variance	NO_3^- (mg g^{-1}dw)		PO_4^{3-} (mg g^{-1}dw)		K^+ (mg g^{-1}dw)		Ca^{2+} (mg g^{-1}dw)		Mg^{2+} (mg g^{-1}dw)		Na^+ (mg g^{-1}dw)		Cl^- (mg g^{-1}dw)	
	Leaves	Roots	Leaves	Roots	Leaves	Roots	Leaves	Roots	Leaves	Roots	Leaves	Roots	Leaves	Roots
Salinity (S)	***	***	***	*	***	***	ns	***	ns	***	***	***	***	***
Omeprazole (OMP)	**	**	ns	ns	ns	*	*	ns	ns	*	ns	ns	*	ns
S × O	ns	*	ns	ns	ns	ns	ns	ns	ns	**	*	ns	*	ns
SALINITY (mM NaCl)														
1	24.33a	26.29a	23.1a	8.81a	39.46a	26.45a	18.99	4.10a	4.80	1.83a	2.82b	1.94b	10.16b	4.19b
75	3.32b	3.24b	16.0b	6.37b	26.42b	9.83b	20.34	2.28b	5.15	1.24b	81.12a	17.89a	173.94a	21.11a
OMEPRAZOLE (μM)														
0	11.96b	9.89b	11.3	6.99	34.23	14.04b	16.95b	2.77	4.75	1.47b	48.63	9.84	99.81a	12.83
10	14.90a	16.09a	19.0	7.10	31.27	19.75a	20.73a	3.12	5.16	1.40b	43.04	9.63	94.28ab	12.32
100	14.61a	18.31a	20.0	8.68	33.32	20.63a	21.31a	3.68	5.01	1.73a	34.25	10.27	82.06b	12.81
S × OMP														
1 mM NaCl × 0 μM OMP	21.19	17.33b	22.8	8.92	40.61	20.76	16.00	3.53	4.62	1.8b	1.9c	1.75	8.29c	3.35
1 mM NaCl × 10 μM OMP	25.84	28.43a	22.7	7.49	37.51	28.11	21.47	3.95	4.91	1.43c	3.67c	1.52	10.89c	3.49
1 mM NaCl × 100 μM OMP	25.96	33.12a	23.9	10.02	40.26	30.48	19.49	4.83	4.86	2.25a	2.9c	2.53	11.29c	5.74
75 mM NaCl × 0 μM OMP	2.73	2.46c	16.4	5.06	27.85	7.32	17.90	2.01	4.88	1.14c	95.35a	17.92	191.33a	22.30
75 mM NaCl × 10 μM OMP	3.96	3.74c	15.4	6.71	25.04	11.39	19.99	2.30	5.41	1.37c	82.41ab	17.73	177.66a	21.15
75 mM NaCl × 100 μM OMP	3.27	3.5c	16.3	7.34	26.37	10.78	23.14	2.53	5.16	1.21c	65.6b	18.01	152.84b	19.88

*ns, *, **, *** Nonsignificant or significant at $P \leq 0.05$, 0.01, and 0.001, respectively. Different letters within each column indicate significant differences according to Duncan's multiple-range test ($P = 0.05$).*

the PCA was carried out. The first two principal components (PCs) were related with Eigen values > 1 and explained 85.2% of the total variance with PC1 and PC2 accounting for 70.5 and 14.7%, respectively (**Figure 7**). PC1 was positively correlated to nitrate and potassium concentration in leaf tissues, plant height, net photosynthetic rate, yield as well as leaf number and area. PC1 was also negatively correlated to both toxic ions as well as to r_s. Moreover, PC2 was positively correlated to Ca^{2+} and Mg^{2+} in leaves, transpiration and root length and root surface, and negatively correlated to RWC basal and apical, r_s and K^+ in leaves and fruit. Furthermore, the score plot of the PCA clearly divided the two nutrient solutions (1 and 75 mM NaCl) along PC1 with nonsaline treatment concentrating most of the plant growth parameters, yield and PO_4^{3-}, K^+, Ca^{2+}, Mg^{2+} in roots and physiological parameters, whereas the saline treatment stands out for toxic ions (Na^+ and Cl^-) (**Figure 7**). The OMP applications were clustered in respect to PC2, with both 10 and 100 μM OMP on the positive side of the PC2 that is characterized by improved physiological status, root characteristics, photosynthetic performance, R/S and yield (at 1 mM NaCl) and higher leaf Mg^{2+} as well as lower Na^+ and Cl^- concentrations in both leaves and roots (at 75 mM NaCl; **Figure 7**).

DISCUSSION

Implications of Omeprazole for Morphological and Physiological Parameters

Vegetable crops respond to excessive sodium chloride concentration in soil or irrigation water with growth inhibition and yield reduction (Tester and Davenport, 2003), and the severity of crop production losses may fluctuate in relation to several interconnected variables such as cultural environment, genetic material (species and/or cultivars) as well as the concentration, time of exposure, and type of salts (Colla et al., 2010). The significant depression of plant growth parameters (plant height, leaf number, leaf area, biomass production) as well as yield reduction with increasing NaCl in the nutrient solution has been reported previously in several greenhouse experiments on potted leafy and fruit vegetables, including tomato (Colla et al., 2006; Savvas et al., 2011; Rouphael et al., 2012, 2016, 2017b; Lucini et al., 2016). Furthermore, high concentration of NaCl in the nutrient solution will induce a broad range of biochemical, physiological, anatomical, and metabolic changes such as impairment of root activity, nutrient imbalance, chlorophyll degradation and decrease of the net photosynthetic rate (Munns, 2002; Munns and Tester, 2008), as observed in the present experiment on tomato plants supplied with 75 mM NaCl. Significant decrease in morphological and physiological traits in NaCl-treated tomato plants occurred; and that effect varied in relation to the OMP application. However, the positive effects of OMP, for example on WUE, were found at the end of the salinity treatment, suggesting a time-dependent action of OMP. It is probable that the WUE improved under salinity thanks to another mechanism adopted by plants to minimize water loss even at low r_s, that is leaf area restriction, a feature shown by both halophytes and non–halophytes under high salinity (Maas and Nieman, 1978).

The expected stimulator effect of OMP, a benzimidazole inhibitor of animal proton pumps, on plant growth parameters (i.e., plant height, leaf number and area, shoot dry biomass),

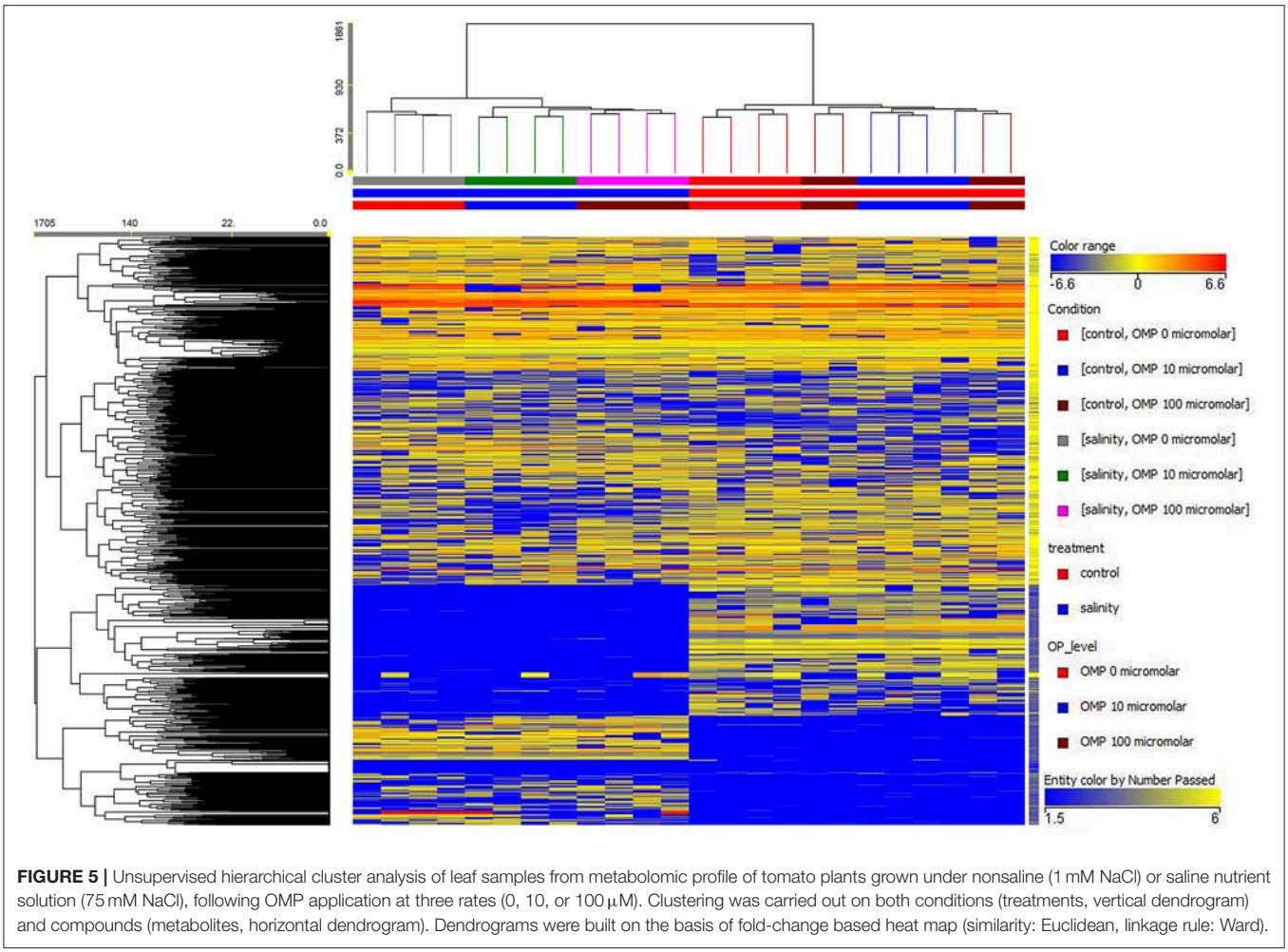


FIGURE 5 | Unsupervised hierarchical cluster analysis of leaf samples from metabolomic profile of tomato plants grown under nonsaline (1 mM NaCl) or saline nutrient solution (75 mM NaCl), following OMP application at three rates (0, 10, or 100 μM). Clustering was carried out on both conditions (treatments, vertical dendrogram) and compounds (metabolites, horizontal dendrogram). Dendrograms were built on the basis of fold-change based heat map (similarity: Euclidean, linkage rule: Ward).

previously reported in Van Oosten et al. (2017), was not evidenced in the absence of salt stress. However, other morphological and physiological parameters were increased by OMP application independently of salinity, in particular root dry weight, root length and surface, R/S, transpiration and photosynthetic net rate. An explanation for this different response, observed against the former study which was performed in the absence of stress, could be attributed to the different growing conditions (hydroponic vs. substrate experiment), variation between the tomato cultivars employed (determinate type "M82" vs. indeterminate type "Seny") and not least the length of the growth cycle (14 vs. 65 days) as the former study was terminated before the plants reached their reproductive stage. On the other hand, the detrimental impact of NaCl was clearly mitigated when 10 and 100 μM of OMP were applied to tomato plants by substrate drench. The improvement of plant growth parameters induced by OMP application could be associated with the stimulation of the root system architecture (increased root dry biomass, total root surface, and length), which may improve nutrient use efficiency and total biomass production. The application of OMP may have also triggered a signal transduction pathway mediated by endogenous phytohormone (i.e., elicitation of root auxin synthesis), which resulted in significant increase of root length and density, thus inducing a "*nutrient acquisition response*" that favored nutrient uptake and translocation.

Omeprazole application could be also responsible for the inhibition of swelling-dependent chloride channels (Schmarda et al., 2000). The presence of non-isosmotic conditions can alter intracellular and extracellular osmolality, generating a passive flow of water thus causing cell swelling or shrinkage (Sardini et al., 2003). In plants, shrinkage is much more dangerous than swelling that is counteracted by vacuolar and cell wall action. When a cell swells, it attempts to restore its original volume by activating channels or transporters in order to release appropriate osmolytes, typically K^+, Cl^- and organic osmolytes. If the osmolyte and/or ion efflux is blocked (OMP effect), it is possible that root cells undergo an enlargement of cell volume thus having a positive effect on root characteristics (total root length and surface area).

Another putative mechanism supporting the inductive role of OMP in stress tolerance is the higher CO_2 assimilation rate, through: (i) better osmotic adjustment, (ii) improved balance between the uptake and loss of water, (iii) higher efficiency in

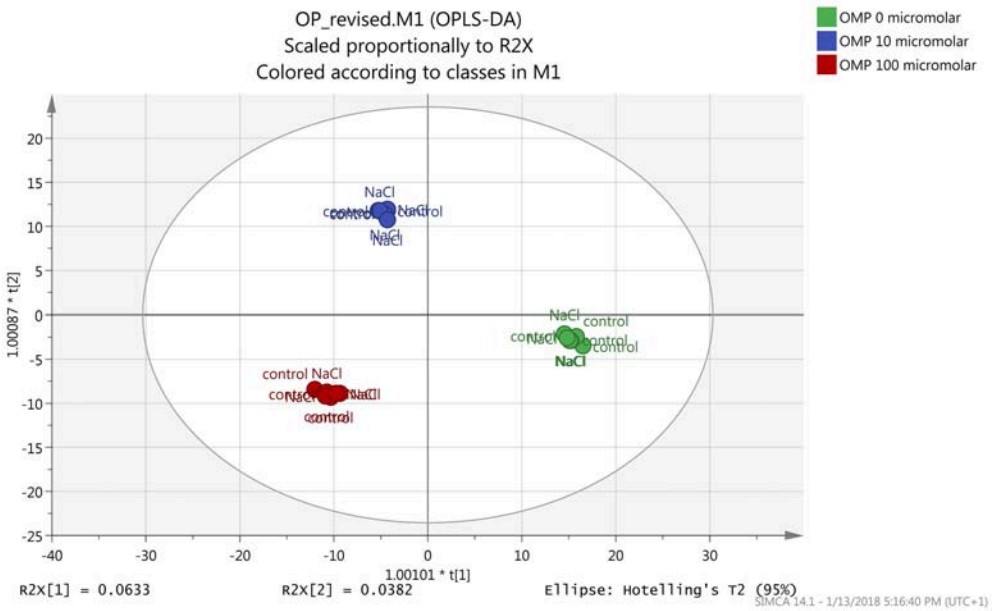


FIGURE 6 | Orthogonal Projections to Latent Structures Discriminant Analysis (OPLS-DA) on tomato leaves metabolome from plants grown under nonsaline (1 mM NaCl) or saline nutrient solution (75 mM NaCl), following OMP application at three rates (0, 10, or 100 μM). Individual replications are given in the class prediction model score plot.

absorbing macro and microelements from the substrate, thus boosting tomato performance. In addition, the application of OMP may improve photosynthesis by reducing the stomatal resistance, as observed in the current experiment as well as by Van Oosten et al. (2017).

Implications of Omeprazole for Ion Homeostasis

The maintenance of ion homeostasis, in which salt overly sensitive (SOS) pathway plays a key role, is a major adaptation strategy against salinity, highly implicated in plant salt tolerance (Soni et al., 2013). In the present study, the high concentration of Na^+ and Cl^- in the nutrient solution depressed cation and anion uptake, translocation and accumulation based on the strong decrease of NO_3^-, PO_4^-, K^+ (in both leaf and root tissues) and Ca^{2+} and Mg^{2+} (in roots) as previously reported by Grattan and Grieve (1999) on a wide range of horticultural commodities. It is well established that Cl^- competition for NO_3^- transporter proteins affects NO_3^- uptake and transport and reduces the loading of NO_3^- into the root xylem (Carillo et al., 2005, and references therein). Moreover, since NO_3^- is necessary to induce nitrate reductase (NR), the first key enzyme of the nitrogen assimilation process, decrease of NO_3^- flux from roots under salinity stress severely affects NR and nitrogen assimilation in leaves (Campbell, 1999). High concentration of Na^+, in turn, impairs not only K^+ translocation from root to shoot but also its uptake by plasma membrane transport (Gao et al., 2016). Moreover, Na^+ can depolarize and damage the plasma membrane favoring K^+ leakage, further decreasing the cytosolic K^+ content (Wang et al., 2013). When Na^+ concentration is much higher than that of K^+, it can substitute K^+ in key enzymatic reactions and damage metabolic pathways in cytosolic compartments. Therefore, plants are less sensitive to the absolute amount of Na^+ than to K^+/Na^+ ratio (Shabala and Cuin, 2008; Cuin et al., 2009). However, salt tolerant tomato plants were able to retain higher Na^+ and Cl^- levels in leaves than in roots, suggesting the presence of an active inclusion mechanism in plants as a trait of salt tolerance (Läuchli and Epstein, 1990; Rodriguez et al., 2005). In fact, taking into account the high genotypic diversity of tomato plants with respect to ion homeostasis, the more tolerant species/accessions are able to accumulate higher amounts of salts in shoots (leaves and stems), while those more sensitive accumulate salts principally in roots (Cuartero and Fernández-Mu-oz, 1998). It has been ascertained that tomato roots can sense and control the Na^+ concentration reaching aerial parts depending on the intensity of the stress, probably thanks to the SOS pathway (Olías et al., 2009).

Accordingly, an intriguing current result was that OMP treatment decreased Na^+ and Cl^- concentration in leaves, especially the 100 μM application, and increased leaf Ca^{2+} concentration. However, K^+/Na^+ ratio did not significantly increase in leaves but also in the roots of OMP treated plants where K^+ slightly increased. These findings suggest the induction of a salt-tolerance mechanism other than vacuolar sequestration of Na^+, which is a common means for decreasing Na^+ concentration in the cytoplasm, thereby contributing to the osmotic adjustment while maintaining water absorption under salt stress conditions (Silva and Gerós, 2009). Mitochondria and plastids can also sequester some Na^+ contributing to its compartmentalization (Conde et al., 2011). At the same time,

TABLE 3 | Leaf metabolites discriminating tomato plants under two salinity levels and treated with omeprazole (OMP) at three rates of application.

Compound	OPLS-DA VIP		[OMP 100 μM] vs. [OMP control], 1 mM NaCl		[OMP 100 μM] vs. [OMP control], 75 mM NaCl	
	Score	Standard error	Log fold-change	Regulation	Log fold-change	Regulation
AMINO ACIDS						
L-asparagine	1.33	0.89	−4.13	Down	−3.18	Down
L-cystathionine	1.33	1.31	0.00	Down	−1.50	Down
L-lysine	1.39	0.73	0.52	Up		
L-saccharopine	1.31	0.45	0.00	Down		
HORMONES						
brassinolide	1.38	0.36	−0.37	Down	−2.98	Down
oxindole-3-acetyl-aspartate-N-beta-glucosyl-beta-1,4-glucose	1.30	0.39	−6.66	Down	−1.75	Down
a 2-oxindole-3-acetyl-hexose	1.64	0.54	−4.40	Down		
indole-3-acetyl-tryptophan	1.56	0.43	6.72	Up		
gibberellin Asub34/sub	1.47	0.17	20.34	Up		
gibberellin Asub98/sub	1.39	0.85	−5.29	Down	−1.35	Down
gibberellin Asub51/sub-catabolite	1.32	0.67	−9.51	Down		
trans-zeatin ribosidetriphosphate	1.38	0.96	0.00	Down	−3.92	Down
(+)-cis-abscisic aldehyde	1.38	1.00	9.30	Up		
dihydroxyphaseic acid	1.36	0.86	5.48	Up	−0.03	Down
methyl jasmonate	1.36	1.08	0.59	Up	−0.01	Down
LIPIDS						
9,10-epoxystearate	1.35	0.65	0.37	Up		
(9R,10S)-dihydroxystearate	1.34	0.66	0.45	Up		
1-18:0-2-18:1-phosphatidylethanolamine	1.43	0.79	1.40	Up		
1-16:0-2-18:3-diacylglycerol-trimethylhomoserine	1.39	0.93	4.94	Up	−1.56	Down
1-18:3-2-16:3-monogalactosyldiacylglycerol	1.31	0.96	0.01	Up	−0.23	Down
1-18:1-2-16:1-monogalactosyldiacylglycerol	1.39	0.57	10.83	Up	−6.62	Down
1-18:1-2-16:0-monogalactosyldiacylglycerol	1.36	1.23	9.91	Up	−0.14	Down
1-18:2-2-18:2-monogalactosyldiacylglycerol	1.31	0.90	−0.21	Down	−0.38	Down
1-18:1-2-16:0-phosphatidylglycerol	1.38	0.91	5.20	Up	−2.57	Down
1,2-dipalmitoyl-phosphatidylcholine	1.37	0.40	−5.29	Down	−3.94	Down
1-18:1-2-18:3-phosphatidylcholine	1.43	0.45	−0.15	Down	−2.57	Down
1-18:3-2-18:2-phosphatidylcholine	1.38	0.58	0.11	Up	−0.19	Down
1-18:1-2-18:1-sn-glycerol-3-phosphocholine	1.37	1.06	0.09	Up	−0.60	
1-18:3-2-18:3-phosphatidylcholine	1.35	0.65	−0.13	Down	−2.41	Down
1-18:3-2-18:1-phosphatidylcholine	1.32	0.68	−0.15	Down	−0.60	Down
linolenate	1.34	0.74	6.84	Up		
(9S,10S)-9,10-dihydroxyoctadecanoate	1.34	0.66	0.26	Up		
4-alpha-carboxy-5-alpha-cholesta-8,24-dien-3-beta-ol	1.31	0.93	18.25	Up	−4.50	Down
SESQUITERPENE LACTONES						
zealexin A1	1.42	0.71	0.05	Up	−1.65	Down
zealexin A3	1.34	0.87	0.44	Up		
parthenolide	1.38	1.00	9.30	Up		
germacra-1(10),4,11(13)-trien-12-oate	1.42	0.71	−5.10	Down	−1.65	Down
3-beta-hydroxycostunolide	1.38	1.00	9.30	Up		
3-hydroxylubimin	1.50	0.55	4.21	Up		Down
2-dehydrolubimin	1.33	0.78	−4.83	Down		
ALKALOIDS						
10-deoxysarpagine	1.38	0.80	−5.41	Down	−1.44	Down

(Continued)

TABLE 3 | Continued

Compound	OPLS-DA VIP		[OMP 100 μM] vs. [OMP control], 1 mM NaCl		[OMP 100 μM] vs. [OMP control], 75 mM NaCl	
	Score	Standard error	Log fold-change	Regulation	Log fold-change	Regulation
vellosimine	1.36	0.37	−0.20	Down		
17-O-acetylajmaline	1.35	1.03	0.08	Up		
1,3,7,9-tetramethylurate	1.39	1.15	−4.22	Down	−0.08	Down
(S)-n-methylcanadine	1.40	1.05	5.16	Up	−0.08	Down
quinidinone	1.40	0.62	0.42	Up	−5.61	Down
cinchoninone	1.36	0.37	−4.84	Down		
lupanine	1.34	0.60	−0.02	Down		
17-oxosparteine	1.34	0.60	−0.02	Down		
PHENOLICS						
glyceollin I/II	1.38	1.06	0.00	Down		
maysin	1.35	0.56	−3.19	Down		
1-naphthol glucoside	1.38	0.87	0.54	Up		
tetramethylmyricetin/tetramethylquercetagetin	1.32	0.67	0.14	Up		
2′-hydroxy 3,6,7,4′-tetramethylquercetagetin	1.32	0.58	0.53	Up	−0.49	Down
cyanidin 3-O-glucoside-7-O-(6-O-(p-hydroxybenzoyl)-glucoside)	1.31	0.65	4.88	Up	−0.07	Down
cyanidin 3-O-glucoside-7-O-(6-O-(4-O(6-O-(p-hydroxybenzoyl)-glucosyl)-oxybenzoyl)-glucoside)	1.49	0.65	−2.81	Down		
cinnamaldehyde	1.43	0.63	14.91	Up		
beta-D-glucosyl-2-hydroxycinnamate	1.40	0.68	0.48	Up	−0.62	Down
PORPHYRIN						
protoporphyrin IX	1.38	0.35	−8.53	Down		
uroporphyrinogen-III	1.41	0.94	−0.11	Down		
CAROTENOIDS						
3,4,3′,4′-tetradehydroisozeaxanthin	1.41	0.55	6.19	Up		
capsanthin	1.34	0.86	0.12	Up	−0.14	Down
sulcatone	1.31	0.58	0.00	Down		
caloxanthin	1.34	0.86	0.12	up	−0.14	Down
5,6-epoxy-3-hydroxy-9-apo-beta;-caroten-9-one	1.36	1.08	0.42	Up	−0.01	Down
zeinoxanthin	1.43	1.05	0.24	Up	−0.08	Down
POLYAMINES AND CONJUGATES						
dihydroxyferuloyl-sinapoyl spermidine	1.38	0.54	0.00	Down	−3.72	Down
acetylspermidine	1.39	0.46				
tyramine	1.69	0.45	0.00	Up	−7.96	Down
cinnamoyltyramine	1.31	0.88	−4.92	Down		
PTERIDINE						
a 5,6,7,8-tetrahydropteridine	1.35	0.35	5.53	Up		
6-hydroxymethyl-7,8-dihydropterin	1.34	1.25	0.21	Up	−2.88	Down
tetrahydropteroyl-α-glutamylglutamate	1.33	0.80	0.03	Up	−5.91	Down
OTHERS						
L-dopachrome	1.33	0.66	−0.08	Down	−1.06	Down
6,7-dimethyl-8-(1-D-ribityl)lumazine	1.33	0.97	0.00	Down	−1.73	Down
(R)-pantoate	1.43	0.47	0.54	Up		
dehydroascorbate (bicyclicform)	1.38	0.49			−0.31	Down
a plastoquinone	1.44	0.82	0.09	Up		
an N-acetyl-D-hexosamine	1.40	0.55	−0.09	Down	−0.82	Down
9-methylthiononylhydroximoyl-glutathione	1.32	0.70	15.32	Up	−4.79	Down
7-methylthioheptyldesulfoglucosinolate	1.31	0.88	5.87	Up		

Compounds were gained through UHPLC-ESI/QTOF-MS metabolomics and selected by OPLS-DA discriminant analysis followed by VIP (Variables of Importance in Projection) analysis. Compounds are grouped in functional classes and provided together with VIP score, VIP score standard error, as well as fold-change analysis.

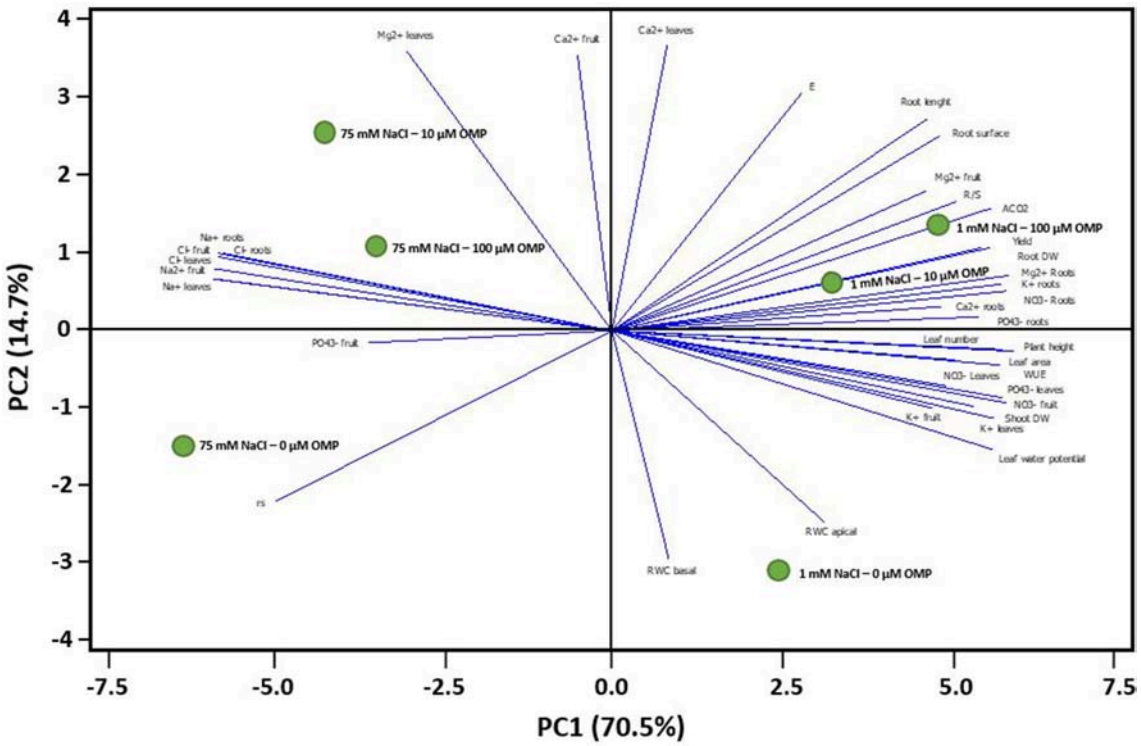


FIGURE 7 | Principal component loading plot and scores of principal component analysis (PCA) of morphological, physiological traits and ion contents of greenhouse tomato grown under nonsaline (1 mM NaCl) or saline nutrient solution (75 mM NaCl), following OMP application at three rates (0, 10, or 100 µM).

cytosolic K^+ concentration can be maintained at a constant level or, thanks to the K^+ stored in the vacuole, even increased, osmoregulating the cell and avoiding the impairment of plant metabolism under salinity.

Implications of Omeprazole for the Metabolomic Profile of Tomato Leaves

The metabolomic profile of tomato leaves was clearly affected by the OMP treatment as highlighted by both unsupervised and supervised multivariate statistics. Hierarchical cluster analysis, i.e., the unsupervised chemometric approach, evidenced two distinct clusters, comprising NaCl saline stress and nonsaline control. Looking at sub-clusters within main clusters, the effect of OMP was still evident, resulting in a mixed cluster in control (comprising both 10 and 100 µM OMP) separate from 0 µM OMP. However, three distinct sub-clusters could be evidenced in the main salinity cluster representing 0, 10, and 100 µM OMP. The former clustering, therefore, evidenced that OMP treatment had an impact on tomato leaf metabolomic profile even under nonsaline conditions. This effect became dose-related when 75 mM NaCl was applied. On these premises, it can be postulated that the effect of OMP far exceeded the osmoregulation of ions. This is coherent with the fact that OMP is known to interfere with P-Type IIC ATPases, a large family of ATP-driven transporters (Shin et al., 2009) that have not been reported in *planta* (Van Oosten et al., 2017). As a further confirmation, Na concentration in salinized roots was unrelated to the OMP treatment. Interestingly, looking at plants grown in 75 mM NaCl, Na^+ concentration in leaves was reduced by OMP in a dose-dependent manner but the K^+/Na^+ ratio was almost unaffected. These findings support the fact that a complex metabolic response might be involved, potentially including hormonal network balance and compounds trafficking.

To investigate further the effect of OMP on tomato leaf metabolome, a supervised tool was carried out. It is reported that OPLS-DA is a powerful supervised approach in metabolomics (Worley and Powers, 2013). Indeed, the utilization of class membership in OPLS-DA allows a better separation between classes in score plot hyperspace, while effectively separating Y-predictive variation from Y-uncorrelated variation in X. The VIP score, being calculated as a weighted sum of the squared correlations between the OPLS-DA components and the original variables, is next able to summarize the contribution a variable provides to the model. The excellent OPLS-DA model parameters achieved starting from UHPLC-ESI/QTOF-MS profiles, suggest that differences were actually represented within our dataset.

Hormone compounds were among the most represented in VIP analysis. A complex fine-tuning of plant hormone profiles is occurring under salt stress conditions, as recently reviewed (Ryu and Cho, 2015). Abscisic acid (ABA) is a key enzyme in regulating the response to saline stress since its increase induces stomatal closure, accumulation of osmolytes and growth defects thus ensuring plant survival under salinity. Indeed, ABA

precursors accumulated in OMP-treated tomato under 1 mM NaCl, suggesting that the treatment might trigger an improved tolerance to salinity. Coherently, a decrease was observed for both auxins (catabolites decreased and an inactive conjugate form increased under 1 mM NaCl) and a cytokinin (a zeatin-riboside derivative in 75 mM NaCl). Auxins are known to cause hypersensitivity to salt stress, likely because they interfere with the salt-mediated remodeling of root architecture (Ryu and Cho, 2015 and references therein). Analogously, cytokinins have a negative role in response to salinity, as their receptors modulate environmental signals and because of their ABA-antagonistic activity (Ryu and Cho, 2015 and references therein). Finally, also a decrease in gibberellins (GA) can trigger salt tolerance, as GA-deficient mutants exhibited enhanced salt stress response (Ryu and Cho, 2015). Notably, GA catabolites were found among discriminant metabolites in our experiments. It must be also pointed out that a complex network of cross-talking enzymes might be considered. As an example, it is known that auxin regulation of GA biosynthesis has a key role in regulating growth between different organs/tissues, and that this aspect relates to survival under salinity conditions (Yamaguchi, 2008). Ethylene is a further hormone known to play a central role in abiotic stress response. Our analytical approach could not detect ethylene, as this is a volatile small metabolite. The brassinosteoid brassinolide was also involved in OMP-related response. Brassinosteroids are involved in plant stress response through cross-talking with ABA, and are supposed to have a positive role in stress tolerance via modulation of stomatal conductance (Ryu and Cho, 2015 and references therein). Unexpectedly, the current trend was not consistent with previous findings, as brassinolide down accumulated in 75 mM NaCl treated tomato plants. Nonetheless, the decrease in L-cystathionine (involved in *de novo* synthesis of ethylene precursor methionine) was found down accumulated in OMP-treated tomato leaves under 75 mM NaCl. Although the changes in phytohormonal profile induced by OMP deserve further investigation, the above-reported information clearly suggests that OMP treatment significantly altered hormonal balance in tomato. Looking at the changes in growth and physiological parameters, these OMP-induced alterations might have contributed toward the increase in salt stress tolerance we observed.

Besides hormonal imbalance, lipids were also involved in the response to OMP treatment. Interestingly, two cutin-related compounds were up accumulated in leaves treated with OMP. Cuticular lipids are reported to be induced by NaCl and drought, with the aim of limiting water losses thanks to their ability to postpone the onset of cellular dehydration (Kosma et al., 2009). Several membrane lipids, mainly glycosylated lipids or phospholipids, were also involved in stress response. The plasma membrane H^+ ATPase pumps play essential roles in signal transduction, cell expansion, stomatal opening, and salt stress response (Sun et al., 2010a). These pumps counteract salt activated K^+ efflux, which is mediated by depolarization activated channels and accelerate H_2O_2 production via NADPH oxidases (Zhang et al., 2017), thus triggering Ca^{2+} influx (Sun et al., 2010b). Subsequently, elevated Ca^{2+} levels activate the SOS signaling pathway (Zhang et al., 2017). SOS pathway alters, among others, the cytoskeleton, root architecture and mineral partitioning (Ji et al., 2013). The changes observed in membrane lipids might be the result of these membrane related processes occurring under salinity. Analogously, the accumulation in phenolics and carotenoids could be related to the increased H_2O_2 production, with the aim of strengthening the antioxidant capacity of leaves under salt stress. Coherently, the oxidized form of ascorbic acid was found to be down accumulated in OMP treated plants under 75 mM NaCl.

Also terpenes and alkaloids are compounds that can be triggered by environmental stress, and by NaCl salinity in particular (Chadwick et al., 2013; Lucini et al., 2015); among terpenes, sesquiterpenoids are reported to possess antioxidant capacity (Chadwick et al., 2013), and therefore might be also implicated in the aforementioned response to oxidative stress at the membrane level.

Spermidine and tyramine polyamine conjugates were also altered by OMP treatment. These compounds can be acylated for regulatory purposes likely altering their biological functionality; they are reported to be involved in a wide range of plant developmental processes such as cell division, flowering, and responses to environmental stress (Luo et al., 2009). Although their specific role in OMP response is still unclear, their recruitment was highlighted in the present results, consistently with previous findings on abiotic stress response (Shi and Chan, 2014; Rouphael et al., 2016). The involvement of pteridine, needed for folate biosynthesis, is also not surprising. Indeed, plant metabolism involves a wide range of interconversion and donation of one-carbon (C1) units, through reactions where folates are essential cofactors. Folates participate moreover in the THF-mediated glycine-serine conversion in photorespiration.

Overall, a very articulated and complex metabolic response was observed in response to OMP treatment, involving hormonal balance and cross-talking, membrane processes and oxidative stress there occurring under salinity, as well as a range of other stress elicited chemical compounds. These processes differ from the classical mechanisms through which plants increase tolerance to salinity. Typically, salt-resistant plants possess an improved capacity for H^+ pumping activity, which enables salinized cells to retain K^+/Na^+ homeostasis and avoid ionic toxicity. According to our results, the processes related to OMP treatment transcend typical ion homeostasis. Although a single well-defined specific mechanism could not be outlined, a hormone-like activity has been postulated. On this basis, the mechanism(s) through which OMP affects tolerance to NaCl salt stress involve different biochemical/metabolic responses. Taken together, these OMP-related changes finally end in an improved capacity of counteracting the detrimental processes triggered by salinity.

CONCLUSIONS

Under climate change scenario, the pressure of abiotic stressors and in particular salinity on vegetable productivity is expected to further challenge food security in the coming decades.

Thus, it is important to explore the potential role of small bioactive molecules resourced from human/animal research in increasing vegetable plant tolerance to conditions of salinity. The metabolic profile of plants was found significantly affected by OMP treatment, and dose-dependent changes in key metabolites were identified under 75 mM NaCl salt stress conditions. OMP was not strictly involved in homeostasis of ions, even though it was able to decrease leaf Na^+ and Cl^- concentration under salinity stress. This is in agreement with the fact that plants do not possess P-Type IIC ATPases, i.e., the known target of OMP. However, this small bioactive molecule appeared to be involved in a signal transduction pathway regulating endogenous hormones responsible for the increase of morphological root parameters, and consequently for the "*nutrient acquisition response.*" Hormonal network was significantly affected by OMP, eliciting increase in ABA, decrease in auxins and cytokinin, as well as a tendency in GA down accumulation. Furthermore, membrane processes were affected by the OMP treatment, involving stimulation of cutin biosynthesis, alteration of membrane lipids and an improved capacity for counteracting radical-mediated oxidative processes via the accumulation of phenolics and carotenoids. Several other stress-related compounds were affected by the OMP treatment, including polyamine conjugates, alkaloids and sesquiterpene lactones. Taken all together, OMP heightens this essential adaptation mechanism and increases tomato nutrient uptake and allocation, photosynthesis and plant performance under salt stress conditions thus improving resource use efficiency and tolerance to salinity. Although large-scale commercial application of OMP to mitigate plant salinity stress might currently not be economically viable, the present findings further corroborate the potential for development of a new class of formulations based on OMP analog molecules.

AUTHOR CONTRIBUTIONS

GR: Had the original idea on testing omeprazole on vegetable crops and contributed in the set up of the experimental protocol; YR: Defined the scientific hypothesis, set up the experimental protocol, coordinated the research and he was significantly involved in writing; LL: Performed the whole the metabolomic analysis, and gave an important contribution on metabolomic results interpretation as well as a significant contribution in writing the manuscript; PC: Contributed in writing the physiological and ion analyses parts and run the PCA and heat map; AP and VC: Worked on the mineral and statistical analysis; CE-N: Was responsible for the greenhouse tasks; GC, MK, and SDP: Contributed in writing and improving the manuscript.

SUPPLEMENTARY MATERIAL

The Supplementary Material for this article can be found online at: https://www.frontiersin.org/articles/10.3389/fpls.2018.00249/full#supplementary-material

Supplementary Table 1 | Analysis of variance and mean comparisons for nitrate, phosphate, potassium, calcium, magnesium, sodium, and chloride in fruits of tomato plants grown under two salinity levels and treated with omeprazole (OMP) at three rates of application.

Supplementary Table 2 | Raw data set as gained by UHPLC-ESI/QTOF-MS metabolomic analysis of tomato leaves under two salinity levels and three omeprazole (OMP) rates of application. Data are presented as raw intensities together with composite spectrum (accurate mass/ion abundance pairwise for each compound).

Supplementary Figure 1 | Orthogonal Projections to Latent Structures Discriminant Analysis (OPLS-DA) on tomato leaves metabolome from plants grown under nonsaline (1 mM NaCl, upper pane) or saline nutrient solution (75 mM NaCl, lower pane), following OMP application at three rates (0, 10, or 100 μM). Individual replications are given in the class prediction model score plot.

REFERENCES

Annunziata, M. G., Ciarmiello, L. F., Woodrow, P., Maximova, E., Fuggi, A., and Carillo, P. (2017). Durum wheat roots adapt to salinity remodeling the cellular content of nitrogen metabolites and sucrose. *Front. Plant Sci.* 7:2035. doi: 10.3389/fpls.2016.02035

Asins, M. J., Villalta, I., Aly, M. M., Olias, R., Álvarez De Morales, P. A. Z., Huertas, R., et al. (2013). Two closely linked tomato HKT coding genes are positional candidates for the major tomato QTL involved in Na^+/K^+ homeostasis. *Plant Cell Environ.* 36, 1171–1191. doi: 10.1111/pce.12051

Batelli, G., Verslues, P. E., Agius, F., Qiu, Q., Fujii, H., Pan, S., et al. (2007). SOS2 promotes salt tolerance in part by interacting with the vacuolar H^+-ATPase and upregulating its transport activity. *Mol. Cell. Biol.* 27, 7781–7790. doi: 10.1128/MCB.00430-07

Blumwald, E., Aharon, G. S., and Apse, M. P. (2000). Sodium transport in plant cells. *Biochim. Biophys. Acta* 1465, 140–151. doi: 10.1016/S0005-2736(00)00135-8

Bose, J., Rodrigo-Moreno, A., Lai, D., Xie, Y., Shen, W., and Shabala, S. (2015). Rapid regulation of the plasma membrane H^+-ATPase activity is essential to salinity tolerance in two halophyte species, *Atriplex lentiformis* and *Chenopodium quinoa*. *Ann. Bot.* 115, 481–494. doi: 10.1093/aob/mcu219

Campbell, W. H. (1999). Nitrate reductase structure, function and regulation: bridging the gap between biochemistry and physiology. *Annu. Rev. Plant Biol.* 50, 277–303. doi: 10.1146/annurev.arplant.50.1.277

Carillo, P., Mastrolonardo, G., Nacca, F., and Fuggi, A. (2005). Nitrate reductase in durum wheat seedlings as affected by nitrate nutrition and salinity. *Funct. Plant Biol.* 32, 209–219. doi: 10.1071/FP04184

Carillo, P., Mastrolonardo, G., Nacca, F., Parisi, D., Verlotta, A., and Fuggi, A. (2008). Nitrogen metabolism in durum wheat under salinity: accumulation of proline and glycine betaine. *Funct. Plant Biol.* 35, 412–426. doi: 10.1071/FP08108

Chadwick, M., Trewin, H., Gawthrop, F., and Wagstaff, C. (2013). Sesquiterpenoids lactones: benefits to plants and people. *Int. J. Mol. Sci.* 14, 12780–12805. doi: 10.3390/ijms140612780

Chelysheva, V. V., Smolenskaya, I. N., Trofimova, M. C., Babakov, A. V., and Muromtsev, G. S. (1999). Role of the 14-3-3 proteins in the regulation of H^+-ATPase activity in the plasma membrane of suspension-cultured sugar beet cells under cold stress. *FEBS Lett.* 456, 22–26. doi: 10.1016/S0014-5793(99)00923-0

Ciarmiello, L. F., Piccirillo, P., Carillo, P., De Luca, A., and Woodrow, P. (2015). Determination of the genetic relatedness of fig (*Ficus carica* L.) accessions using RAPD fingerprint and their agro-morphological characterization. *S. Afr. J. Bot.* 97, 40–47. doi: 10.1016/j.sajb.2014.11.012

Colla, G., Roupahel, Y., Cardarelli, M., and Rea, E. (2006). Effect of salinity on yield, fruit quality, leaf gas exchange, and mineral composition of grafted watermelon plants. *HortScience* 41, 622–627.

Colla, G., Rouphael, Y., Jawad, R., Kumar, P., Rea, E., and Cardarelli, M. (2013). The effectiveness of grafting to improve NaCl and CaCl2 tolerance in cucumber. *Sci. Hortic.* 164, 380–391. doi: 10.1016/j.scienta.2013.09.023

Colla, G., Rouphael, Y., Leonardi, C., and Bie, Z. (2010). Role of grafting in vegetable crops grown under saline conditions. *Sci. Hortic.* 127, 147–155. doi: 10.1016/j.scienta.2010.08.004

Colla, G., Rouphael, Y., Rea, E., and Cardarelli, M. (2012). Grafting cucumber plants enhance tolerance to sodium chloride and sulfate salinization. *Sci. Hortic.* 135, 177–185. doi: 10.1016/j.scienta.2011.11.023

Conde, A., Chaves, M. M., and Gerós, H. (2011). Membrane transport, sensing and signaling in plant adaptation to environmental stress. *Plant Cell Physiol.* 52, 1583–1602. doi: 10.1093/pcp/pcr107

Costantini, E. A., and Lorenzetti, R. (2013). Soil degradation processes in the Italian agricultural and forest ecosystems. *Ital. J. Agron.* 8:28. doi: 10.4081/ija.2013.e28

Cuartero, J., and Fernández-Mu-oz, R. (1998). Tomato and salinity. *Sci. Hortic.* 78, 83–125. doi: 10.1016/S0304-4238(98)00191-5

Cuin, T. A., Bose, J., Stefano, G., Jha, D., Tester, M., Mancuso, S., et al. (2011). Assessing the role of root plasma membrane and tonoplast Na^+/H^+ exchangers in salinity tolerance in wheat: in planta quantification methods. *Plant Cell Environ.* 34, 947–961. doi: 10.1111/j.1365-3040.2011.02296.x

Cuin, T. A., Tian, Y., Betts, S. A., Chalmandrier, R., and Shabala, S. (2009). Ionic relations and osmotic adjustment in durum and bread wheat under saline conditions. *Funct. Plant Biol.* 36, 1110–1119. doi: 10.1071/FP09051

Daliakopoulos, I. N., Tsanis, I. K., Koutroulis, A., Kourgialas, N. N., Varouchakis, A. E., Karatzas, G. P., et al. (2016). The threat of soil salinity: a European scale review. *Sci. Total Environ.* 573, 727–739. doi: 10.1016/j.scitotenv.2016.08.177

Fellenius, E., Berglindh, T., Sachs, G., Olbe, L., Elander, B., Sjöstrand, S. E., et al. (1981). Substituted benzimidazoles inhibit gastric acid secretion by blocking ($H^+ + K^+$) ATPase. *Nature* 290, 159. doi: 10.1038/290159a0

Fuglsang, A. T., Paez-Valencia, J., and Gaxiola, R. A. (2011). "Plant proton pumps: regulatory circuits involving H^+-ATPase and H^+-PPase," in *Transporters and Pumps in Plant Signaling: Signaling and Communication in Plants, Vol. 7*, eds M. Geisler and K. Venema (Heidelberg: Springer-Verlag), 39–64.

Gao, Y., Lu, Y., Wu, M., Liang, E., Li, Y., Zhang, D., et al. (2016). Ability to remove Na^+ and retain K^+ correlates with salt tolerance in two maize inbred lines seedlings. *Front. Plant Sci.* 7:1716. doi: 10.3389/fpls.2016.01716

Gorham, J., Läuchli, A., and Leidi, E. O. (2010). "Plant responses to salinity," in *Physiology of Cotton*, eds J. M. Stewart, D. M. Oosterhuis, J. J. Heitholt, and J. R. Mauney (Dordrecht: Springer), 129–141.

Grattan, S. R., and Grieve, C. M. (1999). Mineral nutrient acquisition and response by plants grown in saline environments. *Handb. Plant Crop Stress* 9, 203–229.

Hasegawa, P. M. (2013). Sodium (Na^+) homeostasis and salt tolerance of plants. *Environ. Exp. Bot.* 92, 19–31. doi: 10.1016/j.envexpbot.2013.03.001

Hasegawa, P. M., Bressan, R. A., Zhu, J. K., and Bohnert, H. J. (2000). Plant cellular and molecular responses to high salinity. *Annu. Rev. Plant Physiol. Plant Mol. Biol.* 51, 463–499. doi: 10.1146/annurev.arplant.51.1.463

Ji, H., Pardo, J. M., Batelli, G., Van Oosten, M. J., Bressan, R. A., and Li, X. (2013). The salt overly sensitive (SOS) pathway: established and emerging roles. *Mol. Plant* 6, 275–286. doi: 10.1093/mp/sst017

Jones, M. M., and Turner, N. C. (1978). Osmotic adjustment in leaves of *sorghum* in response to water deficits. *Plant Physiol.* 61, 122–126. doi: 10.1104/pp.61.1.122

Kaschani, F., and van der Hoorn, R. (2007). Small molecule approaches in plants. *Curr. Opin. Chem. Biol.* 11, 88–98. doi: 10.1016/j.cbpa.2006.11.038

Kosma, D. K., Bourdenx, B., Bernard, A., Parsons, E. P., Lu, S., Joubes, J., et al. (2009). The impact of water deficiency on leaf cuticle lipids of *arabidopsis*. *Plant Physiol.* 151, 1918–1929. doi: 10.1104/pp.109.141911

Lace, B., and Prandi, C. (2016). Shaping small bioactive molecules to untangle their biological function: a focus on fluorescent plant hormones. *Mol. Plant* 9, 1099–1118. doi: 10.1016/j.molp.2016.06.011

Läuchli, A., and Epstein, E. (1990). Plant responses to saline and sodic conditions. *Agric. salinity Assess. Manage.* 71, 113–137.

Lawless, H. T., and Heymann, H. (2010). *Sensory Evaluation of Foods. Principles and Practices, 2nd Edn*. New York, NY: Springer Science + Business.

Li, J., Jia, H., Wang, J., Cao, Q., and Wen, Z. (2014). Hydrogen sulfide is involved in maintaining ion homeostasis via regulating plasma membrane Na^+/H^+ antiporter system in the hydrogen peroxide-dependent manner in salt-stress *Arabidopsis thaliana* root. *Protoplasma* 251, 899–912. doi: 10.1007/s00709-013-0592-x

Lucini, L., Borgognone, D., Rouphael, Y., Cardarelli, M., Bernardi, J., and Colla, G. (2016). Mild potassium chloride stress alters the mineral composition, hormone network, and phenolic profile in artichoke leaves. *Front. Plant Sci.* 7:948. doi: 10.3389/fpls.2016.00948

Lucini, L., Rouphael, Y., Cardarelli, M., Canaguier, R., Kumar, P., and Colla, G. (2015). The effect of a plant-derived biostimulant on metabolic profiling and crop performance of lettuce grown under saline conditions. *Sci. Hortic.* 182, 124–133. doi: 10.1016/j.scienta.2014.11.022

Luo, J., Fuell, C., Parr, A., Hill, L., Bailey, P., Elliott, K., et al. (2009). A novel polyamine acyltransferase responsible for the accumulation of spermidine conjugates in *Arabidopsis* seed. *Plant Cell* 21, 318–333. doi: 10.1105/tpc.108.063511

Maas, E. V., and Nieman, R. H. (1978). "Physiology of plant tolerance to salinity," in *Crop Tolerance to Suboptimal Land Conditions*, ed G. A. Jung (Madison, WI: American Society of Agronomy), 277–299.

Machado, R. M. A., and Serralheiro, R. P. (2017). Soil salinity: effect on vegetable crop growth. Management practices to prevent and mitigate soil salinization. *Horticulturae* 3:30. doi: 10.3390/horticulturae3020030

Munns, R. (2002). Comparative physiology of salt and water stress. *Plant Cell Environ.* 25, 239–250. doi: 10.1046/j.0016-8025.2001.00808.x

Munns, R., and Tester, M. (2008). Mechanisms of salinity tolerance. *Annu. Rev. Plant Biol.* 59, 651–681. doi: 10.1146/annurev.arplant.59.032607.092911

Nakabayashi, R., and Saito, K. (2015). Integrated metabolomics for abiotic stress responses in plants. *Curr. Opin. Plant Biol.* 24, 10–16. doi: 10.1016/j.pbi.2015.01.003

Olías, R., Eljakaoui, Z., Pardo, J. M., and Belver, A. (2009). The Na^+/H^+ exchanger SOS1 controls extrusion and distribution of Na^+ in tomato plants under salinity conditions. *Plant Signal. Behav.* 4, 973–976. doi: 10.4161/psb.4.10.9679

Pardo, J. M., Cubero, B., Leidi, E. O., and Quintero, F. J. (2006). Alkali cation exchangers: roles in cellular homeostasis and stress tolerance. *J. Exp. Bot.* 57, 1181–1199. doi: 10.1093/jxb/erj114

Pretali, L., Bernardo, L., Butterfield, T. S., Trevisan, M., and Lucini, L. (2016). Botanical and biological pesticides elicit a similar induced systemic response in tomato (*Solanum lycopersicum*) secondary metabolism. *Phytochemistry* 130, 56–63. doi: 10.1016/j.phytochem.2016.04.002

Rana, G., and Katerji, N. (2000). Measurement and estimation of actual evapotranspiration in the field under Mediterranean climate: a review. *Eur. J. Agron.* 13, 125–153. doi: 10.1016/S1161-0301(00)00070-8

Rodriguez, P., Torrecillas, A., Morales, M. A., Ortuno, M. F., and Sánchez-Blanco, M. J. (2005). Effects of NaCl salinity and water stress on growth and leaf water relations of Asteriscus maritimus plants. *Environ. Exp. Bot.* 53, 113–123. doi: 10.1016/j.envexpbot.2004.03.005

Rombouts, C., Hemeryck, L. Y., Van Hecke, T., De Smet, S., De Vos, W. H., and Vanhaecke, L. (2017). Untargeted metabolomics of colonic digests reveals kynurenine pathway metabolites, dityrosine and 3-dehydroxycarnitine as red versus white meat discriminating metabolites. *Sci. Rep.* 7:42514. doi: 10.1038/srep42514

Rouphael, Y., Cardarelli, M., Bonini, P., and Colla, G. (2017a). Synergistic action of a microbial-based biostimulant and a plant derived-protein hydrolysate enhances lettuce tolerance to alkalinity and salinity. *Front. Plant Sci.* 8:131. doi: 10.3389/fpls.2017.00131

Rouphael, Y., Cardarelli, M., Rea, E., and Colla, G. (2012). Improving melon and cucumber photosynthetic activity, mineral composition, and growth performance under salinity stress by grafting onto Cucurbita hybrid rootstocks. *Photosynthetica* 50, 180–188. doi: 10.1007/s11099-012-0002-1

Rouphael, Y., Colla, G., Bernardo, L., Kane, D., Trevisan, M., and Lucini, L. (2016). Zinc excess triggered polyamines accumulation in lettuce root metabolome, as compared to osmotic stress under high salinity. *Front. Plant Sci.* 7:842. doi: 10.3389/fpls.2016.00842

Rouphael, Y., Colla, G., Giordano, M., El-Nakhel, C., Kyriacou, M. C., and De Pascale, S. (2017b). Foliar applications of a legume-derived protein hydrolysate elicit dose-dependent increases of growth, leaf mineral composition, yield and fruit quality in two greenhouse tomato cultivars. *Sci. Hortic.* 226, 353–360. doi: 10.1016/j.scienta.2017.09.007

Rouphael, Y., Colla, G., Graziani, G., Ritieni, A., Cardarelli, M., and De Pascale, S. (2017c). Phenolic composition, antioxidant activity and mineral profile in two seed-propagated artichoke cultivars as affected by microbial inoculants and planting time. *Food Chem.* 234, 10–19. doi: 10.1016/j.foodchem.2017.04.175

Rouphael, Y., De Micco, V., Arena, C., Raimondi, G., Colla, G., and De Pascale, S. (2017d). Effect of *Ecklonia maxima* seaweed extract on yield, mineral composition, gas exchange, and leaf anatomy of zucchini squash grown under saline conditions. *J. Appl. Phycol.* 29, 459–470. doi: 10.1007/s10811-016-0937-x

Ryu, H., and Cho, Y. G. (2015). Plant hormones in salt stress tolerance. *J. Plant Biol.* 58, 147–155. doi: 10.1007/s12374-015-0103-z

Sardini, A., Amey, J. S., Weylandt, K. H., Nobles, M., Valverde, M. A., and Higgins, C. F. (2003). Cell volume regulation and swelling-activated chloride channels. *Biochim. Biophys. Acta* 1618, 153–162. doi: 10.1016/j.bbamem.2003.10.008

Savvas, D., Savva, A., Ntatsi, G., Ropokis, A., Karapanos, I., Krumbein, A., et al. (2011). Effects of three commercial rootstocks on mineral nutrition, fruit yield, and quality of salinized tomato. *J. Plant Nutr. Soil Sci.* 174, 154–162. doi: 10.1002/jpln.201000099

Schmarda, A., Dinkhauser, P., Gschwentner, M., Ritter, M., Fürst, J., Scandella, E., et al. (2000). The gastric H, K-ATPase blocker lansoprazole is an inhibitor of chloride channels. *Br. J. Pharmacol.* 129, 598–604. doi: 10.1038/sj.bjp.0703070

Seoane, M., Esperanza, M., and Cid, Á. (2017). Cytotoxic effects of the proton pump inhibitor omeprazole on the non-target marine microalga *Tetraselmis suecica*. *Aquatic Toxicol.* 191, 62–72. doi: 10.1016/j.aquatox.2017.08.001

Shabala, S. (2013). Learning from halophytes: physiological basis and strategies to improve abiotic stress tolerance in crops. *Ann. Bot.* 112, 1209–1221. doi: 10.1093/aob/mct205

Shabala, S., and Cuin, T. A. (2008). Potassium transport and plant salt tolerance. *Physiol. Plant.* 133, 651–669. doi: 10.1111/j.1399-3054.2007.01008.x

Shen, G., Wei, J., Qiu, X., Hu, R., Kuppu, S., Auld, D., et al. (2015). Co-overexpression of AVP1 and AtNHX1 in cotton further improves drought and salt tolerance in transgenic cotton plants. *Plant Biol. Mol. Rep.* 33, 167–177. doi: 10.1007/s11105-014-0739-8

Shi, H., and Chan, Z. (2014). Improvement of plant abiotic stress tolerance through modulation of the polyamine pathway. *J. Integr. Plant Biol.* 56, 114–121. doi: 10.1111/jipb.12128

Shin, J. M., and Kim, N. (2013). Pharmacokinetics and pharmacodynamics of the proton pump inhibitors. *J. Neurogastroenterol.* 19:25. doi: 10.5056/jnm.2013.19.1.25

Shin, J. M., Munson, K., Vagin, O., and Sachs, G. (2009). The gastric HK-ATPase: structure, function, and inhibition. *Pflügers Archiv.* 457, 609–622. doi: 10.1007/s00424-008-0495-4

Silva, P., and Gerós, H. (2009). Regulation by salt of vacuolar H^+-ATPase and H^+-pyrophosphatase activities and Na^+/H^+ exchange. *Plant Signal Behav.* 4, 718–726. doi: 10.4161/psb.4.8.9236

Soni, P., Kumar, G., Soda, N., Singla-Pareek, S. L., and Pareek, A. (2013). Salt overly sensitive pathway members are influenced by diurnal rhythm in rice. *Plant Signal Behav.* 8:e24738. doi: 10.4161/psb.24738

Sun, J., Li, L., Liu, M., Wang, M., Ding, M., Deng, S., et al. (2010a). Hydrogen peroxide and nitric oxide mediate K^+/Na^+ homeostasis and antioxidant defense in NaCl-stressed callus cells of two contrasting poplars. *Plant Cell Tissue Organ Cult.* 103, 205–215. doi: 10.1007/s11240-010-9768-7

Sun, J., Wang, M. J., Ding, M. Q., Deng, S. R., Liu, M. Q., Lu, C. F., et al. (2010b). H2O2 and cytosolic $Ca2^+$ signals triggered by the PM H^+-coupled transport system mediate K^+/Na^+ homeostasis in NaCl-stressed *Populus euphratica* cells. *Plant Cell Environ.* 33, 943–958. doi: 10.1111/j.1365-3040.2010.02118.x

Tavakkoli, E., Fatehi, F., Coventry, S., Rengasamy, P., and McDonald, G. K. (2011). Additive effects of Na^+ and Cl–ions on barley growth under salinity stress. *J. Exp. Bot.* 62, 2189–2203. doi: 10.1093/jxb/erq422

Tavakkoli, E., Rengasamy, P., and McDonald, G. K. (2010). High concentrations of Na^+ and Cl^- ions in soil solution have simultaneous detrimental effects on growth of faba bean under salinity stress. *J. Exp. Bot.* 61, 4449–4459. doi: 10.1093/jxb/erq251

Tester, M., and Davenport, R. (2003). Na^+ tolerance and Na^+ transport in higher plants. *Ann. Bot.* 91, 503–527. doi: 10.1093/aob/mcg058

Tsygankova, V., Andrusevich, Y., Shtompel, O., Pilyo, S., Prokopenko, V., Kornienko, A., et al. (2016). Study of growth regulating activity derivatives of [1,3] Oxazolo [5, 4-d] pyrimidine and N-Sulfonyl Substituted of 1,3-Oxazole on soybean, wheat, flax and pumpkin plants. *Int. J. Chem. Stud.* 4, 106–120.

Van Oosten, M. J., Silletti, S., Guida, G., Cirillo, V., Di Stasio, E., Carillo, P., et al. (2017). A benzimidazole proton pump inhibitor increases growth and tolerance to salt stress in tomato. *Front. Plant Sci.* 8:1220. doi: 10.3389/fpls.2017.01220

Wang, M., Zheng, Q., Shen, Q., and Guo, S. (2013). The critical role of potassium in plant stress response. *Int. J. Mol. Sci.* 14, 7370–7390. doi: 10.3390/ijms14047370

Woodrow, P., Ciarmiello, L. F., Annunziata, M. G., Pacifico, S., Iannuzzi, F., Mirto, A., et al. (2017). Durum wheat seedling responses to simultaneous high light and salinity involve a fine reconfiguration of amino acids and carbohydrate metabolism. *Physiol. Plant.* 159, 290–312. doi: 10.1111/ppl.12513

Worley, B., and Powers, R. (2013). Multivariate analysis in metabolomics. *Curr. Metabolomics* 1, 92–107. doi: 10.2174/2213235X11301010092

Yamaguchi, S. (2008). Gibberellin metabolism and its regulation. *Annu. Rev. Plant Biol.* 59, 225–251. doi: 10.1146/annurev.arplant.59.032607.092804

Zhang, Y., Wang, Y., Sa, G., Zhang, Y., Deng, J., Deng, S., et al. (2017). *Populus euphratica* J3 mediates root K^+/Na^+ homeostasis by activating plasma membrane H+-ATPase in transgenic Arabidopsis under NaCl salinity. *Plant Cell Tiss. Organ Cult.* 131, 75–88. doi: 10.1007/s11240-017-1263-y

Conflict of Interest Statement: The authors declare that the research was conducted in the absence of any commercial or financial relationships that could be construed as a potential conflict of interest.

frontiers in Plant Science

ORIGINAL RESEARCH
published: 07 March 2018
doi: 10.3389/fpls.2018.00301

Arbuscular Mycorrhiza Improves Substrate Hydraulic Conductivity in the Plant Available Moisture Range Under Root Growth Exclusion

Michael Bitterlich[1], Philipp Franken[1,2] and Jan Graefe[1]*

[1] Leibniz Institute of Vegetable and Ornamental Crops, Großbeeren, Germany, [2] The Department of Plant Physiology, Humboldt University of Berlin, Berlin, Germany

Arbuscular mycorrhizal fungi (AMF) proliferate in soils and are known to affect soil structure. Although their contribution to structure is extensively investigated, the consequences of those processes for soil water extractability and transport has, so far, gained surprisingly little attention. Therefore we asked, whether AMF can affect water retention and unsaturated hydraulic conductivity under exclusion of root ingrowth, in order to minimize plant driven effects. We carried out experiments with tomato inoculated with *Rhizoglomus irregulare* in a soil substrate with sand and vermiculite that created variation in colonization by mixed pots with wild type (WT) plants and mycorrhiza resistant (RMC) mutants. Sampling cores were introduced and used to assess substrate moisture retention dynamics and modeling of substrate water retention and hydraulic conductivity. AMF reduced the saturated water content and total porosity, but maintained air filled porosity in soil spheres that excluded root ingrowth. The water content between field capacity and the permanent wilting point (6–1500 kPa) was only reduced in mycorrhizal substrates that contained at least one RMC mutant. Plant available water contents correlated positively with soil protein contents. Soil protein contents were highest in pots that possessed the strongest hyphal colonization, but not significantly affected. Substrate conductivity increased up to 50% in colonized substrates in the physiologically important water potential range between 6 and 10 kPa. The improvements in hydraulic conductivity are restricted to substrates where at least one WT plant was available for the fungus, indicating a necessity of a functional symbiosis for this effect. We conclude that functional mycorrhiza alleviates the resistance to water movement through the substrate in substrate areas outside of the root zone.

Keywords: arbuscular mycorrhiza, unsaturated hydraulic conductivity, water retention, substrate, soil water potential

OPEN ACCESS

Edited by:
Youssef Rouphael, University of Naples Federico II, Italy

Reviewed by:
Paul Hallett, University of Aberdeen, United Kingdom
Raffaella Balestrini, Consiglio Nazionale delle Ricerche (CNR), Italy

****Correspondence:***
Michael Bitterlich
Bitterlich@igzev.de

Specialty section:
This article was submitted to Plant Microbe Interactions, a section of the journal Frontiers in Plant Science

Received: *11 December 2017*
Accepted: *21 February 2018*
Published: *07 March 2018*

Citation:
Bitterlich M, Franken P and Graefe J (2018) Arbuscular Mycorrhiza Improves Substrate Hydraulic Conductivity in the Plant Available Moisture Range Under Root Growth Exclusion. Front. Plant Sci. 9:301. doi: 10.3389/fpls.2018.00301

INTRODUCTION

Biostimulants in agri- and horticulture are defined as substances or microorganisms applied to plants in minute quantities aiming to improve crop quality traits, stress tolerance and nutrient efficiency, without being mineral nutrients, soil improvers or pesticides, which are applied in high quantities (du Jardin, 2015). Biostimulants include humic acids, protein hydrolysates, seaweed

extracts, biopolymers (Colla et al., 2015; du Jardin, 2015), beneficial microbes such as plant growth promoting rhizobacteria (Ruzzi and Aroca, 2015) and arbuscular mycorrhizal fungi (AMF) (Rouphael et al., 2015).

Arbuscular mycorrhizal fungi proliferate in soils beyond the root zone. Thus, extraradical hyphae constitute a network that enhances the soil volume connected to the plant (Smith and Read, 2008). Along with the well investigated phosphorus delivery of AMF to plants from beyond root zone areas (Smith and Read, 2008), AMF influence soil properties. Soil structure, the three-dimensional particle arrangement of organic/mineral units, i.e., aggregates, and pore space is of substantial importance for nutrient, gas and water fluxes in soils (Wu et al., 2014). AMF presence, activity and turnover affects soil structure (Augé et al., 2001; Piotrowski et al., 2004; Rillig and Mummey, 2006; Leifheit et al., 2014). Similar to roots (Bodner et al., 2014), AMF contribute substantially to the hierarchical formation of aggregates by merging smaller aggregates of the sediment load into larger macroaggregates (Tisdall and Oades, 1982; Miller and Jastrow, 2000; Six et al., 2004; Rillig and Mummey, 2006). Increased abundance and stabilization of aggregates by AMF hyphae are frequently observed, well documented and related to their so called 'sticky-string bag' function (Miller and Jastrow, 2000). Those hyphae entangle and enmesh particles due to polysaccharides and other 'sticky' substances on their surface to which solid particles adhere. Hence, hyphae stabilize particle-particle contact and form aggregates (Degens et al., 1996) or maintain particle connectivity by bridging pore spaces (Miller and Jastrow, 2000; Rillig and Mummey, 2006). In addition, turnover of mycelia releases organic substances into soils (Driver et al., 2005; Rillig and Mummey, 2006). Glomalin, a proteinaceous substance produced by AMF, is mainly released during hyphal turnover (Driver et al., 2005). As a part of the slow turnover carbon pool, glomalin accumulates in many soils and is often positively correlated to aggregate stability (Wright and Upadhyaya, 1998), because it sticks particles together and/or hinders disintegration of those organic/mineral units by water infiltration. AMF may also produce hydrophobins, proteins of filamentous fungi that lower the surface tension of water and enables hyphae to break through a water film (Rillig, 2005). Hydrophobins may contribute to observations that AMF can increase soil water repellency (Rillig et al., 2010). Thus, AMF can influence the physical properties of soils in various direct and also indirect ways, i.e., changes to microbial community, host morphology and activity, which may act in concert, in antagonistic or synergistic ways (Rillig and Mummey, 2006). The structure-related changes in physical soil properties and surfactant effects of released biochemicals determine water and solute mobility, as well as transport capacity of the growth soil or substrate. Hence, soils colonized by AMF may possess different constraints to solute and water transport. Currently, effects of AMF on soil structure may be consensus, but the relevance of those effects to plants remains less clear (Querejeta, 2017).

Water and solute transport occurs through the pore space. The hydraulic properties of the pore space are related to primary texture, i.e., particle size distribution, and secondary structure (De Gryze et al., 2006; Querejeta, 2017). Thus, by changing the structure of a soil, AMF can influence the soil's hydraulic properties (Augé et al., 2001; Augé, 2004). Hitherto, comparative quantification of the hydraulic properties of soils with and without mycorrhiza has gained surprisingly little attention (Querejeta, 2017), even though they limit the mass flow of water and solutes to roots and the ability of plants to extract water. Water retention and hydraulic conductivity are quantitative measures for water capacity and mobility, respectively, but water retention has been rarely quantified for mycorrhizal substrates (Augé et al., 2001; Bearden, 2001; Augé, 2004; Daynes et al., 2013) and, to the best of our knowledge, unsaturated hydraulic conductivity of colonized substrates is not yet reported at all. In substrates that contain roots as well as AMF, water retention relations can shift compared to non-colonized substrates (Augé et al., 2001; Bearden, 2001; Daynes et al., 2013). The pioneering study of Augé et al. (2001) revealed that water contents of equally rooted mixtures of sandy soil corresponded to different water potentials. The soil water potential equals the energy plant roots require to extract water from the soil, achieved by root osmotic adjustments. Plant physiological responses to AMF colonization can be related to direct mycorrhizal effects on root hydraulic conductivity and regulation of root aquaporin expression (Porcel et al., 2006; Aroca et al., 2007; Bárzana et al., 2012). Additionally, they could be related to shifts in soil water retention and conductivity due to AMF colonization. Indeed, many studies measuring the physiological response of shoots to soil moisture found that, in order to trigger a comparable response to non-colonized soils, AMF soils had to be drier (reviewed in: Augé, 2001). In another study, mycorrhiza-resistant bean mutants showed improved stomatal conductance when grown in a mycorrhizal soil (Augé, 2004). In order to avoid or delay wilting, plants adjust stomatal aperture to the soil water status (Tardieu and Simonneau, 1998). Hence, the observations by Augé (2004) point to a soil-originated effect.

Compartments that exclude root and allow fungal ingrowth have not yet been used to assess water retention and conductivity. Yet, they could give a better idea of the impact of extraradical hyphae in non-rooted substrates on plant water supply. On a small scale, non-rooted areas like these probably occur in every mycorrhizal scenario. Root water uptake can form water depletion gradients between rooted and non-rooted areas. Measuring the hydraulic properties inside the root-free compartments could serve as a surrogate for water extractability and water transport away from those areas. We investigated whether AMF can change water retention and hydraulic conductivity in root-free substrates. To test this, we designed an experiment using the root-free compartments and mixed cultures of host and non-host tomato genotypes in order to create a variation of substrate colonization, similar to previous approaches (Neumann and George, 2005a; Hallett et al., 2009). We chose a substrate mix that allows for growing plants hydroponically. It resembles two or three component substrate mixtures, comprising sand and vermiculite that are frequently encountered in fundamental mycorrhizal research (e.g., Azcón, 1989; El-Atrach et al., 1989; Kim et al., 1997; Boldt et al., 2011; Porcel et al., 2015, 2016; Rasmussen et al., 2016; Ruiz-Lozano et al., 2016) and, for inoculum production (Silva et al., 2007).

We used a hydroponic system to minimize the effect of reduced accessible substrate volume in non-mycorrhizal (NM) pots. We frequently replaced nutrients that were taken up with low quantities of readily available nutrients. This way, the additional volume accessible by hyphal ingrowth in mycorrhizal pots only has a marginal impact on plant nutrition and, thus, reduces nutrient-related growth responses. Moreover, AMF effects on soil hydraulic properties are expectably most pronounced in coarsely textured environments (Leifheit et al., 2014; Querejeta, 2017). We used tomato as a host, a rather non-responsive plant to AMF in terms of growth (e.g., Smith et al., 2004), to minimize differences in root-compartment contact and irrigated daily to minimize pronounced dry-wet cycles in those compartments.

MATERIALS AND METHODS

Experimental Design, Plant Growth, and Mycorrhizal Development

We planted two *Solanum lycopersicum* cv. 76R wild type (WT) plants and the mutant resistant to mycorrhizal colonization (RMC) (Barker et al., 1998) in 7.5 L pots in intra- (WT/WT; RMC/RMC) and inter-genotypic (WT/RMC) combination and inoculated half of the pots with a commercial inoculum containing *Rhizoglomus irregulare* (INOQ GmbH, Schnega, Germany). Four replicates were randomly set up in the greenhouse, grown for 8 weeks and irrigated daily with 600 mL nutrient solution (De Kreij et al., 1997); N: 10.32 mM; P: 0.07 mM; K: 5.5 mM; Mg: 1.2 mM; S: 1.65 mM; Ca: 2.75 mM; Fe: 0.02 mM; pH: 6.2; EC: 1.6 mS) containing 10% of standard phosphate to guarantee good fungal colonization. Additional 400–600 mL of deionized water were added daily, so that total irrigation maintained 13–16% volumetric water content (10–30 kPa). The pots contained a sterilized mixture (2:2:1; v/v/v) of fine sand (0.2–1 mm; Euroquarz, Ottendorf-Okrilla, Germany), vermiculite (Agra-Vermiculite, Rhenen, Netherlands) and 2 mm sieved local sandy soil (per 100 g soil: 5.3 mg P; 5.4 mg K; 1.9 mg Mg; 1.1 mg N; organic matter 1.3% DW; pH 6.1; 82% sand; 14% silt; 4% clay). The reproducible substrate allowed hydroponic fertilization to better control nutrient availability. The soil added served as a source for aggregation nuclei from some smaller sediment and homogeneously distributed organic matter. Two 250 mL compartments were introduced into the pots with a 30 μm nylon mesh that excludes root ingrowth. One was used to extract extraradical mycelia [containing a mixture of glass beads and soil as in Neumann and George (2005b)] to verify extraradical spreading and the other containing the potting mixture to assess moisture retention properties.

The pots were inoculated with 0.5 L of the *R. irregulare* inoculum. For the non-mycorrhizal treatment the inoculum was filtered with deionized water and autoclaved for 15 min at 121°C. The filtrate and the sterilized inoculum were added to the pots in equal amounts (0.5 L). The compartments were not inoculated.

Bradford-reactive soil protein content (BRSP) was quantified (triplicates; 2 g subsamples of air dried substrates) after Wright and Upadhyaya (1996) as a measure for glomalin, an important compound for aggregate stability with surfactant properties. Trypan blue staining was carried out after Koske and Gemma (1989) for intrardical staining of AMF and intraradical colonization was quantified on 100 root pieces with the grid line intersection method after Giovannetti and Mosse (1980).

For shoot phosphorus (P), 250 mg of pulverized dry material was oxygenized for 20 min with 5 mL HNO_3 (65%) and 3 mL H_2O_2 (30%). After 1 h of microwave decomposition and filtration (MN 615, Macherey-Nagel, Germany) P concentration of filtrates were analyzed with an EPOS analyser (ascorbic-acid method, EPOS 5060-55, Eppendorf, Germany).

Assessment of Substrate Hydraulic Properties

The bulk density in compartments was 1.2 g cm^{-3}, calculated as the oven-dried (24 h; 105°C) weight per unit volume. Air filled porosity is determined as the difference of dry porosity and the saturated water content (Θ_{SAT}). Before planting, standard soil sampling cores (V = 250 mL, h = 5 cm) were introduced into pots (n = 4 per treatment and planting combination) in a way that the cylinder diameter covered the central section of the substrate filling level and the depth of the cylinder covered the radius from the center to the rim of the pot.

After harvesting, the simplified evaporation method (Schindler, 1980) was applied on the sampling cores introduced into the substrate fraction that excluded root proliferation. The principle of the simplified evaporation method is a continuous drying of a soil or substrate sample under controlled laboratory conditions. This measurement was carried out with a commercial HYPROP system (UMS GmbH, München, Germany) using standard sampling cores (V = 250 mL, h = 5 cm). For the measurement in the HYPROP system, the soil cores were weighed, water saturated overnight and two tensiometers were introduced into the substrate using carefully prepared holes. The water tension of both tensiometers (h_1, h_2, and hPa) was logged every 10 min during evaporation and the substrate samples were weighed at least two times a day. Using the mean soil water potential (Ψ_S and hPa) over both tensiometers and the weight loss from evaporation, a retention function of the mean volumetric water content [$\Theta(\Psi_S)$] of the prepared soil sample can be derived (Peters and Durner, 2008). The measurement lasted until the know air entry point of the tensiometers ceramic (Al_2 O_3 and 8800 hPa) was passed, when tension drops off to 0 hPa. This specific can be used to extent the typical measurement range of tensiometers from 1000 to 8800 hPa (Peters and Durner, 2008). Afterward, samples were dried (105°C, 24 h) and weighed again to determine substrate dry mass.

Assuming half of the water flow for evaporation deriving from the upper cylinder height and a linear gradient of volumetric water content (Θ) from bottom to top, a function for the hydraulic conductivity $K(h)$ can be estimated as:

$$K(h_i) = \frac{0.5q}{\frac{\Delta h}{z_1 - z_2} - 1} \quad (1)$$

where q is the water flow, Δh is the mean tension difference of the two tensiometers and z_i are the depths of the tensiometers (Peters and Durner, 2008).

Although made for soils, water retention models can also be used for substrate mixes (Fonteno, 1992). Thus, several water retention models were tested. In order to evaluate the functional relationship between Θ and Ψ_S, a bimodal model for water retention (Durner, 1994) was fitted with the HYPROP-DES software (UMS GmbH, München, Germany). The adopted model allows for a mixture of two pore size distributions (n_1, n_2) (Eq. 2). This model choice was based on the Akaike Information Criteria (AICc) for finite sample sizes (Akaike, 1974), which penalizes model complexity, so candidate models with minimum AICc are preferred.

$$S_e(h) = \sum_{i=1}^{2} \omega_i \left(\frac{1}{1 + (\alpha_i |\psi_S|)^{n_i}}\right)^{1-\frac{1}{n_i}} \quad (2)$$

S_e is the effective saturation defined as $S_e = (\Theta - \Theta_r)/(\Theta_{SAT} - \Theta_r)$ (Mualem, 1976) with Θ_r and Θ_{SAT} as the saturated and residual volumetric water content, respectively. ω_i is a weighting factor for the specific mixture component, n_i is the pore size distribution parameter and α_i is the reciprocal potential at the air entry water tension of the substrate sample. The input is the geometric mean of tensions of both tensiometers (Ψ_S).

The retention function was coupled to a model of for hydraulic conductivities (K) in unsaturated porous media (Mualem, 1976):

$$K(h) = K_s S_e^{\tau} \left(\frac{\int_0^{S_e} h^{-1}\, dS_e(h)}{\int_0^1 h^{-1} dS_e(h)} \right)^2 \quad (3)$$

where K_S and τ are the saturated conductivity and a pore tortuosity parameter, respectively. Overall nine parameters were identified simultaneously from combined retention and conductivity data: Θ_S, Θ_r, w_2, α_1, α_2, n_1, n_2, K_S, and τ. Parameter estimation was carried out as described in Peters and Durner (2008).

Statistical analysis (α = 0.05; normal distribution, homogeneity of variances, ANOVA, *t*-test and regressions) and figures were done with STATISTICA 12 Software (StatSoft, Tulsa, OK, United States).

RESULTS

As anticipated, plant growth and P content were not changed by inoculation with *R. irregulare* (**Table 1**). Substrate characteristics in the root-free compartments were largely unaffected by plant combinations. Porosity and the saturated water content (Θ_{SAT}) were reduced in mycorrhizal substrates. Unaltered air filled porosity indicates that *R. irregulare* reduced the effective wettable pore space. Bradford-reactive soil protein contents (BRSP) neither changed upon plant genotype combination nor upon inoculation. Changes in Θ_{SAT} were not related to BRSP contents ($R^2 = 0.10$, $P > 0.05$).

In pots that contained two RMC mutants, we only observed surface colonization of roots (**Figure 1**). Non-mycorrhizal (NM) roots were free of mycorrhiza. Colonization of the bulk root system was reduced in the inter-genotype planting combination compared to roots from pots with two WT plants. Although only surface colonization was detected, we found a small amount of

TABLE 1 | Substrate and plant parameters of non-mycorrhizal (NM) pots and pots inoculated with *Rhizoglomus irregulare* (AM) for the intra- and inter-genotypic plant combinations containing either two wild type tomato plants (WT/WT), two resistant mutants (RMC/RMC) or one WT and one resistant mutant (WT/RMC).

Variable	Inoculation	Pot combination			ANOVA		
		WT/WT	WT/RMC	RMC/RMC	Pot	Inoculation	H × I
Substrate							
Total dry porosity [cm^3 cm^{-3}]	NM	0.56 ± 0.02	0.54 ± 0.01	0.56 ± 0.01	0.516	**0.026**	0.727
	AM	0.53 ± 0.01	0.53 ± 0.01	0.53 ± 0.01			
Θ_{SAT} [cm^3 cm^{-3}]	NM	0.50 ± 0.01	0.49 ± 0.01	0.50 ± 0.01	0.652	**0.031**	0.885
	AM	0.47 ± 0.01	0.48 ± 0.01	0.49 ± 0.01			
Air filled porosity [cm^3 cm^{-3}]	NM	0.06 ± 0.02	0.05 ± 0.01	0.06 ± 0.01	0.784	0.698	0.906
	AM	0.06 ± 0.02	0.05 ± 0.01	0.05 ± 0.01			
K_{SAT} [log10 cm d^{-1}]	NM	2.00 ± 0.06	2.01 ± 0.19	2.14 ± 0.19	0.414	0.856	0.443
	AM	1.91 ± 0.09	2.23 ± 0.12	2.07 ± 0.08			
BRSP [mg cm^{-3}]	NM	0.38 ± 0.08	0.42 ± 0.06	0.43 ± 0.05	0.771	0.258	0.204
	AM	0.47 ± 0.09	0.46 ± 0.06	0.40 ± 0.04			
Plant							
Plant dry weight [g]	NM	148 ± 3.94	149 ± 8.35	159 ± 6.13	0.913	0.822	0.455
	AM	151 ± 10.4	154 ± 4.88	147 ± 8.09			
Root dry weight [g]	NM	37.5 ± 3.22	36.4 ± 4.41	45.9 ± 6.22	0.911	0.837	0.204
	AM	40.1 ± 5.15	42.0 ± 4.38	35.5 ± 3.30			
Shoot phosphorus [% DW]	NM	0.28 ± 0.01	0.27 ± 0.02	0.26 ± 0.01	0.619	0.206	0.849
	AM	0.25 ± 0.02	0.26 ± 0.01	0.25 ± 0.01			

Θ_{SAT} and K_{SAT} denote the saturated water content and conductivity, respectively. Means (±SE) and significant P-values are highlighted in bold (two way ANOVA, α = 0.05, n = 4).

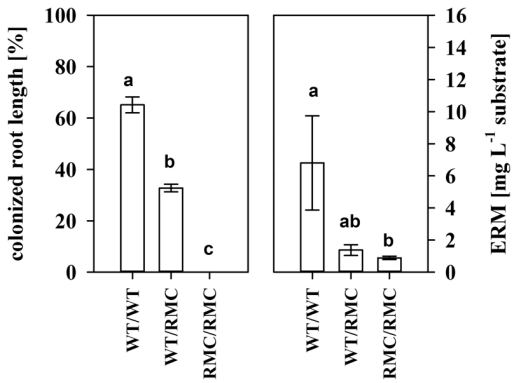


FIGURE 1 | Colonized root length (left) and the extracted extraradical mycelia (ERM) dry matter of pots inoculated with *Rhizoglomus irregulare* for the intra- and inter-genotypic plant combinations containing either two wild type tomato plants (WT/WT), two resistant mutants (RMC/RMC) or one WT and one resistant mutant (WT/RMC). No colonization was observed in non-mycorrhizal pots. Means (±SE) are shown and values with the same letter are not significantly different (one way ANOVA, Tukey HSD, $\alpha = 0.05$, $n = 4$).

hyphae also in root-free compartments from pots with two RMC mutants. Since we did not find any hyphae in NM compartments and the compartments were not inoculated, those hyphae have to originate from growing fungi.

The water retention and hydraulic conductivity analyses were carried out in a pot specific manner because, in a few instances, variation in individual assessments caused inflections in treatment-wise model fits that diverged from individual curvatures.

The plant relevant range in most scenarios complies with water potentials from field capacity (FC; Ψ_S = 6 kPa) to the permanent wilting point (PWP; Ψ_S = 1500 kPa). Within this range, the water content of colonized substrates was gradually lower with every RMC plant added to the pots, indicating that those substrates can be more thoroughly depleted of water (**Figure 2**). Θ at FC was equal in NM and AMF substrates, but was lower at the PWP. This difference was most pronounced in RMC/RMC pots. However, the total plant available water content between FC and PWP was not different in colonized substrates and was equally related to the amount of BRSP in NM and AMF substrates (**Figure 3**). The mycorrhizal effect that relates to the shift in water retention was observed between Ψ_S = 3 and 6 kPa. Here, the slope of the curve was steeper in AMF substrates ($P = 0.042$), which was not related to the planting combination ($P = 0.458$). Exemplarily, an additional depletion of approximately 2.4% water content was required in colonized WT/WT pots to reduce Ψ_S by 3 kPa, whereas less than 1% of additional reduction in Θ was required in the other planting combinations.

Considering equal suction and water content, hydraulic conductivity determines substrate the water flow capacity. We found unsaturated hydraulic conductivity (K) improved in colonized substrates between Ψ_S = 6 kPa and Ψ_S = 10 kPa (**Figure 4**). The effect was most pronounced in WT/WT pots and gradually decreased with the replacement of one or two WT plants with RMC mutants. This range is the most relevant for plant water uptake in most scenarios, because water is held against gravitation and easily extractable in high quantities. This effect was truly a mycorrhizal one. Although variability in K was also partially explained by BRSP [$K(\Psi_S = 6$ kPa): $R^2 = 0.31$; $P < 0.05$; $K(\Psi_S = 10$ kPa): $R^2 = 0.41$; $P < 0.05$; $N = 24$], the mycorrhizal improvements in K were still significant [K(6 kPa): $P = 0.028$; K(10 kPa): $P = 0.041$], when BRSP was used as the continuous predictor to account for BRSP variability. To some degree, K in mycorrhizal substrates depends on more than the protein content and, interestingly, this positive effect seemed to require a functional symbiosis. Expressed on an absolute basis, true enhancements were only observed in pots where at least one WT was growing, but totally absent in the RMC/RMC pots (**Figure 5**). At higher water potentials values those mycorrhizal influences were less pronounced.

DISCUSSION

We succeeded to obtain plants of similar size with equal nutritional status, which was important to minimize the plant's influence on root-free compartments. Non-mycorrhizal treatments for every pot combination were required, because the substrate without roots is nevertheless in physical contact with the surrounding rooted substrate. It is, thus, not entirely uncoupled from plant activity, i.e., via substrate mass flow driven by transpiration, as indicated in a similar approach with the same genotypes (Hallett et al., 2009). However, we did not detect any changes in substrate-related traits upon the planting combination. Hence, we assume that the impact of plants on root-free compartments was equal in all combinations.

We found also mycelia in pots where only resistant mutants grew. This is possible, because spore germination of AMF mainly depends on soil moisture and temperature (Daniels and Trappe, 1980) and pre-symbiotic hyphae can proliferate rather undirected over distances of almost 1 cm (Powell, 1976). That would cover half of the sampling core depth, when colonized from both entry sides. We assume this also happened in compartments subjected to water retention assessments.

Mechanisms Affecting Substrate Hydraulic Characteristics

When AMF penetrate soils or substrates, they grow in pores and on the surface of particles. In this way, they influence the size of the pore space and change its physico-chemical properties by releasing biochemicals, inducing particle redistribution and altering the degree of particle surface coverage with organic material. Penetrating AMF can induce aggregation of particles, i.e., affect soil structure, by entangling and enmeshing solid particles and by releasing polysaccharides and other 'sticky' substances to which particles adhere. They furthermore change surface wettability, because organic materials possess other wetting properties than mineral particles. Aggregation affects pore size distribution (Daynes et al., 2013), pore geometry and, surfactant effects influence the wettable pore space and water extractability. In the bulk pore space (quantified as porosity from

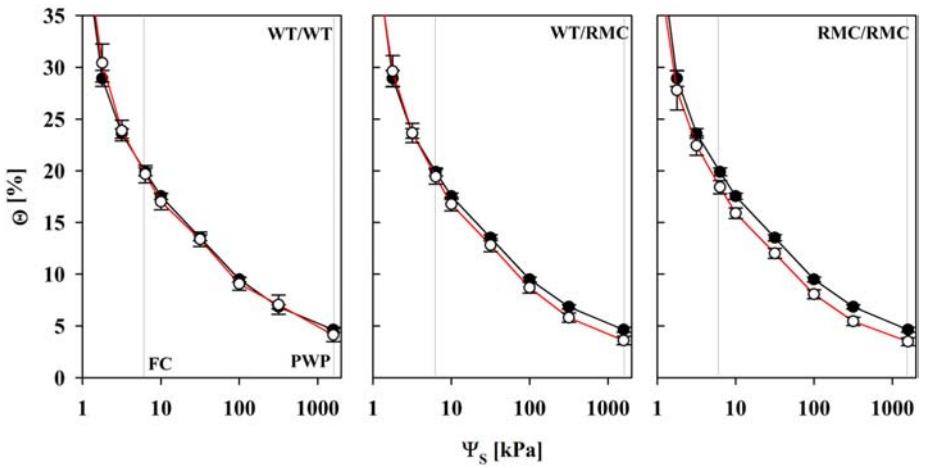


FIGURE 2 | Water retention curves of non-mycorrhizal (black line) pots and of pots inoculated with *R. irregulare* (red line) for intra- (WT/WT; RMC/RMC) and inter-genotypic (WT/RMC) plant combinations from field capacity (FC) to the permanent wilting point (PWP). Θ and Ψ_S denote the volumetric water content and the substrate water potential, respectively. Means (n = 4; ± SE) are shown, deriving from individual pot specific fits. There was a significant mycorrhizal effect at $\Theta_{100\ kPa}$ (P = 0.016) and $\Theta_{1500\ kPa}$ (P = 0.015). Marginal significances were observed for $\Theta_{10\ kPa}$ (P = 0.054) and $\Theta_{300\ kPa}$ (P = 0.096). Significant differences between planting combinations or factor interaction were not detected (two way ANOVA, α = 0.05, n = 4).

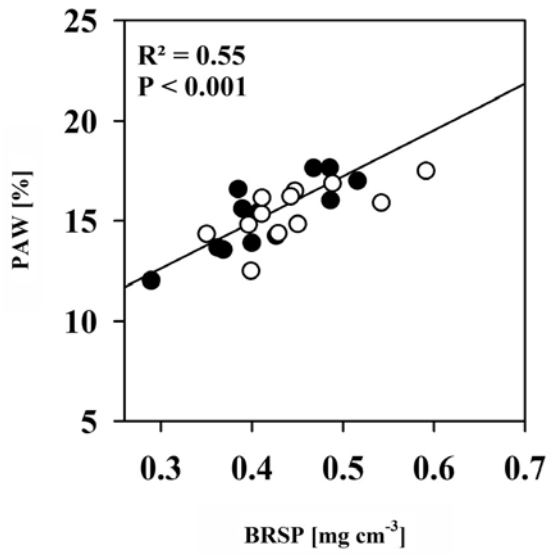


FIGURE 3 | The relationship between plant available water contents (PAW) and Bradford-reactive soil protein contents (BRSP) for non-mycorrhizal (black dots) pots and of pots inoculated with *R. irregulare* (white dots) for all intra- and inter-genotypic plant combinations combined per inoculation treatment. Significant differences between planting combinations, mycorrhizal inoculation or factor interaction were not detected (two way ANOVA, α = 0.05, n = 4).

the ratio of dry bulk density and particle density), water and solute transport occurs. Its water storage and transport capacity is measured by water retention and hydraulic conductivity characteristics that depend on those structural and surfactant effects.

Porosity increases when organic matter is removed by combustion (McCarthy et al., 2008). AMF are part of the organic matter pool. Moreover, AMF can increase water repellency (Rillig et al., 2010) and may therefore be responsible for the slight reduction in the total wettable pore space, i.e., the saturated water content (Θ_{SAT}). One could argue that the reduction in Θ_{SAT} upon substrate colonization were merely due to the volume of fungal biomass present. However, to achieve this, more than 22000 spores (with a radius of 60 μm) or 63 km of hyphae (with a diameter of 10 μm) per cm^3 of substrate would be required. These are unlikely dimensions. Still, there could be a small impact because spores and hyphae will at least be partially attached to the solid matrix, potentially blocking small pore necks, and could impede access to larger continuous pore spaces (Rillig et al., 2007). This would require that those pores had been air filled, when samples were harvested. Factors determining water repellency change the liquid-solid phase contact angle (Letey et al., 1962; Hallett, 2008) and potentially reduce the effective wettable pore space. This may have been induced by AMF. Indeed, water contents near saturation drop with repellency (Hallett, 2008). This is consistent with our observed restoration of air filled porosity and could also have hindered infiltration of pores during saturation, which were only partially clogged by a hydrophobic fungal structure. Water repellency effects are especially pronounced in coarsely textured substrates, where the main driver for repellency, i.e., organic matter including AMF, covers a relatively high proportion of solid surface (Doerr et al., 2000; Hallett, 2008). In principle, the substrate we used falls into this category as it was dominated by coarse sand and large vermiculite particles (<200 μm). Still, the differences, although significant, have been small and possibly could have been abolished upon longer duration of water contact during saturation (Doerr et al., 2000).

The shape of the water retention curves of soils are determined by the primary, i.e., particle size distribution, and the secondary structure of the pore system, the latter being shaped by

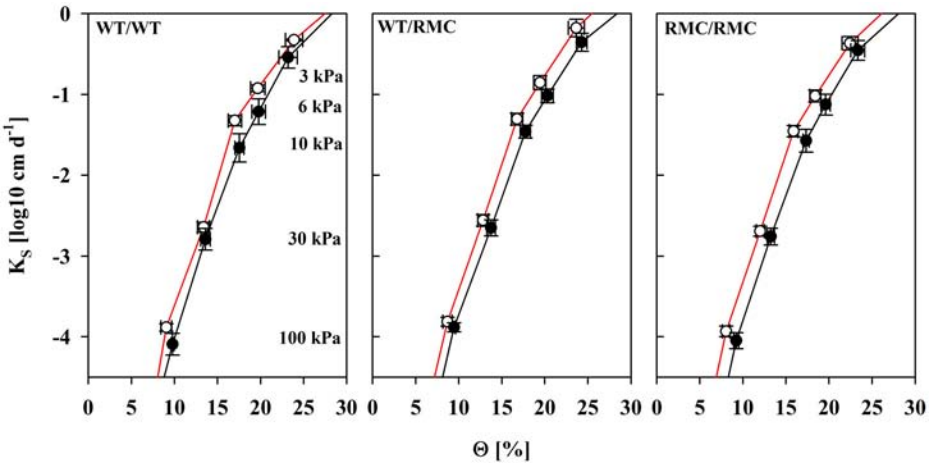


FIGURE 4 | Substrate hydraulic conductivity (*K*) as a function of the volumetric water content (Θ) of non-mycorrhizal (black line) pots and of pots inoculated with *R. irregulare* (red line) for intra- (WT/WT; RMC/RMC) and inter-genotypic (WT/RMC) plant combinations. Means (n = 4; ±SE) are shown, deriving from individual pot specific fits. There was a significant mycorrhizal effect at $K_{6\ kPa}$ (P = 0.040) and $\Theta_{10\ kPa}$ (P = 0.034). Marginal significances were observed for $\Theta_{3\ kPa}$ (P = 0.072) and $\Theta_{100\ kPa}$ (P = 0.067). Significant differences between planting combinations or factor interaction were not detected (two way ANOVA, α = 0.05, n = 4).

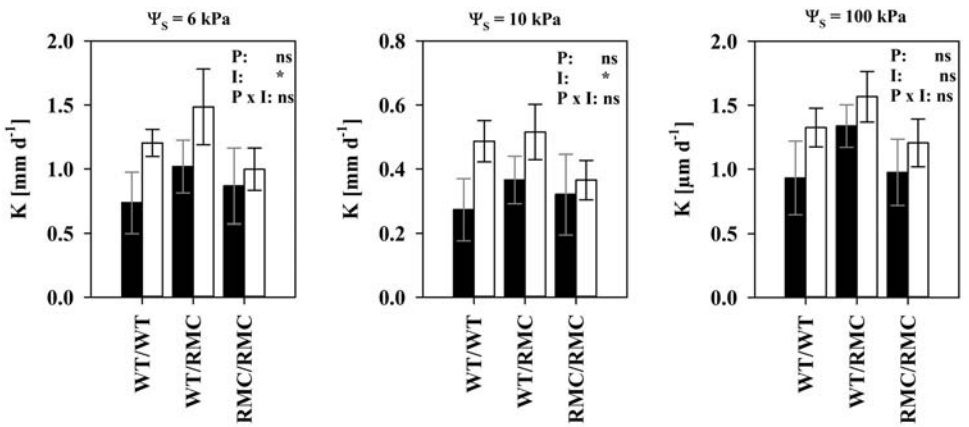


FIGURE 5 | Substrate hydraulic conductivity (*K*) at three different levels of substrate water potential (pF) of non-mycorrhizal (black columns) pots and of pots inoculated with *R. irregulare* (white columns) for the intra- and inter-genotypic plant combinations containing either two wild type tomato plants (WT/WT), two resistant mutants (RMC/RMC) or one WT and one resistant mutant (WT/RMC). Means (n = 4; ±SE) are shown, deriving from individual pot specific fits. Asterisks denote significant differences between non-mycorrhizal and mycorrhizal pots. Significant differences between planting combinations or factor interaction were not detected (two way ANOVA, α = 0.05, $^{ns}P > 0.05$, $^{*}P < 0.05$).

aggregation and formation of microchannels (Durner, 1992). Depending on size and genetic development of aggregates, shifts of water retention due to the structural pore system are located between Ψ_S = 0.3 and 10 kPa (Durner, 1992). AMF influence the mean aggregate diameter (Daynes et al., 2013). The size and amount of aggregates alters the proportions of inter-aggregate and enclosed intra-aggregate pores of the total pore space (Regelink et al., 2015). Those discrete structural changes to pore size distribution in the bulk pore system peak in narrow to moderate ranges (Durner, 1992) and require a detailed look. Indeed, a steeper slope of [$\Theta(\Psi_S)$] within the expected range (here within Ψ_S = 3–6 kPa) was observed in colonized substrates. This is consistent with other reports on a sandy soil mixture (Augé et al., 2001), a vertisol (Bearden, 2001) and a substrate deriving from coarse spoil (Daynes et al., 2013) under root ingrowth. We show here for the first time that this pattern also appears under root exclusion. Because this was most pronounced in WT/WT pots in the current experiment, changes in the structural pore system seemed to be related to the viability or activity of the symbiosis.

Soil protein contents, here quantified as BRSP, are often positively correlated with aggregation and subsequently with AMF colonization (e.g., Rillig et al., 2002; Wu et al., 2012). Within aggregates, pores of particular sizes exist that hold water against gravitation and are emptied within the plant-available moisture range (Hallett et al., 2009; Regelink et al., 2015). If AMF promotes aggregation, increases in water contents between FC and PWP would hence be expected. Consistently, BRSP contents

were positively correlated with plant available water contents in the moisture range between FC and PWP (**Figure 3**). However, neither BRSP contents nor plant available water contents were influenced by AMF. Hence, there was no effect of AMF on total plant available water. We only found the tendency that BRSP improves upon substrate colonization. In general, however, levels were very variable. BRSP accumulates in soils because of its low turnover rate and is mainly released by AMF during hyphae turnover (Driver et al., 2005). We have provided a substantial amount of BRSP with the soil (as indicated by contents from NM pots). This was more important for water holding capacity than the possibly minute quantities added by AMF. Due to the rather short duration of the experiment, BRSP release from hyphae turnover was probably low.

Interestingly, in substrates from WT/RMC and RMC/RMC pots, lower water contents were observed in the plant-available moisture range, but not in WT/WT pots. In pots containing a viable symbiosis, the water contents between FC and PWP were conserved, which may indicate structural stabilization under the absence of roots. The reduction of water contents between FC and PWP in WT/RMC and RMC/RMC pots in comparison to their NM counterparts indicates a destabilization of structure and losses of structural pore volume. We can only speculate about underlying mechanisms. One scenario could be that this observation is related to the absence of access to plant derived carbon in a-symbiotic fungi. A-symbiotic AMF will lack plant carbon to produce compounds required to adhere to particles or cover surfaces, but may invest resources from spores in proliferation toward susceptible hosts. It follows that effects of stickiness may be outweighed by penetration. Macroaggregates formed by fungi are transient and underlie turnover (Six et al., 2004). We may have provided such aggregates by the addition of soil (like BRSP), which could have been partially disintegrated by penetration of less 'sticky' a-symbiotic fungi. This would increase the access to previously enclosed intra-aggregate pores and water may become accessible upon disintegration. Another possibility coming from the same effect is that a-symbiotic hyphae could induce less pore enclosure when growing inside aggregates or on the surface of particles. Although, the scenario is speculation and requires further research, it would also explain the maintenance of water retention in NM substrates that not possessed penetrating organisms and would not require observable changes in protein contents.

On coarsely textured substrates like sandy soils, AMF effects are mainly related to the sticky-string bag function (Miller and Jastrow, 2000) inducing entanglement and enmeshment processes with hyphae bridging large pore spaces between particles (Clough and Sutton, 1978; Forster, 1979). This is a realistic scenario also for our substrate and may be explanatory for the stimulation of unsaturated hydraulic conductivity between Ψ_S = 6 and 10 kPa. We show that here for the first time. Unsaturated hydraulic conductivity is mainly determined by the few largest water filled pores and in addition, depends on pore tortuosity and pore connectivity (Durner, 1994). Unsaturated conductivity can change although water retention remains the same (Durner, 1992), which is exactly what we observed in WT/WT pots. The shape and organization of voids in aggregated soils differs from unstructured porous systems. As an example, a net of planar voids will show a quite different hydrological behavior than a pore space between spherical aggregates, although both may possess similar retention characteristics (Durner, 1992). Under the present conditions around field capacity, large pores are already air filled and hyphae may constitute a connection for water and/or a less tortuous pathway for water. A less tortuous water pathway would also appear, when hyphae have a smoothing effect on particle profiles. Like other microbes, AMF may also produce compounds that are hydrophobic when dry, but strongly hydrophilic when wet (Hallett, 2008). This could increase conductivity from those water filled pores. Due to the dimension of hyphae, it is unlikely that just intrahyphal water flow explains this observed promotion, which was as large as 50% in this study. This is further supported by the fact that substrates from pots with two resistant mutants completely lack that promotion. This is interesting and worth studying in detail in the future. The absence of stimulation of K in substrates with a-symbiotic hyphae may point to the necessity of a functional symbiosis that has access to plant derived carbon as a source for production of exudates and sticky substances.

Further studies can elucidate the proposed mechanisms of this discussion, e.g., by applying imaging technologies on the microscale. The superior aim of this study was to quantify AMF impacts on substrate hydraulic properties under conditions similar to those frequently encountered in mycorrhizal research that target physiological host responses. Stabilization of water retention and improvements of unsaturated hydraulic conductivity outside root zone areas were apparently most pronounced in systems with the most viable symbiosis. It would be fascinating to combine outcomes of soil hydraulic properties with molecular techniques, elucidating the expression of genes required to produce those AMF compounds that influence the physical properties of soils in viable symbioses. In particular it would be intriguing to investigate whether the expression of genes encoding for production of glycoproteins and hydrophobins requires a symbiotic association with plants. More importantly, we did not find a scenario, where substrate hydraulic properties of colonized pots equaled that of non-colonized ones. This may have important consequences for plant reactions, which will, however, differ with the setting.

Relevance for Plants

It is consensus that plants grow differently on different soils under otherwise equal conditions. This may also apply for AMF plants in the same soil or substrate with different properties. Our findings underpin the extension of the ambit of plants by AMF hyphae, not only for P acquisition, but also for their hydraulic environment in the growing medium. Especially the stimulation of K may be of importance. Viewing at the hyphal compartment as a root-free substrate microstructure, a root close to those substrate proportions can create a water potential gradient by water uptake. Water would then move along the evolving gradient at higher rates into the vicinity of roots in colonized substrates. Alternatively, a shallower water potential gradient between both substrate proportions is necessary to induce the same water flow. Potential plant water uptake can

then increase without the necessity of intrahyphal translocation, which has been estimated to be insignificant (George et al., 1992). This can have important ecological implications, because this would require less investment in root osmotic adjustments to realize the same water flow to roots or effectively enlarges the rhizosphere in colonized substrates with a viable symbiosis. Improvements in hydraulic conductivity via reduced tortuosity or increased connectivity may also appear quicker than hydraulic effects deriving from a hierarchical development of structure, because it may depend less on release of organic material in turnover processes.

Plants sense moisture stress and adjust transpiration via stomatal movement to avoid exhaustive behavior with the resources available (Tardieu and Simonneau, 1998) and mycorrhizal plants often show increased stomatal conductance (Augé et al., 2015). It appears logic, that a substrate with changed water retention and hydraulic conductivity induces a different stress response in the plant. Water contents and hydraulic conductivity are important inputs in plant based stomata (e.g., Tardieu and Simonneau, 1998) and root water uptake models (e.g., van Lier et al., 2006; de Jong van Lier et al., 2008). In addition to direct effects of mycorrhiza on root morphology, root hydraulic conductivity and plant aquaporin expression (e.g., Porcel et al., 2006; Aroca et al., 2007; Bárzana et al., 2012; Ruiz-Lozano et al., 2016) modulating plant water uptake, there may be effects downstream to roots that contribute to the often observed stimulation of plant water, nutrient uptake and drought tolerance in AMF plants. Both, mycorrhizal roots capable of more efficient water uptake and less resistance to water flow in the substrate, may be required for the frequent observation of higher soil drying rates in mycorrhizal systems.

The conservation of water retention and stimulation of unsaturated hydraulic conductivity in AMF symbioses can be a beneficial effect that qualifies AMF as biostimulants in plant production systems. If this also occurs in soils in the field, AMF constitute an effective enlargement of the rhizosphere for water and solute acquisition from the periphery in addition to P. In pot productions systems, this could stimulate water and solute acquisition per unit time, when fertilization and irrigation regimes avoid detrimental degrees of water and nutrient depletion.

AUTHOR CONTRIBUTIONS

MB conducted the experiments, analyzed the data, and wrote the manuscript. PF and JG supervised the work, helped developing the experiments, revised the manuscript, and contributed to writing.

FUNDING

MB was supported by an Elsa Neumann grant of Berlin. The project has received funding from the Ministry of Consumer Protection, Food and Agriculture of the Federal Republic of Germany, from the Ministry for Science, Research and Culture of the State of Brandenburg, and from the Thuringian Ministry of Infrastructure and Agriculture.

ACKNOWLEDGMENTS

We would like to thank Birgit Löffelbein and Anna Vetter for excellent technical assistance. We thank Dr. Susan Barker and Prof. Sally Smith from the University of Adelaide for provision of the tomato RMC mutant.

REFERENCES

Akaike, H. (1974). A new look at the statistical model identification. *IEEE Trans. Automatic Control* Ac-19, 716–723. doi: 10.1109/TAC.1974.1100705

Aroca, R., Porcel, R., and Ruiz-Lozano, J. M. (2007). How does arbuscular mycorrhizal symbiosis regulate root hydraulic properties and plasma membrane aquaporins in *Phaseolus vulgaris* under drought, cold or salinity stresses? *New Phytol.* 173, 808–816. doi: 10.1111/j.1469-8137.2006.01961.x

Augé, R. M. (2001). Water relations, drought and vesicular-arbuscular mycorrhizal symbiosis. *Mycorrhiza* 11, 3–42. doi: 10.1007/s005720100097

Augé, R. M. (2004). Arbuscular mycorrhizae and soil/plant water relations. *Can. J. Soil Sci.* 84, 373–381. doi: 10.4141/S04-002

Augé, R. M., Stodola, A. J. W., Tims, J. E., and Saxton, A. M. (2001). Moisture retention properties of a mycorrhizal soil. *Plant Soil* 230, 87–97. doi: 10.1023/A:1004891210871

Augé, R. M., Toler, H. D., and Saxton, A. M. (2015). Arbuscular mycorrhizal symbiosis alters stomatal conductance of host plants more under drought than under amply watered conditions: a meta-analysis. *Mycorrhiza* 25, 13–24. doi: 10.1007/s00572-014-0585-4

Azcón, R. (1989). Selective interaction between free-living rhizosphere bacteria and vesiculararbuscular mycorrhizal fungi. *Soil Biol. Biochem.* 21, 639–644. doi: 10.1016/0038-0717(89)90057-6

Barker, S. J., Stummer, B., Gao, L., Dispain, I., Oconnor, P. J., and Smith, S. E. (1998). A mutant in *Lycopersicon esculentum* Mill. with highly reduced VA mycorrhizal colonization - isolation and preliminary characterisation. *Plant J.* 15, 791–797. doi: 10.1046/j.1365-313X.1998.00252.x

Bárzana, G., Aroca, R., Paz, J. A., Chaumont, F., Martinez-Ballesta, M. C., Carvajal, M., et al. (2012). Arbuscular mycorrhizal symbiosis increases relative apoplastic water flow in roots of the host plant under both well-watered and drought stress conditions. *Ann. Bot.* 109, 1009–1017. doi: 10.1093/aob/mcs007

Bearden, B. N. (2001). Influence of arbuscular mycorrhizal fungi on soil structure and soil water characteristics of vertisols. *Plant Soil* 229, 245–258. doi: 10.1023/A:1004835328943

Bodner, G., Leitner, D., and Kaul, H. P. (2014). Coarse and fine root plants affect pore size distributions differently. *Plant Soil* 380, 133–151. doi: 10.1007/s11104-014-2079-8

Boldt, K., Pörs, Y., Haupt, B., Bitterlich, M., Kühn, C., Grimm, B., et al. (2011). Photochemical processes, carbon assimilation and RNA accumulation of sucrose transporter genes in tomato arbuscular mycorrhiza. *J. Plant Physiol.* 168, 1256–1263. doi: 10.1016/j.jplph.2011.01.026

Clough, K. S., and Sutton, J. C. (1978). Direct observation of fungal aggregates in sand dune soil. *Can. J. Microbiol.* 24, 333–335. doi: 10.1139/m78-056

Colla, G., Nardi, S., Cardarelli, M., Ertani, A., Lucini, L., Canaguier, R., et al. (2015). Protein hydrolysates as biostimulants in horticulture. *Sci. Hortic.* 196, 28–38. doi: 10.3389/fpls.2017.02202

Daniels, B. A., and Trappe, J. M. (1980). Factors affecting spore germination of the vesicular-arbuscular mycorrhizal fungus, *Glomus epigaeus*. *Mycologia* 72, 457–471. doi: 10.2307/3759519

Daynes, C. N., Field, D. J., Saleeba, J. A., Cole, M. A., and McGee, P. A. (2013). Development and stabilisation of soil structure via interactions between organic matter, arbuscular mycorrhizal fungi and plant roots. *Soil Biol. Biochem.* 57, 683–694. doi: 10.1016/j.soilbio.2012.09.020

De Gryze, S., Jassogne, L., Six, J., Bossuyt, H., Wevers, M., and Merckx, R. (2006). Pore structure changes during decomposition of fresh residue: X-ray tomography analyses. *Geoderma* 134, 82–96. doi: 10.1016/j.geoderma.2005.09.002

de Jong van Lier, Q., Van Dam, J. C., Metselaar, K., De Jong, R., and Duijnisveld, W. H. M. (2008). Macroscopic root water uptake distribution using a matric flux potential approach. *Vadose Zone J.* 7, 1065–1078. doi: 10.2136/vzj2007.0083

De Kreij, C., Voogt, W., van den Bos, A. L., and Baas, R. (1997). *Voedingsoplossingen voor de Teelt van Tomaat in Gesloten Teeltsystemen*. Naaldwijk: Brochure VG Tomaat.

Degens, B. P., Sparling, G. P., and Abbott, L. K. (1996). Increasing the length of hyphae in a sandy soil increases the amount of water-stable aggregates. *Appl. Soil Ecol.* 3, 149–159. doi: 10.1016/0929-1393(95)00074-7

Doerr, S. H., Shakesby, R. A., and Walsh, R. P. D. (2000). Soil water repellency: its causes, characteristics and hydro-geomorphological significance. *Earth Sci. Rev.* 51, 33–65. doi: 10.1016/S0012-8252(00)00011-8

Driver, J. D., Holben, W. E., and Rillig, M. C. (2005). Characterization of glomalin as a hyphal wall component of arbuscular mycorrhizal fungi. *Soil Biol. Biochem.* 37, 101–106. doi: 10.1016/j.soilbio.2004.06.011

du Jardin, P. (2015). Plant biostimulants: definition, concept, main categories and regulation. *Sci. Hortic.* 196, 3–14. doi: 10.1016/j.scienta.2015.09.021

Durner, W. (1992). "Predicting the unsaturated hydraulic conductivity using multi-porosity water retention curves," in *Proceedings of the International Workshop Indirect Methods for Estimating the Hydraulic Properties of Unsaturated Soils*, eds M. T. van Genuchten, F. J. Leij, and L. J. Lund (Riverside, CA: University of California), 185–202.

Durner, W. (1994). Hydraulic conductivity estimation for soils with heterogeneous pore structure. *Water Resour. Res.* 30, 211–223. doi: 10.1029/93WR02676

El-Atrach, F., Vierheilig, H., and Ocampo, J. A. (1989). Influence of non-host plants on vesicular-arbuscular mycorrhizal infection of host plants and on spore germination. *Soil Biol. Biochem.* 21, 161–163. doi: 10.1016/0038-0717(89)90026-6

Fonteno, W. C. (1992). Problems & considerations in determining physical properties of horticultural substrates. *Acta Hortic.* 342, 197–204.

Forster, S. M. (1979). Microbial aggregation of sand in an embryo dune system. *Soil Biol. Biochem.* 11, 537–543. doi: 10.1016/0038-0717(79)90014-2

George, E., Haussler, K. U., Vetterlein, D., Gorgus, E., and Marschner, H. (1992). Water and nutrient translocation by hyphae of *Glomus mosseae*. *Can. J. Bot.* 70, 2130–2137. doi: 10.1139/b92-265

Giovannetti, M., and Mosse, B. (1980). Evaluation of techniques for measuring vesicular arbuscular mycorrhizal infection in roots. *New Phytol.* 84, 489–500. doi: 10.1111/j.1469-8137.1980.tb04556.x

Hallett, P. D. (2008). A brief overview of the causes, impacts and amelioration of soil water repellency: a review. *Soil Water Res.* 3, 521–528.

Hallett, P. D., Feeney, D. S., Bengough, A. G., Rillig, M. C., Scrimgeour, C. M., and Young, I. M. (2009). Disentangling the impact of AM fungi versus roots on soil structure and water transport. *Plant Soil* 314, 183–196. doi: 10.1007/s11104-008-9717-y

Kim, K. Y., Jordan, D., and McDonald, G. A. (1997). Effect of phosphate-solubilizing bacteria and vesicular-arbuscular mycorrhizae on tomato growth and soil microbial activity. *Biol. Fertil. Soils* 26, 79–87. doi: 10.1007/s003740050347

Koske, R. E., and Gemma, J. N. (1989). A modified procedure for staining roots to detect VA mycorrhizas. *Mycol. Res.* 92, 486–488. doi: 10.1016/S0953-7562(89)80195-9

Leifheit, E. F., Veresoglou, S. D., Lehmann, A., Morris, E. K., and Rillig, M. C. (2014). Multiple factors influence the role of arbuscular mycorrhizal fungi in soil aggregation: a meta-analysis. *Plant Soil* 374, 523–537. doi: 10.1007/s11104-013-1899-2

Letey, J., Osborn, J., and Pelishek, R. E. (1962). The influence of the water-solid contact angle on water movement in soil. *Hydrol. Sci. J.* 7, 75–81. doi: 10.1080/02626666209493272

McCarthy, J. F., Ilavsky, J., Jastrow, J. D., Mayer, L. M., Perfect, E., and Zhuang, J. (2008). Protection of organic carbon in soil microaggregates via restructuring of aggregate porosity and filling of pores with accumulating organic matter. *Geochim. Cosmochim. Acta* 72, 4725–4744. doi: 10.1016/j.gca.2008.06.015

Miller, R. M., and Jastrow, J. D. (2000). "Mycorrhizal fungi influence soil structure," in *Arbuscular Mycorrhizas: Physiology and Function*, ed. Y. Kapulnik (Berlin: Springer), 3–18. doi: 10.1007/978-94-017-0776-3_1

Mualem, Y. (1976). A new model for predicting the hydraulic conductivity of unsaturated porous media. *Water Resour. Res.* 12, 513–522. doi: 10.1029/WR012i003p00513

Neumann, E., and George, E. (2005a). Does the presence of arbuscular mycorrhizal fungi influence growth and nutrient uptake of a wild-type tomato cultivar and a mycorrhiza-defective mutant, cultivated with roots sharing the same soil volume? *New Phytol.* 166, 601–609.

Neumann, E., and George, E. (2005b). Extraction of extraradical arbuscular mycorrhizal mycelium from compartments filled with soil and glass beads. *Mycorrhiza* 15, 533–537.

Peters, A., and Durner, W. (2008). Simplified evaporation method for determining soil hydraulic properties. *J. Hydrol.* 356, 147–162. doi: 10.1016/j.jhydrol.2008.04.016

Piotrowski, J. S., Denich, T., Klironomos, J. N., Graham, J. M., and Rillig, M. C. (2004). The effects of arbuscular mycorrhizas on soil aggregation depend on the interaction between plant and fungal species. *New Phytol.* 164, 365–373. doi: 10.1111/j.1469-8137.2004.01181.x

Porcel, R., Aroca, R., Azcón, R., and Ruiz-Lozano, J. M. (2006). PIP aquaporin gene expression in arbuscular mycorrhizal *Glycine max* and *Lactuca sativa* plants in relation to drought stress tolerance. *Plant Mol. Biol.* 60, 389–404. doi: 10.1007/s11103-005-4210-y

Porcel, R., Aroca, R., Azcon, R., and Ruiz-Lozano, J. M. (2016). Regulation of cation transporter genes by the arbuscular mycorrhizal symbiosis in rice plants subjected to salinity suggests improved salt tolerance due to reduced Na^+ root-to-shoot distribution. *Mycorrhiza* 26, 673–684. doi: 10.1007/s00572-016-0704-5

Porcel, R., Redondo-Gómez, S., Mateos-Naranjo, E., Aroca, R., Garcia, R., and Ruiz-Lozano, J. M. (2015). Arbuscular mycorrhizal symbiosis ameliorates the optimum quantum yield of photosystem II and reduces non-photochemical quenching in rice plants subjected to salt stress. *J. Plant Physiol.* 185, 75–83. doi: 10.1016/j.jplph.2015.07.006

Powell, C. L. (1976). Development of mycorrhizal infections from *Endogone* spores and infected root segments. *Trans. Br. Mycol. Soc.* 66, 439–445. doi: 10.1016/S0007-1536(76)80214-8

Querejeta, J. I. (2017). "Soil water retention and availability as mediated by mycorrhizal symbiosis: consequences for individual plants, communities, and ecosystems," in *Mycorrhizal Mediation of Soil: Fertility, Structure, and Carbon Storage*, eds N. C. Johnson, C. A. Gehring, and J. Jansa (Amsterdam: Elsevier Inc.), 299–317.

Rasmussen, S. R., Füchtbauer, W., Novero, M., Volpe, V., Malkov, N., Genre, A., et al. (2016). Intraradical colonization by arbuscular mycorrhizal fungi triggers induction of a lipochitooligosaccharide receptor. *Sci. Rep.* 6:29733. doi: 10.1038/srep29733

Regelink, I. C., Stoof, C. R., Rousseva, S., Weng, L., Lair, G. J., Kram, P., et al. (2015). Linkages between aggregate formation, porosity and soil chemical properties. *Geoderma* 247, 24–37. doi: 10.1016/j.geoderma.2015.01.022

Rillig, M. C. (2005). A connection between fungal hydrophobins and soil water repellency? *Pedobiologia* 49, 395–399. doi: 10.1016/j.pedobi.2005.04.004

Rillig, M. C., Caldwell, B. A., Wösten, H. A., and Sollins, P. (2007). Role of proteins in soil carbon and nitrogen storage: controls on persistence. *Biogeochemistry* 85, 25–44. doi: 10.1007/s10533-007-9102-6

Rillig, M. C., Mardatin, N. F., Leifheit, E. F., and Antunes, P. M. (2010). Mycelium of arbuscular mycorrhizal fungi increases soil water repellency and is sufficient to maintain water-stable soil aggregates. *Soil Biol. Biochem.* 42, 1189–1191. doi: 10.1016/j.soilbio.2010.03.027

Rillig, M. C., and Mummey, D. L. (2006). Mycorrhizas and soil structure. *New Phytol.* 171, 41–53. doi: 10.1111/j.1469-8137.2006.01750.x

Rillig, M. C., Wright, S. F., and Eviner, V. T. (2002). The role of arbuscular mycorrhizal fungi and glomalin in soil aggregation: comparing effects of five plant species. *Plant Soil* 238, 325–333. doi: 10.1023/A:1014483303813

Rouphael, Y., Franken, P., Schneider, C., Schwarz, D., Giovannetti, M., Agnolucci, M., et al. (2015). Arbuscular mycorrhizal fungi act as biostimulants in horticultural crops. *Sci. Hortic.* 196, 91–108. doi: 10.1016/j.scienta.2015.09.002

Ruiz-Lozano, J. M., Aroca, R., Zamarreño, Á. M., Molina, S., Andreo-Jiménez, B., Porcel, R., et al. (2016). Arbuscular mycorrhizal symbiosis induces strigolactone biosynthesis under drought and improves drought tolerance in lettuce and tomato. *Plant Cell Environ.* 39, 441–452. doi: 10.1111/pce.12631

Ruzzi, M., and Aroca, R. (2015). Plant growth-promoting rhizobacteria act as biostimulants in horticulture. *Sci. Hortic.* 196, 124–134. doi: 10.1016/j.jenvman.2016.11.060

Schindler, U. (1980). Ein schnellverfahren zur messung der wasserleitfähigkeit im teilgesättigten Boden an stechzylinderproben. *Arch. Acker Pflanzenbau Bodenkd.* 24, 1–7.

Silva, F. S. B., Yano-Melo, A. M., and Maia, L. C. (2007). Production and infectivity of inoculum of arbuscular mycorrhizal fungi multiplied in a substrate supplemented with Tris-HCl buffer. *Braz. J. Microbiol.* 38, 752–755. doi: 10.1590/S1517-83822007000400030

Six, J., Bossuyt, H., Degryze, S., and Denef, K. (2004). A history of research on the link between (micro) aggregates, soil biota, and soil organic matter dynamics. *Soil Tillage Res.* 79, 7–31. doi: 10.1016/j.still.2004.03.008

Smith, S. E., and Read, D. J. (2008). *Mycorrhizal Symbiosis*. London: Academic Press.

Smith, S. E., Smith, F. A., and Jakobsen, I. (2004). Functional diversity in arbuscular mycorrhizal (AM) symbioses: the contribution of the mycorrhizal P uptake pathway is not correlated with mycorrhizal responses in growth or total P uptake. *New Phytol.* 162, 511–524. doi: 10.1111/j.1469-8137.2004.01039.x

Tardieu, F., and Simonneau, T. (1998). Variability among species of stomatal control under fluctuating soil water status and evaporative demand: modelling isohydric and anisohydric behaviours. *J. Exp. Bot.* 49, 419–432. doi: 10.1093/jxb/49.Special_Issue.419

Tisdall, J. M., and Oades, J. (1982). Organic matter and water−stable aggregates in soils. *Eur. J. Soil Sci.* 33, 141–163. doi: 10.1111/j.1365-2389.1982.tb01755.x

van Lier, Q. J., Metselaar, K., and Van Dam, J. C. (2006). Root water extraction and limiting soil hydraulic conditions estimated by numerical simulation. *Vadose Zone J.* 5, 1264–1277. doi: 10.2136/vzj2006.0056

Wright, S. F., and Upadhyaya, A. (1996). Extraction of an abundant and unusual protein from soil and comparison with hyphal protein of arbuscular mycorrhizal fungi. *Soil Sci.* 161, 575–586. doi: 10.1097/00010694-199609000-00003

Wright, S. F., and Upadhyaya, A. (1998). A survey of soils for aggregate stability and glomalin, a glycoprotein produced by hyphae of arbuscular mycorrhizal fungi. *Plant Soil* 198, 97–107. doi: 10.1023/A:1004347701584

Wu, Q. S., Cao, M. Q., Zou, Y. N., and He, X. H. (2014). Direct and indirect effects of glomalin, mycorrhizal hyphae, and roots on aggregate stability in rhizosphere of trifoliate orange. *Sci. Rep.* 4:5823. doi: 10.1038/srep05823

Wu, Q. S., He, X. H., Zou, Y. N., He, K. P., Sun, Y. H., and Cao, M. Q. (2012). Spatial distribution of glomalin-related soil protein and its relationships with root mycorrhization, soil aggregates, carbohydrates, activity of protease and glucosidase in the rhizosphere of *Citrus unshiu*. *Soil Biol. Biochem.* 45, 181–183. doi: 10.1016/j.soilbio.2011.10.002

Conflict of Interest Statement: The authors declare that the research was conducted in the absence of any commercial or financial relationships that could be construed as a potential conflict of interest.

frontiers in Plant Science

ORIGINAL RESEARCH
published: 26 March 2018
doi: 10.3389/fpls.2018.00323

Exogenous Abscisic Acid Promotes Anthocyanin Biosynthesis and Increased Expression of Flavonoid Synthesis Genes in *Vitis vinifera* × *Vitis labrusca* Table Grapes in a Subtropical Region

Renata Koyama[1], Sergio R. Roberto[1], Reginaldo T. de Souza[2], Wellington F. S. Borges[1], Mauri Anderson[3], Andrew L. Waterhouse[3], Dario Cantu[3], Matthew W. Fidelibus[3] and Barbara Blanco-Ulate[4]**

[1] Department of Agronomy, Londrina State University, Londrina, Brazil, [2] Embrapa Grape and Wine, Bento Gonçalves, Brazil, [3] Department of Viticulture and Enology, University of California, Davis, Davis, CA, United States, [4] Department of Plant Sciences, University of California, Davis, Davis, CA, United States

OPEN ACCESS

Edited by:
Giuseppe Colla,
Università degli Studi della Tuscia, Italy

Reviewed by:
Zhanwu Dai,
Institut National de la Recherche Agronomique, France
Chiara Cirillo,
University of Naples Federico II, Italy

***Correspondence:**
Sergio R. Roberto
sroberto@uel.br
Barbara Blanco-Ulate
bblanco@ucdavis.edu

Specialty section:
This article was submitted to Crop and Product Physiology, a section of the journal Frontiers in Plant Science

Received: 23 December 2017
Accepted: 27 February 2018
Published: 26 March 2018

Citation:
Koyama R, Roberto SR, de Souza RT, Borges WFS, Anderson M, Waterhouse AL, Cantu D, Fidelibus MW and Blanco-Ulate B (2018) Exogenous Abscisic Acid Promotes Anthocyanin Biosynthesis and Increased Expression of Flavonoid Synthesis Genes in Vitis vinifera × Vitis labrusca Table Grapes in a Subtropical Region. Front. Plant Sci. 9:323. doi: 10.3389/fpls.2018.00323

Hybrid (*Vitis vinifera* × *Vitis labrusca*) table grape cultivars grown in the subtropics often fail to accumulate sufficient anthocyanins to achieve good uniform berry color. Growers of *V. vinifera* table grapes in temperate regions generally use ethephon and, more recently, (*S*)-*cis*-abscisic acid (*S*-ABA) to overcome this problem. The objective of this study was to determine if *S*-ABA applications at different timings and concentrations have an effect on anthocyanin regulatory and biosynthetic genes, pigment accumulation, and berry color of the Selection 21 cultivar, a new *V. vinifera* × *V. labrusca* hybrid seedless grape that presents lack of red color when grown in subtropical areas. Applications of *S*-ABA 400 mg/L resulted in a higher accumulation of total anthocyanins and of the individual anthocyaninsanthocyanins: delphinidin-3-glucoside, cyanidin-3-glucoside, peonidin-3-glucoside, and malvidin-3-glucoside in the berry skin and improved the color attributes of the berries. Treatment with two applications at 7 days after véraison (DAV) and 21 DAV of *S*-ABA 400 mg/L resulted in a higher accumulation of total anthocyanins in the skin of berries and increased the gene expression of *CHI*, *F3H*, *DFR*, and *UFGT* and of the *VvMYBA1* and *VvMYBA2* transcription factors in the seedless grape cultivar.

Keywords: seedless table grape hybrid, berry quality, anthocyanin biosynthesis, MYB transcription factors, plant growth regulators, *S*-ABA

INTRODUCTION

Table grapes (*Vitis* spp.) have become an important fresh commodity in Brazil for both internal market and exportation. Over the period of 2000–2016, Brazil presented an increase of ∼150% in table grape production, reaching around 970,000 MT in 2016 (Food and Agriculture Organization [Fao], 2018). The northern region of Paraná state is one of the main areas of table grape production. The mild winter and subtropical conditions of this region permit two crops of grapes per year, which allow Brazilian growers to time their production to coincide with market windows

of other countries and compete for more advantageous prices. However, in these subtropical regions, berry ripening and harvest often occur during the rainy season, which is not ideal for *Vitis vinifera* cultivars because excess rain and moisture compromise the overall quality of the berries (Biasoto et al., 2014). Therefore, Brazilian table grape production is starting to incorporate American (mostly *Vitis labrusca*) and/or hybrid (*V. vinifera* × *V. labrusca*) grape cultivars that are better adapted to warm and rainy climates. Another disadvantage of growing table grapes in subtropical areas is that high temperatures during ripening can inhibit anthocyanin biosynthesis in the berries from *V. labrusca* and hybrid cultivars (Rybka et al., 2015). This results in inadequate fruit color, and thereby a decrease in market acceptance and the potential economic value of the commodity (Roberto et al., 2012). The seedless table grape Selection 21, a new hybrid of *V. vinifera* × *V. labrusca* recently developed by the Grape Genetic Breeding Program of Embrapa Grape and Wine, Brazil, obtained from the cross of [Arkansas 1976 × ("Niagara White" × "Venus")] × "BRS Linda," is a clear example of a cultivar that lacks red color development when grown in subtropical regions.

The plant growth regulator ethephon, an ethylene-releasing agent, has long been known to improve berry color when applied at véraison (the onset of grape ripening) (Jensen et al., 1975; Roberto et al., 2013). More recently, the application of (*S*)-*cis*-abscisic acid (*S*-ABA) has also been shown to stimulate anthocyanin accumulation and thereby improve berry color (Peppi et al., 2006; Roberto et al., 2012). *S*-ABA appears to be more effective than ethephon in enhancing grape color (Peppi et al., 2006; Roberto et al., 2012) and it has other potential benefits compared to ethephon, including a shorter postharvest interval, and an exemption from tolerance in most countries. The introduction of *S*-ABA as an active ingredient in a commercial plant growth regulator (ProTone®) prompted many studies on *V. vinifera* cultivars under temperate climate conditions. Such studies have shown that the efficacy of *S*-ABA varies with the cultivar (Sandhu et al., 2011), the *S*-ABA concentration (Peppi et al., 2006), the time of application (Ferrara et al., 2015) and the environmental conditions (Reynolds et al., 2016).

Abscisic acid is an important regulator of ripening and anthocyanin biosynthesis in grape berries (Kuhn et al., 2013; Yang and Feng, 2015). Studies have shown that exogenous application of *S*-ABA can significantly increase the activity of a wide range of genes involved in anthocyanin biosynthesis (He et al., 2010). Most of these studies tested the effects of a single application of *S*-ABA before or during véraison. However, studies of the effects of *S*-ABA several applications at different concentrations and timings following véraison are still needed to optimize the use of this plant growth regulator in table grape cultivation (Peppi et al., 2008; Koyama et al., 2010).

In grapes, the anthocyanin biosynthesis pathway involves multiple steps that are controlled by MYB transcription factors, such as VvMYBA1 and VvMYBA2 (Rinaldo et al., 2015). In red varieties, the *VvMYBA1* gene is only expressed after véraison. Both VvMYBA1 and VvMYBA2 regulate anthocyanin biosynthesis during ripening by strictly controlling the expression of the canonical UDP-glucose:flavonoid 3-*O*-glucosyltransferase gene (*UFGT*; He et al., 2010). Determining how long grape berries are competent to induce the expression of anthocyanin biosynthetic genes may help determine the optimal time, number, and frequency of *S*-ABA applications. Currently, little is known about the potential benefits of multiple applications, which may be desirable if a single application results in an insufficient response.

The aim of the present study was to determine the effects of *S*-ABA applications at different concentrations and times on the quality and biochemical properties of berries from the seedless grape Selection 21 hybrid during three growing seasons in the region of Paraná, Brazil. We evaluated a variety of parameters including: (i) grape color development, (ii) berry phenolic profiles, and (iii) gene expression of transcriptional regulators and biosynthetic enzymes of the anthocyanin pathway after treatments with *S*-ABA. The results of this report indicate that two *S*-ABA applications during and after véraison extend the competency of grape berries to respond to ABA and induce the accumulation of anthocyanins, resulting in higher grape berry coloration.

MATERIALS AND METHODS

Plant Material and Grapevine Growth Conditions

The study was conducted during three consecutive seasons (2013, 2014, and 2015) in a commercial vineyard located in Marialva, state of Paraná (PR), Brazil (latitude 23°29′52.8″S, longitude 51°47′58″0 W, 570 masl), using 4-year-old vines of hybrid seedless grape Selection 21 (*V. vinifera* × *V. labrusca*) grafted onto IAC 766 Campinas rootstock. According to the Köppen classification, the climate of the region is Cfa (subtropical), with an average temperature below 18°C in the coldest month (mesothermic) and above 22°C in the hottest month and an average annual rainfall of 1,596 mm. The region's soil is classified as dystroferric red latosol (Bhering and Santos, 2008).

The vines were trained using a bilateral overhead trellis system, where vines were spaced at 2.5 m × 2.5 m (1,600 vines per hectare), and each vine had 6.25 m^2 total canopy area. Cane pruning was performed during the 2013, 2014, and 2015 seasons and was followed by application of 3% hydrogenated cyanamide to the two apical buds to induce and standardize sprouting. The number of canes per vine was evenly adjusted to 40 (20 per arm) and the number of shoots per vine was also established to 40 (1 bearing shoot per cane). Considering that a grape bunch of the Selection 21 weighs on average 460 g, the load per vine is 18.40 kg, which represents an estimated yield of 29.44 tons/ha.

Furthermore, to avoid drifting, a non-treated vine was left as side border between two treated vines, which almost duplicated the experimental area. In each plot, all grape bunches were treated (n = 40 per vine or plot), and the bunch samples (n = 10, five per side) were collected from random positions at each side of the canopy to account for intracanopy variability. Control plants were not subjected to any treatment, instead, they were sprayed

with water at the same time and following the same procedures as the *S*-ABA treatments.

S-ABA Applications

The effects of applying *S*-ABA isomer at different concentrations and times were evaluated in terms of berry quality traits. ProTone® (Valent BioSciences, Libertyville, IL, United States), the commercial growth regulator used in this study, has an active ingredient concentration of 100 g/L *S*-ABA.

As shown in **Figure 1**, the initial treatments tested in the 2013 and 2014 seasons corresponded to: (i) control or water spray, (ii) 200 mg/L *S*-ABA application at 7 days after véraison (DAV), (iii) 400 mg/L *S*-ABA application at 7 DAV, (iv) 200 mg/L *S*-ABA application at 7 DAV plus an additional application at 21 DAV, and (v) 400 mg/L *S*-ABA at 7 DAV plus an additional application at 21 DAV. In the 2015 season, only the control and treatments of 400 mg/L *S*-ABA with one or two applications were performed and analyzed. Berry samples from the 2015 season were collected from each treatment at four different times: 7 DAV (collected 1 h before treatment application), 14 DAV, 28 DAV, and 35 DAV for further targeted analyses (**Figure 1**). For all seasons, a randomized complete block experimental design was used, with five treatments and three to four replicates, and with each plot consisting of one vine (see the previous section for details on trellis system).

Véraison was determined by measuring soluble solid content (SSC) and firmness (using a Texture Analyzer, details below) of grape berries randomly sampled in the experimental vineyard. At véraison, the mean grape SSC concentration was 9°Bx, and 20% of the berries in more than 50% of the grape clusters presented softening (Cantín et al., 2007). The berries presented a mean of 11°Bx at 7 DAV, the time of the first *S*-ABA application, and a mean of 13°Bx at 21 DAV, the time of the second *S*-ABA application.

Ten grape clusters representative of each plot were marked prior to treatment application. For treatment applications, grape clusters were sprayed in the morning using a knapsack sprayer at a pressure of 568.93 psi (39.22 bar) with JA1 hollow cone nozzle tips at a volume of 800 L/ha to provide complete and uniform coverage. In addition, 0.3 mL/L of Break-Thru® (Evonik Industries, Germany) a non-ionic surfactant was added to all treatments. During the trials, the standard regional cultivation practices with regard to nutrition, weed control, and pest and disease management were used.

Berry Physiological Measurements

Clusters of each plot were manually harvested when SSC stabilized (∼15°Bx). The clusters were cleaned, and damaged berries were discarded. Color coverage (% red color) of the bunches was determined using 10 grape clusters per plot by visually rating the clusters on a scale of 1–5 using the following scale: (1) 0–20%, (2) 21–40%, (3) 41–60%, (4) 61–80%, and (5) 81–100% coverage (Roberto et al., 2012). The same grape clusters used for evaluating color coverage measurements were used for berry sampling. For physicochemical analyses, two berries were collected from the upper, middle, and lower portion of each grape bunch, yielding a total of 70 berries per plot. Total anthocyanins and color index of red grapes (CIRG) were determined in berry samples from all seasons. The following variables were analyzed only for the 2013 and 2014 seasons: color coverage, total polyphenols, and berry firmness. All physiological analyzes were performed in the Laboratory of Fruit Analysis of the Agricultural Research Center, Londrina State University, Brazil.

The total anthocyanin concentration of the berries was determined using 30 berries per plot, which were frozen and stored at −20°C. The berry skins were removed using tweezers, taking care to remove only the skin, without pulp. The skins were washed once with water, followed by washing in deionized water and drying with absorbent paper. A 5-g skin sample was then placed in a polystyrene tube containing 50 mL of acidified methanol (1% HCl) and stored in the dark for 48 h at room temperature. The tubes were then removed from the dark and stirred manually for 5 s. Absorbance was determined using a Genesys 10S spectrophotometer (Thermo Fisher Scientific Inc., MA, United States) at 520 nm with the solvent as blank. The results were expressed in milligram malvidin-3-glucoside per gram of skin (mg/g; Peppi et al., 2006).

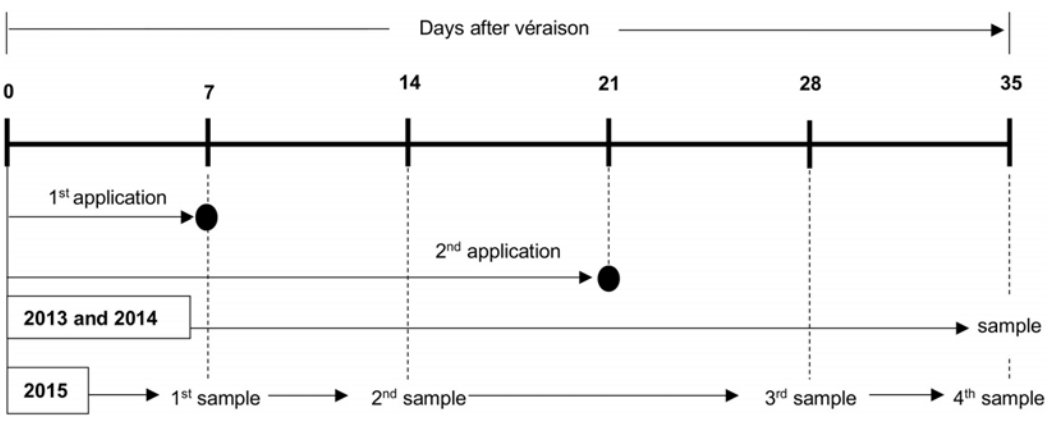


FIGURE 1 | Sample collection schedule for grape clusters treated with *S*-ABA during the 2013, 2014, and 2015 seasons.

The CIRG was determined using 10 berries per plot with a CR-10 colorimeter (Konica Minolta, Japan), using the CIELAB color system. The following variables were determined for the berry equatorial section: lightness (L^*), saturation (C^*), and hue ($h°$). CIRG was then determined using the following equation: CIRG = $(180 - h°)/(L^* + C^*)$ (Carreño et al., 1995).

Total polyphenol determination was performed using 30 berries per plot based on a modified Folin–Ciocalteu method. In summary, the absorbance of each sample was measured after 15 min at 765 nm using a Genesys 10S spectrophotometer (Thermo Fisher Scientific Inc., MA, United States) against a blank sample prepared with water instead of the extract. Determination of total polyphenol was calculated from the calibration curve obtained with gallic acid. The results were expressed in milligram total polyphenols per 100 g of sample (mg/100 g; Bucic-Kojic et al., 2007).

The berry firmness was performed with a TA.XT2i Texture Analyzer (Stable Micro Systems, Surrey, United Kingdom), at 25 ± 1°C, analyzing the equatorial position of 10 berries with pedicels per plot. Each berry was placed on the base of the equipment and compressed using a cylindrical probe with a diameter of 35 mm parallel to the base. A constant force of 0.05 N at a speed of 1.0 mm/s was applied to promote the cracking of the sample. The berry firmness (N) was then determined (Borges et al., 2012).

Gene Expression Analyses

In the 2015 season, three grape clusters of uniform size and at the same phenological stage were identified in each plot, and two berries were collected randomly from each bunch (n = 6 per plot) at each sampling time. The berry skins were removed, frozen, kept at −80°C, and transferred to the Department of Viticulture and Enology of the University of California, Davis, CA, United States, for further analyses. The skins were then placed in liquid nitrogen and ground using a TissueLyser II (Qiagen, CA, United States).

RNA was extracted using 0.5 g of ground tissue (skin) based on the protocol described by Blanco-Ulate et al. (2013). RNA concentration and purity were determined using a NanoDrop 2000c Spectrophotometer (Thermo Fisher Scientific Inc., DE, United States), and RNA integrity was checked by electrophoresis on 1.5% agarose gel. Reverse transcription (synthesis of the cDNA first strand) was performed using 1 μg of RNA and M-MLV Reverse Transcriptase (Promega Corp., Madison, WI, United States) according to the manufacturer's instructions. qRT-PCRs were performed using the SYBR® Green PCR Master Mix kit (Applied Biosystems®, CA, United States). The PCR program consisted of 70°C for 10 min, 36 cycles at 42°C for 2 s, and 37°C for 50 min. *VvActin* (*VIT_04s0044g00580*) was used as the reference gene and processed in parallel with the genes of interest. Gene sequences used for primer design were obtained from the GenBank of the National Center for Biotechnology Information using Primer-BLAST software (Ye et al., 2012; **Table 1**). The relative levels of target gene expression were calculated using the formula $2^{(\text{Reference gene CT} - \text{Gene of interest CT})}$. The linearized values correspond to the relative gene expression within a given sample and are comparable across genes. Four biological replicates of *S*-ABA treated and control grape berries were used to obtain the relative gene expression data.

Anthocyanin Profiling

The same berry samples used for gene expression analyses were used for quantification of individual anthocyanins using high-pressure liquid chromatography (HPLC). A total of 0.1 g of ground tissue (skin) for each treatment and control was freeze-dried and the anthocyanins were extracted using the protocol described in Chassy et al. (2012). Four biological replicates were used to quantify the main grape anthocyanins: delphinidin-3-glucoside, cyanidin-3-glucoside, petunidin-3-glucoside, peonidin-3-glucoside, and malvidin-3-glucoside.

RESULTS

Application of abscisic acid (*S*-ABA) increased the total anthocyanin concentration in berry skins of the seedless grape

TABLE 1 | Primers designed for quantitative PCR analyses.

Gene name	Accession number (NCBI)	Sequence of forward (F) and reverse (R) primers		TM (melting temperature)
CHI	FJ468358	F	5′-CAGTCACCGCAGTTCAGGTC-3′	64.5
		R	5′-GGAAGAGGTCGTTGGTGGAG-3′	64.5
F3H	X75965	F	5′-ATGGCGCCTACGACACTGAC-3′	64.5
		R	5′-ATGGCTGGAAACGATGAAGCC-3′	62.6
DFR	pBS510	F	5′-ACCTGTAGATGGCAAGACCTAGA-3′	60.3
		R	5′-GAACTCTCATTTCCGGCACATTG-3′	60.4
LDOX	NM_001281218	F	5′-GACAGCTTGAGTGGGAGGAC-3′	60.4
		R	5′-AGTCGCTTGGTGTCTTAGGC-3′	58.4
UFGT	JF522529	F	5′-TGGTGGCTGACGCATTCAT-3′	60.2
		R	5′-CCCCATCTCTGCTGCCATATC-3′	64.5
VvMYBA1	AB097923	F	5′-TTATCGCAAGCCTCAGGACAG-3′	62.6
		R	5′-TCCCAGAAGCCCACATCAA-3′	60.2
VvMYBA2	AB073013	F	5′-GATGTGGGCTTCTGGGATAC-3′	62.4
		R	5′-AGGGAGTAGAGTATGAATGCAAGA-3′	61.2

Selection 21 during the 2013 and 2014 seasons, regardless of the *S*-ABA concentration and time of application (**Table 2**). However, berries that received 400 mg/L of *S*-ABA at 7 and 21 DAV had significantly higher, almost two to three times more, anthocyanin concentrations than other treatments.

According to the CIRG, berries from control treatments had a green to a yellow color (CIRG < 2) in both seasons (**Table 2**). In 2013, berries treated with one or two applications of 200 mg/L *S*-ABA or one application of 400 mg/L *S*-ABA at 7 DAV, and those in the 2014 season that were treated with one application of 200 mg/L *S*-ABA developed a pink color (2 < CIRG < 4). Remarkably, berries of the 2013 season treated with two applications of 400 mg/L *S*-ABA and berries of the 2014 season treated with one or two 400 mg/L *S*-ABA applications, developed a stronger red color (4 < CIRG < 5; **Figure 2**). For both the 2013 and 2014 seasons, color coverage was lowest in control grapes and highest in grapes treated with two applications of 400 mg/L *S*-ABA.

Increase in total polyphenols was evident in grapes subjected to two 400 mg/L *S*-ABA applications during the 2013 and 2014 seasons. These berries also presented the lowest mean berry firmness (**Table 3**). Importantly, the increased softening due to *S*-ABA application did not result in higher frequency of cracked berries in any of the studied seasons. Qualitative assessment of berry cracking was performed visually.

Further analyses of the effect of 400 mg/L *S*-ABA treatments on CIRG, total and individual anthocyanins concentrations, and gene expression of transcription factors and biosynthetic enzymes were performed with grape berries collected from the 2015 trial. Measurements of CIRG confirmed previous results obtained during the 2013 and 2014 seasons, at the time of harvest (35 DAV), grapes treated with two *S*-ABA applications had the highest CIRG values (**Figure 3**). Grape bunches from the control treatment presented pink berries (CIRG = 3.4), whereas those treated with one or two applications of *S*-ABA had red berries (CIRG = 4.5 and CIRG = 4.8, respectively). As determined in the previous seasons, berries treated with 400 mg/L *S*-ABA also presented higher total anthocyanin content than the control at 14 and 28 DAV (**Table 4**). At 28 DAV, grapes treated with one (7 DAV) or two (7 and 21 DAV) applications of 400 mg/L *S*-ABA presented total anthocyanin concentrations almost three times higher than the control. Even 3 weeks after the first application (28 DAV), berries treated with only one *S*-ABA application showed a total anthocyanin content similar to those treated with two *S*-ABA applications. Nonetheless, the second application of 400 mg/L *S*-ABA significantly affected the total anthocyanin accumulation at the time of harvest (35 DAV, 4 weeks after the first *S*-ABA application and 2 weeks after the second *S*-ABA application).

S-ABA altered the concentrations and proportions of individual anthocyanins in berries from the seedless grape Selection 21 (**Table 4**). With the exception of petunidin-3-glucoside, *S*-ABA application significantly improved the concentrations of all the measured anthocyanins. Cyanidin-3-glucoside and peonidin-3-glucoside levels increased at 14 DAV, 1 week after the first *S*-ABA application. The second *S*-ABA application stimulated the accumulation of the anthocyanin delphinidin-3-glucoside at 28 DAV, yielding differences relative to both the control and to the samples treated with only one *S*-ABA application. At 28 DAV, the concentrations of peonidin-3-glucoside and malvidin-3-glucoside increased after exogenous *S*-ABA application but were not further increased by the second application. At the time of harvest (35 DAV), peonidin-3-glucoside and cyanidin-3-glucoside were the dominant pigments present after all treatments. Delphinidin-3-glucoside, cyanidin-3-glucoside, and peonidin-3-glucoside presented higher accumulation following the second application of 400 mg/L *S*-ABA, but the number of applications did not affect the accumulation of malvidin-3-glucoside.

As presented in **Figure 4**, treatment with 400 mg/L *S*-ABA significantly increased the expression of the transcription factors *VvMYBA1* and *VvMYBA2* and the expression of the biosynthetic genes *CHI*, *F3H*, *DFR*, and *UFGT* 1 week after the first application (14 DAV). Three weeks after the first *S*-ABA application (28 DAV), expression of *CHI*, *F3H*, and *DFR* genes remained high, but this was not observed for the transcription factors *VvMYBA1* and *VvMYBA2* or the *UFGT* gene. Four weeks after the first *S*-ABA application (35 DAV), no significant differences were observed in the expression of genes or transcription factors between berries that received one *S*-ABA application and those that received the control treatment. The two applications of 400 mg/L *S*-ABA induced expression of the genes *CHI*, *F3H*, *DFR*, and *UFGT* and the transcription factors *VvMYBA1* and *VvMYBA2* at 14 and 28 DAV (**Figure 4**). *F3H* expression was the most affected by *S*-ABA application, displaying higher levels than the control until the final stages of berry maturation at 35 DAV,

TABLE 2 | Total anthocyanin, color index for red grapes (CIRG), and color coverage of Selection 21 grape berries treated with *S*-ABA.

Treatment (concentration in mg/L)	Total anthocyanin (mg/g)		CIRG		Color coverage[1]	
	2013	2014	2013	2014	2013	2014
Control	0.18 ± 0.07 d	0.12 ± 0.03 d	1.49 ± 0.49 c	1.80 ± 0.12 b	1.00 ± 0.00 d	1.00 ± 0.00 c
S-ABA 200 (7 DAV)	0.39 ± 0.14 cd	0.59 ± 0.14 c	2.15 ± 0.29 bc	3.71 ± 0.61 a	1.75 ± 0.50 c	3.00 ± 0.50 b
S-ABA 400 (7 DAV)	0.57 ± 0.15 bc	0.86 ± 0.06 b	2.78 ± 0.53 b	4.48 ± 0.28 a	3.25 ± 0.50 b	4.25 ± 0.75 ab
S-ABA 200 (7 DAV) + 200 (21 DAV)	0.68 ± 0.02 b	0.74 ± 0.04 bc	3.07 ± 0.33 b	4.34 ± 0.13 a	2.75 ± 0.50 b	3.75 ± 0.38 ab
S-ABA 400 (7 DAV) + 400 (21 DAV)	1.15 ± 0.04 a	1.18 ± 0.01 a	4.14 ± 0.54 a	4.51 ± 0.13 a	4.75 ± 0.50 a	5.00 ± 0.00 a

Mean values within columns followed by different letters differ significantly at the 5% probability level according to Tukey's test. DAV, days after véraison. [1]Color coverage: (1) 0–20%; (2) 21–40%; (3) 41–60%; (4) 61–80%; (5) 81–100% coverage. 2013 and 2014 seasons.

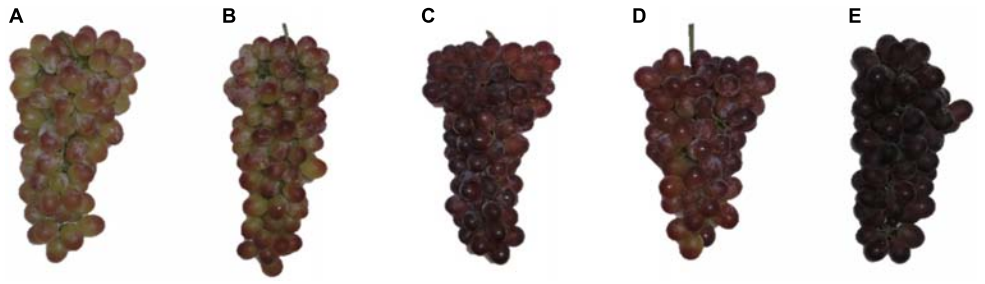


FIGURE 2 | Representative clusters of seedless grape Selection 21 subjected to various treatments with *S*-ABA. **(A)** Control; **(B)** *S*-ABA 200 mg/L 7 days after véraison (DAV); **(C)** *S*-ABA 400 mg/L 7 DAV; **(D)** *S*-ABA 200 mg/L 7 DAV + 200 mg/L 21 DAV; **(E)** *S*-ABA 400 mg/L 7 DAV + 400 mg/L 21 DAV.

whereas the remaining genes presented no differences from the control at harvest. Overall, the gene expression results indicate that a second *S*-ABA application contributed to the maintenance of the expression of the transcription factors *VvMYBA1* and *VvMYBA2* and the genes *F3H* and *UFGT* at higher levels than in the control for an extended period of time.

DISCUSSION

Effects of *S*-ABA on Anthocyanin Concentration, Berry Color, and Firmness

Exogenous application of *S*-ABA improves the color of table grapes by stimulating the anthocyanin synthesis and accumulation in the grape skin (Jeong et al., 2004; Cantín et al., 2007). Our results are similar to those reported previously for "Flame Seedless" grapes, in which applications of 300 mg/L ABA during or after véraison were more effective at increasing anthocyanin concentrations than application before véraison (Peppi et al., 2006). In 'Crimson Seedless' grapes, anthocyanin concentrations increased with application of 400 mg/L *S*-ABA at 17 DAV, but the response varied widely between seasons depending on *S*-ABA dosage and time of application (Leão et al., 2015). Exogenous *S*-ABA application is thought to simulate plant stress responses and accelerate ripening processes (Balint and Reynolds, 2013). High ABA concentrations are believed to be perceived by grapevines as a drought stress signal (Castellarin et al., 2007). Subsequently, water stress leads to changes in grape secondary metabolism, significantly increasing flavonoid levels and, especially, anthocyanin biosynthesis (Zhang et al., 2016).

In our study, in addition to increasing anthocyanin concentration, exogenous *S*-ABA improved both berry color intensity and uniformity. This is important because the visual assessment of berry color characteristics determine, in part, the commercial value of table grapes (Peppi et al., 2006, 2007). Grape clusters with more intense and uniform berry color have higher consumer acceptance. Improved color characteristics such as increased color coverage of grape berries, and uniformity of berry color within a cluster, were also observed in "Benitaka" and "Rubi" table grapes following application of 400 mg/L *S*-ABA at 7 DAV and 15 days before harvest (Roberto et al., 2012, 2013). In our experiments, berries from the seedless grape Selection 21 treated with one or two applications of 400 mg/L *S*-ABA presented higher CIRG values than the controls. Besides, improving cluster color and attractiveness, exogenous *S*-ABA can potentially decrease the time to harvest, a feature that is very advantageous for grape commercialization (Cantín et al., 2007; Ferrara et al., 2015).

The observed increase in anthocyanin concentration resulting from *S*-ABA application does not necessarily result in an increase in total polyphenol content; polyphenols include phenolic acids, stilbenes, coumarins, tannins, and flavonoids (Roubelakis-Angelakis, 2009), as reported in "Alachua" muscadine grapes (Sandhu et al., 2011) and in "Isabel" grapes (Koyama et al., 2014). Environmental factors such as temperature, rainfall, and altitude could also influence berry polyphenol concentrations. In

TABLE 3 | Total polyphenols and firmness of Selection 21 grape berries treated with *S*-ABA.

Treatment (concentration in mg/L)	Total polyphenol content (mg/100 g)		Firmness (N)	
	2013	2014	2013	2014
Control	15.9 ± 3.8	13.4 ± 2.5 b	33.9 ± 5.0 a	25.3 ± 2.5 a
S-ABA 200 (7 DAV)	13.1 ± 3.0	12.8 ± 1.5 b	30.2 ± 3.1 a	23.7 ± 1.3 a
S-ABA 400 (7 DAV)	15.5 ± 1.7	14.3 ± 0.7 b	26.7 ± 2.9 b	19.3 ± 1.9 bc
S-ABA 200 (7 DAV) + 200 (21 DAV)	15.8 ± 1.9	14.4 ± 1.2 b	26.4 ± 1.3 b	20.4 ± 1.6 bc
S-ABA 400 (7 DAV) + 400 (21 DAV)	18.3 ± 1.1	18.3 ± 1.3 a	19.9 ± 2.0 c	15.7 ± 0.5 c

Mean values within columns followed by different letters differ significantly at the 5% probability level according to Tukey's test. DAV, days after véraison. 2013 and 2014 seasons.

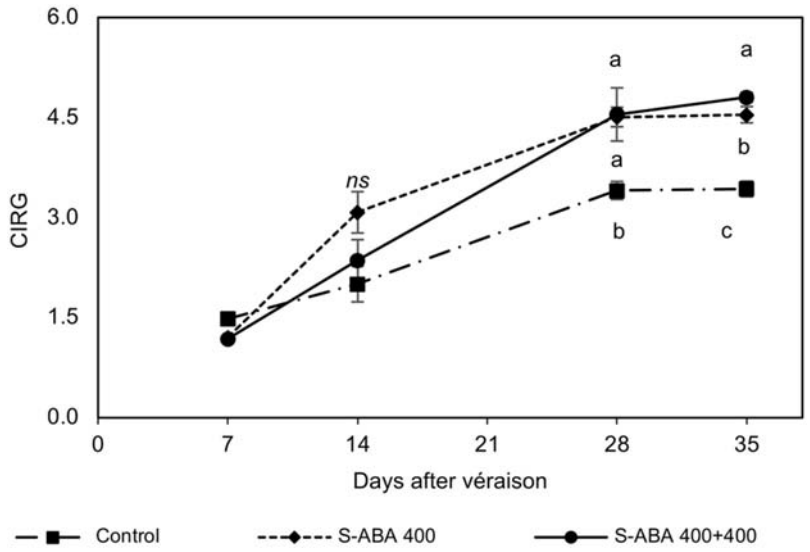


FIGURE 3 | CIRG of Selection 21 grape berries subjected to treatments with *S*-ABA during the 2015 season. Control: no *S*-ABA application; *S*-ABA 400: *S*-ABA 400 mg/L 7 days after véraison (DAV); *S*-ABA 400 + 400: *S*-ABA 400 mg/L 7 DAV + 400 mg/L 21 DAV. The means followed by different letters differ significantly at the 5% probability level according to Tukey's test. ns, non-significant.

addition, the berry ripening stage is directly correlated to the concentration and proportion of several polyphenols that impact the organoleptic properties, nutritional value, and antioxidant capacities of the grapes (Zhu et al., 2016).

S-ABA application can negatively affect berry firmness, an important characteristic for the successful postharvest handling of grapes for the fresh fruit market because it influences transportability and shelf life (Batista et al., 2015). ABA application is known to cause loosening and decreased rigidity of the cell wall, resulting in fruit softening and a higher probability of berry cracking (Thomas et al., 2008; Wada et al., 2008; Gambetta et al., 2010). Treatment of grapes with exogenous *S*-ABA can result in changes in the regulation of proline-rich cell wall proteins and in the induction of cell wall degrading genes, such as polygalacturonases that promote pectin solubilization and depolymerization (Koyama et al., 2010). The effect of *S*-ABA on berry firmness was also observed in "Flame Seedless" grapes, where it caused softening similar to that caused by ethephon application (Peppi et al., 2006), as well as in 'Crimson Seedless' (Lurie et al., 2009) and "Red Globe" grapes (Peppi et al., 2007). Therefore, it is still necessary to evaluate if the benefits of applying exogenous ABA to improve berry color can outweigh a potential reduction in the shelf life of treated grapes.

The Effect of Multiple Applications of *S*-ABA on Individual Anthocyanins Concentration, Gene Expression, and Transcript Factors Expression

Multiple applications of exogenous ABA can promote anthocyanin accumulation for longer periods of time (Giribaldi et al., 2010). It is possible that more than one ABA application could induce a milder response at later grape phenological stages or that the effects of a second application could take more time to be evident. In this study, the second application of 400 mg/L *S*-ABA significantly increased the total anthocyanin content at the time of harvest, which supported the latter hypothesis and confirmed that two *S*-ABA applications had a more pronounced effect than only one application. The higher total anthocyanin concentration observed with *S*-ABA application appeared to result from a transient effect of *S*-ABA, because the anthocyanin concentration of grapes that received only one *S*-ABA application remained essentially constant between 28 and 35 DAV. It may, therefore, be inferred that the action of *S*-ABA decreases over time and that its levels increase with a second application, allowing maintenance of its activity.

Three applications of 400 mg/L *S*-ABA at 1-week intervals prior to véraison resulted in an earlier accumulation of anthocyanin in "Cabernet Sauvignon" grapes, but no differences in anthocyanin concentration at harvest were observed in grapes that received different treatments (Wheeler et al., 2009). The increase in endogenous ABA concentration in grape berries occurs at the beginning of véraison and extends until the establishment of maturation when endogenous ABA concentrations peak. ABA concentration then decreases until harvest, the period over which the decrease occurs ranges from 13 to 20 days depending on the cultivar (Davies et al., 1997; Wheeler et al., 2009). Application of exogenous *S*-ABA close to véraison, when ABA naturally reaches its highest concentration in berries, was shown to be more effective in increasing anthocyanin accumulation than application at other times (Berli et al., 2011). *S*-ABA application significantly increased endogenous ABA levels 7 days after application in "Carménère" grapes, 40 days later, the ABA levels in the treated berries remained higher than those from control (Villalobos-González et al., 2016).

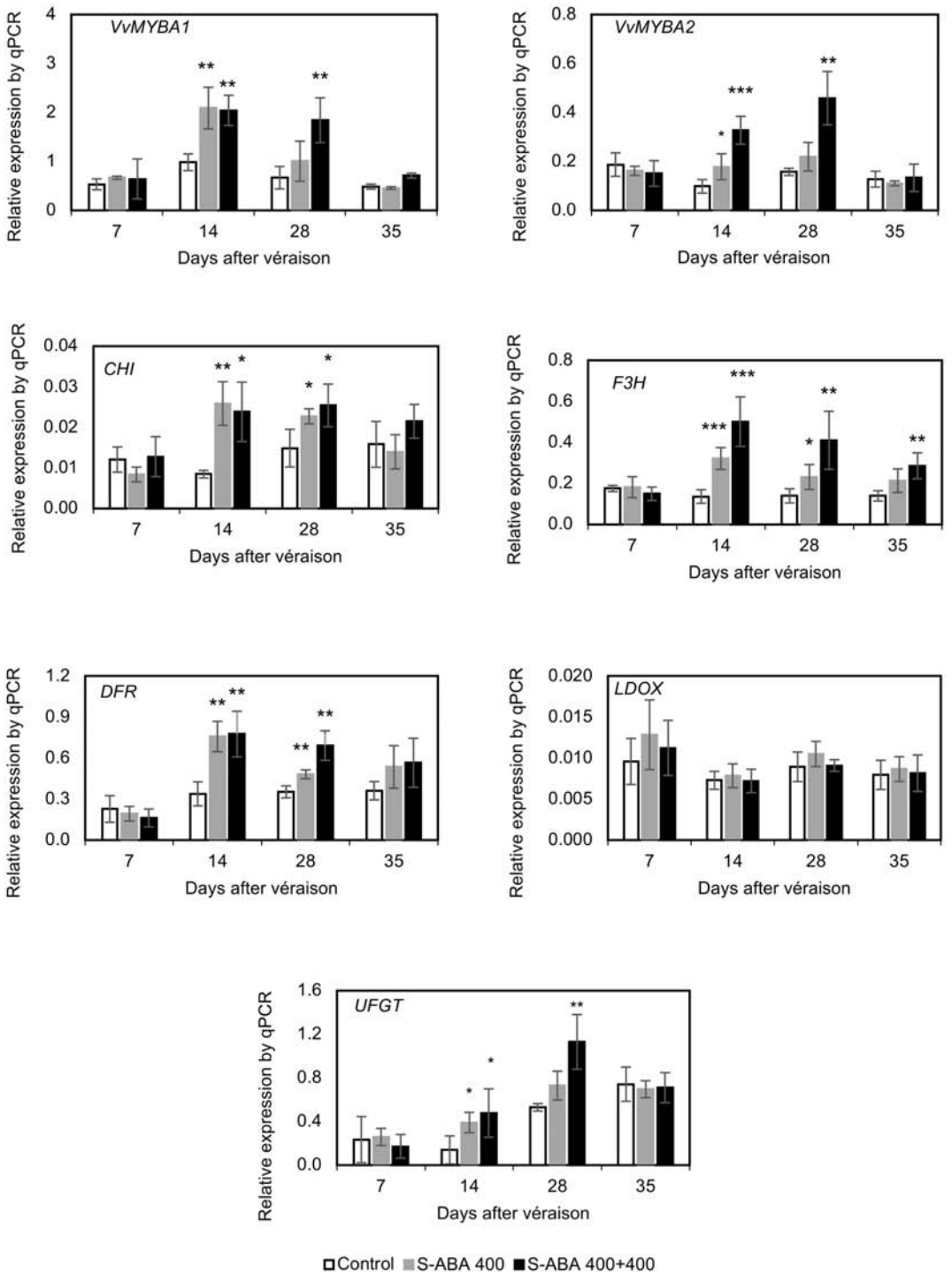


FIGURE 4 | Effects of *S*-ABA application on the expression of *VvMYBA1* and *VvMYBA2* and the transcription levels of the flavonoid biosynthetic genes in grape berries from the Selection 21 hybrid throughout development in the 2015 season. Student's *t*-test was used to determine significant differences between the treatments and the control at $*p \leq 0.05$, $**p \leq 0.01$, and $***p \leq 0.001$. Control, no *S*-ABA application; *S*-ABA 400, *S*-ABA 400 mg/L 7 days after véraison (DAV); *S*-ABA 400 + 400, *S*-ABA 400 mg/L at 7 DAV + 400 mg/L at 21 DAV.

Berry color is affected both by total anthocyanin concentration and by anthocyanin composition. Different anthocyanins have specific characteristics with respect to color and stability (Jeong et al., 2004). The five main grape anthocyanins differ from each other in the number and position of the hydroxyl (OH) and methyl (CH_3) groups on the B-ring. Cyanidin and peonidin are

TABLE 4 | Total and individual anthocyanin content of Selection 21 grape berries treated with *S*-ABA during 2015 season.

Sampling	Treatment (concentration in mg/L)	Total anthocyanin content (mg/g)	Anthocyanin content (mg/L)				
			Delphinidin-3-glucoside	Cyanidin-3-glucoside	Petunidin-3-glucoside	Peonidin-3-glucoside	Malvidin-3-glucoside
7 DAV	Control	0.07 ± 0.01	–	–	2.53 ± 0.43	–	–
	S-ABA 400 (7 DAV)	0.06 ± 0.01	–	–	2.05 ± 0.38	–	–
	S-ABA 400 (7 DAV) + 400 (21 DAV)	0.06 ± 0.01	–	–	1.53 ± 0.39	–	–
14 DAV	Control	0.23 ± 0.09b	–	0.08 ± 0.32c	3.07 ± 0.83	1.33 ± 0.48b	–
	S-ABA 400 (7 DAV)	0.50 ± 0.13a	–	4.85 ± 0.43a	2.35 ± 1.00	7.80 ± 0.05a	–
	S-ABA 400 (7 DAV) + 400 (21 DAV)	0.40 ± 0.13a	–	2.80 ± 0.86b	2.08 ± 0.07	5.10 ± 1.60a	–
28 DAV	Control	0.56 ± 0.17b	0.10 ± 0.01b	7.53 ± 2.33b	2.03 ± 0.33	12.57 ± 4.94b	0.23 ± 0.88b
	S-ABA 400 (7 DAV)	1.54 ± 0.08a	0.12 ± 0.03b	20.58 ± 1.01a	1.40 ± 0.58	45.93 ± 3.48a	3.13 ± 0.98a
	S-ABA 400 (7 DAV) + 400 (21 DAV)	1.57 ± 0.05a	0.38 ± 0.03a	26.23 ± 2.43a	0.95 ± 0.57	40.03 ± 7.17a	2.40 ± 0.01a
35 DAV	Control	0.70 ± 0.04c	0.10 ± 0.01c	9.70 ± 0.77c	1.60 ± 0.94	15.88 ± 2.91c	0.25 ± 0.06b
	S-ABA 400 (7 DAV)	1.52 ± 0.02b	1.52 ± 0.50b	21.58 ± 1.82b	1.00 ± 0.13	46.40 ± 1.86b	5.35 ± 1.58a
	S-ABA 400 (7 DAV) + 400 (21 DAV)	1.65 ± 0.01a	3.11 ± 0.62a	29.70 ± 1.19a	0.93 ± 0.23	54.47 ± 2.36a	4.60 ± 2.07a

Mean values within columns followed by different letters differ significantly at the 5% probability level according to Tukey's test. ns, non-significant. Control, no S-ABA application; 400, S-ABA 400 mg/L 7 days after véraison (DAV); 400 + 400, S-ABA 400 mg/L at 7 DAV + 400 mg/L S-ABA at 21 DAV. –, not detected.

dihydroxylated precursors of red anthocyanins in grape skin, whereas delphinidin, petunidin, and malvidin are trihydroxylated precursors of blue and purple anthocyanins (Liang et al., 2009; Azuma et al., 2015). Accumulation of individual anthocyanins in grapes may be induced by *S*-ABA application and varies with the cultivar. In "Noble" and "Alachua" muscadine grapes, application of *S*-ABA during véraison and again at 8 DAV for "Noble" or again at 13 DAV for "Alachua" resulted in higher levels of accumulation of all evaluated anthocyanins in "Noble" grapes but not in "Alachua" grapes, which only presented higher accumulation of peonidin-3-diglucoside compared to the control (Sandhu et al., 2011). Changes in the proportions of individual anthocyanins resulting from *S*-ABA application were also observed in "Cabernet Sauvignon" grapes, both in berries and in wine (González et al., 2012). In "Isabel" grapes, application of *S*-ABA increased the accumulation of individual anthocyanins both in must and in processed whole juice (Yamamoto et al., 2015). Similar results were observed in wines prepared from "Merlot" grapes treated with a racemic mixture of ABA (*S*-ABA and *R*-ABA). This treatment resulted in changes in the proportions of anthocyanins, increased total phenol and flavonol content, and increased antioxidant activity (Koyama et al., 2014). However, it should be considered that application of racemic mixtures of enantiomers may result in a range of plant responses because *R*-ABA is not found in plants and is less active and less effective than *S*-ABA. The two enantiomeric forms may have different effects on gene expression and on physiological responses (Lacampagne et al., 2010; Zhang, 2014).

Anthocyanin accumulation in grape berries during véraison is probably triggered by increased sugar and ABA concentrations in the berry skin, which activate the expression of genes involved in anthocyanin biosynthesis (Castellarin et al., 2015). The activation threshold for genes involved in anthocyanin production was reported to be between 9 and 10°Bx (Keller, 2015). *S*-ABA application at 7 DAV, when anthocyanin biosynthetic genes are normally induced, followed by a second application at 21 DAV, when endogenous ABA concentrations are close to maximal or are beginning to decrease, can upregulate their expression even further or maintain them at a constant level for a longer period of time.

Anthocyanins are produced through multiple pathways that are controlled by MYB transcription factors. These transcription factors are responsive to ABA and are associated with the regulation of the biosynthetic genes *CHI*, *F3H*, *DFR*, *LDOX*, and *UFGT* (Yu et al., 2012; Oglesby et al., 2016; Olivares et al., 2017). The transcription factors *VvMYBA1* and *VvMYBA2* activate anthocyanin biosynthesis in grapevines and are not functional in white grape cultivars (Rinaldo et al., 2015). Transcription factors affect the ratio of tri-/dihydroxylated anthocyanins through trans-regulation of flavonoid 3-hydroxylase (*F3′H*) and flavonoid 3′5′-hydroxylase (*F3′,5′H*) gene expression (Owen et al., 2009). During anthocyanin biosynthesis, F3H is responsible for the hydroxylation of naringenin at position 3′, generating dihydrokaempferol, a dihydroflavonol that can be hydrolyzed at position 3′ or 5′ of the B-ring by the enzymes F3′H or F3′,5′H, which are responsible for the hydroxylation of the B-ring of flavonoids. F3′H activity promotes accumulation

of the cyanidin and peonidin anthocyanin groups, whereas F3′,5′H activity results in the production of delphinidin and its derivatives petunidin and malvidin. These two enzymes compete in controlling di- and trihydroxylated anthocyanin synthesis (Roubelakis-Angelakis, 2009). In our study, treatment of hybrid grapes with two applications of 400 mg/L *S*-ABA primarily favored the accumulation of delphinidin-3-glucoside and malvidin-3-glucoside (**Table 4**); therefore, such treatment decreased the difference between the concentrations of di- and trihydroxylated anthocyanins in the grapes. This is consistent with previous results obtained for "Aki Queen" grapes (*Vitis labruscana*), in which the application of *S*-ABA stimulated the gene expression of *F3′,5′H* relative to *F3′H*. In addition, the concentrations of petunidin and malvidin increased in the berries, thereby increasing the proportion of trihydroxylated anthocyanins and decreasing the proportions of cyanidin and peonidin anthocyanins relative to the total anthocyanins (Katayama-Ikegami et al., 2016). In this study, the expression of the main enzymes leading to anthocyanin biosynthesis were analyzed. Future experiments to study changes in expression of F3′H and F3′,5′H encoding genes are still required to gain a better insight into the impact of exogenous ABA applications on the differential accumulation of specific anthocyanins.

Our results indicate that application of *S*-ABA increased the expression of the *UFGT* gene and the transcription factors at 28 DAV, but this was not observed for the treatment with only one *S*-ABA application. Anthocyanin accumulation begins when all genes involved in the biosynthetic pathway are induced, especially *UFGT* (Davies et al., 1997). Anthocyanidins (anthocyanins lacking sugar moieties) are unstable and are easily degraded to colorless compounds; therefore, before anthocyanins are transported, they must be stabilized by the addition of a glucose residue at position 3 of the C-ring (Castellarin et al., 2007). The enzyme UFGT catalyzes the final step of anthocyanin biosynthesis, therefore UFGT has been considered by many authors to be a critical enzyme in anthocyanin biosynthesis (Davies et al., 1997).

Temporary stimulation of gene transcription is believed to be related to a decrease in *S*-ABA concentration over time. In 'Crimson Seedless' grapes, a constant decrease in *S*-ABA levels with a half-life time of 14.7 days was observed in treated grape berries (Ferrara et al., 2013). The natural decrease in ABA concentration, along with the decrease in *S*-ABA levels, may, therefore, lead to decreased activity of some genes, depending on the *S*-ABA concentration in the plant. Expression of the *UFGT* gene increased considerably 7 days after *S*-ABA application in 'Crimson Seedless' grapes but decreased 3 weeks after treatment, becoming similar to the control (Koyama et al., 2010). In "Cabernet Sauvignon" grapes treated with ±*cis*, *trans*-ABA, expression analysis of anthocyanin biosynthetic genes revealed that the maximum expression levels were only reached 10–17 days after application and that they then rapidly decreased (Lacampagne et al., 2010). ABA *cis*- and *trans*-isomers differ in the orientation of the carboxyl group at carbon 2. Only the ABA *cis*-isomer is biologically active, and it accounts for almost all of the ABA produced in plant tissues. However, unlike the *S* and *R* enantiomers, the *cis*- and *trans*-isomers can be interconverted in plant tissue (Keller, 2015).

Most of the studies on *S*-ABA involved *V. vinifera* cultivars were done in temperate zones and testing a single application (Peppi et al., 2006, 2008; Koyama et al., 2010; Ferrara et al., 2015; Zhu et al., 2016). In this study, we evaluated the response of a new *V. vinifera* × *V. labrusca* hybrid grape cultivar grown in a subtropical area to multiple *S*-ABA applications. This hybrid often shows lack of color development; therefore, our results confirm the effectiveness of *S*-ABA to improve the color of ripening berries, even under warm climate conditions. The application of *S*-ABA to berries of the seedless grape Selection 21 increased the total anthocyanin concentration, changed the proportion of individual anthocyanins, improved their color attributes, and increased the expression of transcription factors and anthocyanin biosynthetic genes. Two applications of 400 mg/L *S*-ABA, at 7 and 21 DAV, resulted in the best results in terms of color increment and total anthocyanin concentration, favored the accumulation of trihydroxylated anthocyanins, and increased the expression of transcription factors and of the genes *F3H* and *UFGT*.

These results not only show that *S*-ABA is a valuable tool for improving the color of red grapes in warm areas, where color deficiency is frequently observed, but also suggest that *S*-ABA may be useful in grape breeding programs by permitting the selection and release of new cultivars with natural poor color, but other desirable characteristics such as high yield and resistance to common diseases.

CONCLUSION

Exogenous application of *S*-ABA increased the total anthocyanin accumulation in the berry skin of hybrid grapes. Two applications of 400 mg/L *S*-ABA, at 7 and 21 DAV, resulted in increased concentrations of the anthocyanins delphinidin-3-glucoside, cyanidin-3-glucoside, peonidin-3-glucoside, and malvidin-3-glucoside, increased expression of the biosynthetic genes *CHI*, *F3H*, *DFR*, and *UFGT*, and increased expression of the transcription factors *VvMYBA1* and *VvMYBA2*.

AUTHOR CONTRIBUTIONS

RK, SR, RdS, AW, DC, MF, and BB-U conceived and designed the experiments. RK, SR, and WB performed the experiments. RK, SR, DC, MF, and BB-U wrote the manuscript. RK, MA, WB, and BB-U analyzed the data. All authors have read and approved the final version of the manuscript.

FUNDING

This work was supported by the Coordination for the Improvement of Higher Education Personnel (CAPES) and National Council for Scientific and Technological Development (CNPq), Brazil.

REFERENCES

Azuma, A., Ban, Y., Sato, A., Kono, A., Shiraishi, M., Yakushiji, H., et al. (2015). MYB diplotypes at the color locus affect the ratios of tri/di-hydroxylated and methylated/non-methylated anthocyanins in grape berry skin. *Tree Genet. Genomes* 11:31. doi: 10.1007/s11295-015-0855-0

Balint, G., and Reynolds, A. G. (2013). Impact of exogenous Abscisic acid on vine physiology and grape composition of Cabernet Sauvignon. *Am. J. Enol. Vitic.* 64, 74–87. doi: 10.5344/ajev.2012.12075

Batista, P. F., Lima, M. A. C., Leão, P. S., Souza, F. F., and Alves, R. E. (2015). Divergência genética entre variedades de videiras do Banco Ativo de Germoplasma da Embrapa Semiárido. *Rev. Cienc. Agron.* 46, 800–808. doi: 10.5935/1806-6690.20150068

Berli, F. J., Fanzone, M., Piccoli, P., and Bottini, R. (2011). Solar UV-B and ABA are involved in phenol metabolism of *Vitis vinifera* L. Increasing biosynthesis of berry skin polyphenols. *J. Agric. Food Chem.* 59, 4874–4884. doi: 10.1021/jf200040z

Bhering, S. B., and Santos, H. G. (2008). *Mapa de Solos do Estado do Paraná: Legenda Atualizada*. Rio de Janeiro: IAPAR.

Biasoto, A. C. T., Netto, F. M., Marques, E. J. N., and da Silva, M. A. A. P. (2014). Acceptability and preference drivers of red wines produced from *Vitis labrusca* and hybrid grapes. *Food Res. Int.* 62, 456–466. doi: 10.1016/j.foodres.2014.03.052

Blanco-Ulate, B., Vincenti, E., Powell, A. L., and Cantu, D. (2013). Tomato transcriptome and mutant analyses suggest a role for plant stress hormones in the interaction between fruit and *Botrytis cinerea*. *Front. Plant Sci.* 4:e142. doi: 10.3389/fpls.2013.00142

Borges, R. D. S., Roberto, S. R., Yamashita, F., Olivato, J. B., and Assis, A. M. D. (2012). Sensibilidade ao rachamento de bagas das videiras 'Concord', 'Isabel' e 'BRS Rúbea'. *Rev. Bras. Frutic.* 34, 814–822. doi: 10.1590/S0100-29452012000300022

Bucic-Kojic, A., Planinić, M., Tomas, S., Bilić, M., and Velić, D. (2007). Study of solid-liquid extraction kinetics of total polyphenols from grapes seeds. *J. Food Eng.* 86, 236–242. doi: 10.1016/j.jfoodeng.2006.10.027

Cantín, C. M., Fidelibus, M. W., and Crisosto, C. H. (2007). Application of abscisic acid (ABA) at veraison véraison advanced red color development and maintained postharvest quality of 'Crimson Seedless' grapes. *Postharvest Biol. Technol.* 46, 237–241. doi: 10.1016/j.postharvbio.2007.05.017

Carreño, J., Martínez, A., Almela, L., and Fernández-López, J. A. (1995). Proposal of an index for the objective evaluation of the colour of red table grapes. *Food Res. Int.* 28, 373–377. doi: 10.1016/0963-9969(95)00008-A

Castellarin, S. D., Gambetta, G. A., Wada, H., Krasnow, M. N., Cramer, G. R., Peterlunger, E., et al. (2015). Characterization of major ripening events during softening in grape: turgor, sugar accumulation, abscisic acid metabolism, colour development, and their relationship with growth. *J. Exp. Bot.* 67, 709–722. doi: 10.1093/jxb/erv483

Castellarin, S. D., Pfeiffer, A., Sivilotti, P., Degan, M., Peterlunger, E., and Di Gaspero, G. (2007). Transcriptional regulation of anthocyanin biosynthesis in ripening fruits of grapevine under seasonal water deficit. *Plant Cell Environ.* 30, 1381–1399. doi: 10.1111/j.1365-3040.2007.01716.x

Chassy, A. W., Adams, D. O., Laurie, V. F., and Waterhouse, A. L. (2012). Tracing phenolic biosynthesis in *Vitis vinifera* via *in situ* C-13 labeling and liquid chromatography-diode array detector-mass spectrometer/mass spectrometer detection. *Anal. Chim. Acta* 747, 51–57. doi: 10.1016/j.aca.2012.08.012

Davies, C., Boss, P. K., and Robinson, S. P. (1997). Treatment of grape berries, a nonclimacteric fruit with a synthetic auxin, retards ripening and alters the expression of developmentally regulated genes. *Plant Physiol.* 115, 1155–1161. doi: 10.1104/pp.115.3.1155

Ferrara, G., Mazzeo, A., Matarrese, A. M. S., Pacucci, C., Pacifico, A., Gambacorta, G., et al. (2013). Application of abscisic acid (*S*-ABA) to 'Crimson Seedless' grape berries in a Mediterranean climate: effects on color, chemical characteristics, metabolic profile, and *S*-ABA concentration. *J. Plant Growth Regul.* 32, 491–505. doi: 10.1007/s00344-012-9316-2

Ferrara, G., Mazzeo, A., Matarrese, A. M. S., Pacucci, C., Punzi, R., Faccia, M., et al. (2015). Application of abscisic acid (*S*-ABA) and sucrose to improve colour, anthocyanin content and antioxidant activity of cv. Crimson Seedless grape berries. *J. Grape Wine Res.* 21, 18–29. doi: 10.1111/ajgw.12112

Food and Agriculture Organization [Fao]. (2018). *FAOSTAT. Resource Database, Crops*. Available at: http://www.fao.org/faostat/en/#data/QC [accessed February 19, 2018].

Gambetta, G. A., Matthews, M. A., Shaghasi, T. H., Mcelrone, A. J., and Castellarin, S. D. (2010). Sugar and abscisic acid signaling orthologs are activated at the onset of ripening in grape. *Planta* 232, 219–234. doi: 10.1007/s00425-010-1165-2

Giribaldi, M., Hartung, W., and Schubert, A. (2010). The effects of abscisic acid on grape berry ripening are affected by the timing of treatment. *J. Int. Vine Wine Sci.* 44, 9–15.

González, Á. S., Olea, P., Bordeu, E., Alcalde, J. Á., and Gény, L. (2012). S-abscisic acid, 2-chloroethylphosphonic acid and indole-3-acetic acid treatments modify grape (*Vitis vinifera* L. 'Cabernet Sauvignon') hormonal balance and wine quality. *Vitis J. Grapevine Res.* 51, 45–52.

He, F., Mu, L., Yan, G. L., Liang, N. N., Pan, Q. H., Wang, J., et al. (2010). Biosynthesis of anthocyanins and their regulation in colored grapes. *Molecules* 15, 9057–9091. doi: 10.3390/molecules15129057

Jensen, F. L., Kissler, J. J., Peacock, W. L., and Leavitt, G. M. (1975). Effect of ethephon on color and fruit characteristics of 'Tokay' and 'Emperor' table grapes. *Am. J. Enol. Vitic.* 26, 79–81.

Jeong, S. T., Goto-Yamamoto, N., Kobayashi, S., and Esaka, M. (2004). Effects of plant hormones and shading on the accumulation of anthocyanins and the expression of anthocyanin biosynthetic genes in grape berry skins. *Plant Sci.* 167, 247–252. doi: 10.1016/j.plantsci.2004.03.021

Katayama-Ikegami, A., Sakamoto, T., Shibuya, K., and Katayama, T. (2016). Effects of abscisic acid treatment on berry coloration and expression of flavonoid biosynthesis genes in grape. *Am. J. Plant Sci.* 7, 1325–1336. doi: 10.4236/ajps.2016.79127

Keller, M. (2015). *The Science of Grapevines: Anatomy and Physiology*, 2nd Edn. Amsterdam: Elsevier.

Koyama, K., Sadamatsu, K., and Goto-Yamamoto, N. (2010). Abscisic acid stimulated ripening and gene expression in berry skins of the Cabernet Sauvignon grape. *Funct. Integr. Genomics* 10, 367–381. doi: 10.1007/s10142-009-0145-8

Koyama, R., Assis, A. M., Yamamoto, L. Y., Borges, W. F., Sá Borges, R., and Prudêncio, S. H. (2014). Exogenous abscisic acid increases the anthocyanin concentration of berry and juice from 'Isabel' grapes (*Vitis labrusca* L.). *HortScience* 49, 460–464.

Kuhn, N., Guan, L., Dai, Z. W., Wu, B. H., Lauvergeat, V., Gomès, E., et al. (2013). Berry ripening: recently heard through the grapevine. *J. Exp. Bot.* 65, 4543–4559. doi: 10.1093/jxb/ert395

Lacampagne, S., Gagné, S., and Gény, L. (2010). Involvement of abscisic acid in controlling the proanthocyanidin biosynthesis pathway in grape skin: new elements regarding the regulation of tannin composition and Leucoanthocyanidin Reductase (LAR) and Anthocyanidin Reductase (ANR) activities and express. *J. Plant Growth Regul.* 29, 81–90. doi: 10.1007/s00344-009-9115-6

Leão, P. C. S., Lima, M. A. C., Costa, J. P. D., and Trindade, D. C. G. (2015). Abscisic acid and ethephon for improving red color and quality of crimson seedless grapes grown in a tropical region. *Am. J. Enol. Vitic.* 66, 37–45. doi: 10.5344/ajev.2014.14041

Liang, Z., Yang, C., Yang, J., Wu, B., Wang, L., Cheng, J., et al. (2009). Inheritance of anthocyanins in berries of *Vitis vinifera* grapes. *Euphytica* 167, 113–125. doi: 10.1007/s10681-008-9868-1

Lurie, S., Ovadia, R., Nissim-Levi, A., Oren-Shamir, M., Kaplunov, T., Zutahy, Y., et al. (2009). Abscisic acid improves colour development in 'Crimson Seedless' grapes in the vineyard and on detached berries. *J. Hortic. Sci. Biotechnol.* 84, 639–644. doi: 10.1080/14620316.2009.11512579

Oglesby, L., Ananga, A., Obuya, J., Ochieng, J., Cebert, E., and Tsolova, V. (2016). Anthocyanin accumulation in muscadine berry skins is influenced by the expression of the MYB Transcription factors, *MybA1*, and *MYBCS1*. *Antioxidants* 5:35. doi: 10.3390/antiox5040035

Olivares, D., Contreras, C., Muñoz, V., Rivera, S., González-Agüero, M., Retamales, J., et al. (2017). Relationship among color development, anthocyanin and pigment-related gene expression in 'Crimson Seedless' grapes treated with abscisic acid and sucrose. *Plant Physiol. Biochem.* 115, 286–297. doi: 10.1016/j.plaphy.2017.04.007

Owen, S. J., Lafond, M. D., Bowen, P., Bogdanoff, C., Usher, K., and Abrams, S. R. (2009). Profiles of abscisic acid and its catabolites in developing Merlot grape (*Vitis vinifera*) berries. *Am. J. Enol. Vitic.* 60, 277–284.

Peppi, M. C., Fidelibus, M. W., and Dokoozlian, N. (2006). Abscisic acid application timing and concentration affect firmness, pigmentation, and color of 'Flame Seedless' grapes. *HortScience* 41, 1440–1445.

Peppi, M. C., Fidelibus, M. W., and Dokoozlian, N. K. (2007). Application timing and concentration of abscisic acid affect the quality of 'Redglobe' grapes. *J. Hortic. Sci. Biotechnol.* 82, 304–310. doi: 10.1080/14620316.2007.11512233

Peppi, M. C., Walker, M. A., and Fidelibus, M. W. (2008). Application of abscisic acid rapidly upregulated UFGT gene expression and improved color of grape berries. *Vitis J. Grapevine Res.* 47, 11–14.

Reynolds, A., Robbins, N., Lee, H. S., and Kotsaki, E. (2016). Impacts and interactions of abscisic acid and gibberellic acid on sovereign Coronation and Skookum seedless table grapes. *Am. J. Enol. Vitic.* 67, 327–338. doi: 10.5344/ajev.2016.15108

Rinaldo, A. R., Cavallini, E., Jia, Y., Moss, S. M. A., Mcdavid, D. A. J., Hooper, L. C., et al. (2015). A grapevine anthocyanin acyltransferase, transcriptionally regulated by VvMYBA, can produce most acylated anthocyanins present in grape skins. *Plant Physiol.* 169, 1897–1916. doi: 10.1104/pp.15.01255

Roberto, S. R., Assis, A. M., Yamamoto, L. Y., Miotto, L. C. V., Koyama, R., Sato, A. J., et al. (2013). Ethephon use and application timing of abscisic acid for improving color of 'Rubi' table grape. *Pesqui. Agropecu. Bras.* 48, 797–800. doi: 10.1590/S0100-204X2013000700013

Roberto, S. R., Assis, A. M., Yamamoto, L. Y., Miotto, L. C. V., Sato, A. J., Koyama, R., et al. (2012). Application timing and concentration of abscisic acid improve color of 'Benitaka' table grape. *Sci. Hortic.* 142, 44–48. doi: 10.1016/j.scienta.2012.04.028

Roubelakis-Angelakis, K. A. (2009). *Grapevine Molecular Physiology and Biotechnology*, 2nd Edn. Dordrecht: Springer. doi: 10.1007/978-90-481-2305-6

Rybka, A. C. P., Freitas, S. T., Netto, A. F., and Biasoto, A. C. T. (2015). Central composite rotatable design approach to optimize 'Italia' raisin drying conditions. *Comun. Sci.* 6, 454–462. doi: 10.14295/cs.v6i4.993

Sandhu, A. K., Gray, D. J., Lu, J., and Gu, L. (2011). Effects of exogenous abscisic acid on antioxidant capacities, anthocyanins, and flavonol contents of *Muscadine grape* (*Vitis rotundifolia*) skins. *Food Chem.* 126, 982–988. doi: 10.1016/j.foodchem.2010.11.105

Thomas, T. R., Shackel, K. A., and Matthews, M. A. (2008). Mesocarp cell turgor in *Vitis vinifera* L. berries throughout development and its relation to firmness, growth, and the onset of ripening. *Planta* 228, 1067–1076. doi: 10.1007/s00425-008-0808-z

Villalobos-González, L., Peña-Neira, A., Ibáñez, F., and Pastenes, C. (2016). Long-term effects of abscisic acid (ABA) on the grape berry phenylpropanoid pathway: gene expression and metabolite content. *Plant Physiol. Biochem.* 105, 213–223. doi: 10.1016/j.plaphy.2016.04.012

Wada, H., Shackel, K. A., and Matthews, M. A. (2008). Fruit ripening in *Vitis vinifera*: apoplastic solute accumulation accounts for pre-veraison véraison turgor loss in berries. *Planta* 227, 1351–1361. doi: 10.1007/s00425-008-0707-3

Wheeler, S., Loveys, B., Ford, C., and Davies, C. (2009). The relationship between the expression of abscisic acid biosynthesis genes, accumulation of abscisic acid and the promotion of *Vitis vinifera* L. berry ripening by abscisic acid. *J. Grape Wine Res.* 15, 195–204. doi: 10.1111/j.1755-0238.2008.00045.x

Yamamoto, L. Y., Assis, A. M., Roberto, S. R., Bovolenta, Y. R., Nixdorf, S. L., García-Romero, E., et al. (2015). Application of abscisic acid (*S*-ABA) to cv. Isabel grapes (*Vitis vinifera* × *Vitis labrusca*) for color improvement: effects on color, phenolic composition and antioxidant capacity of their grape juice. *Food Res. Int.* 77, 572–583. doi: 10.1016/j.foodres.2015.10.019

Yang, F. W., and Feng, X. Q. (2015). Abscisic acid biosynthesis and catabolism and their regulation roles in fruit ripening. *Phyton* 84, 444–453.

Ye, J., Coulouris, G., Zaretskaya, I., Cutcutache, I., Rozen, S., and Madden, T. L. (2012). Primer-BLAST: a tool to design target-specific primers for polymerase chain reaction. *BMC Bioinformatics* 13:134. doi: 10.1186/1471-2105-13-134

Yu, M., Liu, H. F., and Wang, J. (2012). Effects of ABA on expression of genes related to anthocyanin biosynthesis in grapevine. *J. Fruit Sci.* 29, 29–35.

Zhang, D. P. (2014). *Abscisic Acid: Metabolism, Transport and Signaling*, 1st Edn. Dordrecht: Springer.

Zhang, N., Sun, Q., Li, H., Li, X., Cao, Y., Zhang, H., et al. (2016). Melatonin improved anthocyanin accumulation by regulating gene expressions and resulted in high reactive oxygen species scavenging capacity in cabbage. *Front. Plant Sci.* 7:197. doi: 10.3389/fpls.2016.00197

Zhu, L., Zhang, Y., Zhang, W., and Lu, J. (2016). Effects of exogenous abscisic acid on phenolic characteristics of red *Vitis vinifera* grapes and wines. *Food Sci. Biotechnol.* 25, 361–370. doi: 10.1007/s10068-016-0051-5

Conflict of Interest Statement: The authors declare that the research was conducted in the absence of any commercial or financial relationships that could be construed as a potential conflict of interest.

frontiers in Plant Science

ORIGINAL RESEARCH
published: 06 April 2018
doi: 10.3389/fpls.2018.00363

Exogenous Applications of Brassinosteroids Improve Color of Red Table Grape (*Vitis vinifera* L. Cv. "Redglobe") Berries

*Alexis E. Vergara[1], Katy Díaz[2], Rodrigo Carvajal[2], Luis Espinoza[2], José A. Alcalde[1] and Alonso G. Pérez-Donoso[1]**

[1] Departamento de Fruticultura y Enología, Facultad de Agronomía e Ingeniería Forestal, Pontificia Universidad Católica de Chile, Santiago, Chile, [2] Departamento de Química, Universidad Técnica Federico Santa María, Valparaíso, Chile

Color and other quality parameters of "Redglobe" grape (*Vitis vinifera* L.) berries were evaluated after treatment with brassinosteroid (BR) analogs. Three BRs analogs (24-epibrassinolide, Triol, or Lactone) were applied at three concentrations (0.0, 0.4, or 0.8 mg·L^{-1}), at the onset of veraison. A commercial formulation (B-2000®) was also applied, at a recommended rate of 0.06 mg·L^{-1}. The tested BR analogs were effective improving berry color (evaluated as color index for red grapes, CIRG), increasing the levels of soluble solids and anthocyanins, and changing the types of anthocyanins present without altering other quality and yield parameters. The effects of BR analogs on color enhancement could be explained by an increase in soluble solids content and/or anthocyanin content. Treatment with 24-epibrassinolide (at 0.4 mg·L^{-1}) or the commercial formulation tended to favor the production of dihydroxylated anthocyanins, which are responsible for the red and pink colors of grape berries. Results indicate that the use of BRs constitutes a potential tool in the production of table grapes. This is the first report of this enhancement effect in a productive context.

Keywords: anthocyanins, berry color, brassinosteroids, color index for red grapes (CIRG), quality

OPEN ACCESS

Edited by:
Giuseppe Colla, Università degli Studi della Tuscia, Italy

Reviewed by:
Zhanwu Dai, Institut National de la Recherche Agronomique (INRA), France
Sergio Mugnai, Erasmus University Rotterdam, Netherlands

****Correspondence:***
Alonso G. Pérez-Donoso
agperez@uc.cl

Specialty section:
This article was submitted to Crop and Product Physiology, a section of the journal Frontiers in Plant Science

Received: *11 December 2017*
Accepted: *05 March 2018*
Published: *06 April 2018*

Citation:
Vergara AE, Díaz K, Carvajal R, Espinoza L, Alcalde JA and Pérez-Donoso AG (2018) Exogenous Applications of Brassinosteroids Improve Color of Red Table Grape (Vitis vinifera L. Cv. "Redglobe") Berries. Front. Plant Sci. 9:363. doi: 10.3389/fpls.2018.00363

INTRODUCTION

The assessment of table grape quality is traditionally based on sensory attributes such as flavor, aroma, texture, and color. Numerous studies together with demographic observations have demonstrated the importance of color as the main quality indicator on which consumer acceptance of fruit is based (Clydesdale, 1993).

Often, minimum quality standards can be obtained by performing good management practices in the vineyard (Cameron, 1994; Schrader et al., 1994), but in the case of grape varieties such as "Redglobe," which has large-sized berries and clusters, these practices can easily lead to overproduction. Under these conditions, "Redglobe" berries fail to develop suitable red color (Kliewer and Weaver, 1971; Schrader et al., 1994; Kliewer and Dokoozlian, 2005). In addition, the production of table grapes in Chile is conducted in areas with warm climatic and/or microclimatic conditions. These high temperature conditions inhibit the accumulation of anthocyanins (Mori et al., 2005), negatively affecting the development of berry color (Spayd et al., 2002; Mori et al., 2005; Kuhn et al., 2014). Thus, a series of techniques that tend to correct and/or prevent these problems have been implemented. These techniques include the use of growth regulators such as ethephon and/or abscisic acid (ABA) during veraison; these techniques have been widely

used on varieties such as "Flame Seedless" and "Redglobe." However, softening problems have been reported in "Redglobe" berries as a consequence of ethephon and ABA applications, especially at higher doses. In addition, the results of such treatments are not consistent between growing seasons, and these treatments can lead to the expression of darker colors in berries, which renders "Redglobe" berries less attractive in their target markets (Peppi et al., 2006, 2007; Peppi and Fidelibus, 2008). The side effects associated with the use of ethephon and/or ABA can be reduced by the use of lower doses of these compounds; however, those lower doses are also less effective at increasing the anthocyanin content (Peppi et al., 2007) and berry color.

From this point of view, the alternatives to growth regulators for preventing and/or correcting color development problems appear to be limited. Therefore, new growth regulators must be identified and characterized for inclusion in management programs. In this sense, brassinosteroids (BRs) are associated with the ripening of berries (Symons et al., 2006), and exogenous application of 24-epibrassinolide (a BR analog) increases the accumulation of phenolic compounds (Luan et al., 2013; Xi et al., 2013) such as anthocyanins, which are secondary metabolites that determine the color of the berries.

Brassinosteroids represent a group of hormones first isolated from pollen extracts of *Brassica napus* L. (Mitchell et al., 1970). The isolation of brassinolide, the most active of these hormones (Grove et al., 1979), and the identification of its receptor (Wang et al., 2001) made it possible to study this hormone in various species, including grape species. Symons et al. (2006) reported that BRs are involved in the ripening of "Cabernet Sauvignon" berries. Recent studies have shown that BRs are involved in the accumulation of sugar during the ripening of "Cabernet Sauvignon" (Xu et al., 2015). On the other hand, exogenous applications of 24-epibrassinolide during veraison are effective at increasing sugar accumulations, reducing total acidity at harvest and significantly increasing the total anthocyanin content in "Cabernet Sauvignon" (Luan et al., 2013; Xi et al., 2013). Although the observed increase in total anthocyanins may be associated with an increase in the color of grape berries, this phenomenon has not been proven in any table grape variety, especially red ones, for which berry color is one of the main attributes of quality (Clydesdale, 1993). In addition, all studies related to the ripening of berries and to the effect of BRs on ripening have used only one analog (24-epibrassinolide), limiting the understanding and scope that growth regulators analogous to BRs may have within a productive context. Therefore, the objective of the present work was to evaluate the effects of the exogenous applications of different BR analogs on the development and final quality of "Redglobe" berries.

MATERIALS AND METHODS

Plant Material and Experimental Conditions

The experiments were carried out during 2014–2015 and 2015–2016 growing seasons in a commercial vineyard located in Santa María (Aconcagua Province, Valparaíso Region, Chile; 32°43′00.9"S 70°37′56.7"W). The climate corresponding to the study site is Mediterranean, which consists of a dry season during the summer (from December to February).

Sixteen-year-old self-rooted "Redglobe" grapevines (*Vitis vinifera* L.) of similar vigor, health, and fruit load (38 ± 1.1 clusters·plant^{-1}) were used in the study. The vines were spaced 3.5 m within rows and 3.5 m between rows, and the rows were oriented in southwest–northeastern direction. The vines were trained on an overhead trellis (parronal español) with four main arms and were pruned on a spur-cane system; each cane contained six to seven buds. In both seasons plants were under commercial management and subjected to plant growth regulator application program showing in **Table 1**. However, the plants used in the experiments did not receive the application of ethephon, thus avoiding its interference on the development of fruit color.

Treatments

In the season 2014–2015 three analogs of BRs, 24-epibrassinolide (E; Phyto Technology Laboratories®, United States), 3α-22(*S*), 23-trihydroxy-24-nor-5α-cholan-6-one (Triol; T), and 3α-hydroxy-20-R-B-homo-7-oxa-5α-cholestan-6-one (Lactone; L) at concentrations of 0.0 (control), 0.4, and 0.8 mg·L^{-1} as well as a commercial formulation of BRs, B-2000® (B-2000; IONA, Chile), at concentration of 0.06 mg·L^{-1} (equivalent to 60 mg·ha^{-1} using 1000 liters of solution per ha, i.e., dose recommended by manufacturer) were applied with a backpack sprayer to "Redglobe" clusters during berry softening at the beginning of veraison (January 7, 2015) at a rate of approximately 1.2 L·plant^{-1} until runoff, ensuring that all berry surfaces were covered. The details of the treatments are shown in **Table 2**, and the chemical structures of the analogs are shown in **Figure 1**. The side chain of the structure of the analog Lactone is not shown, as it may be patented and/or protected by copyright; hence, the side chain was replaced with an R.

The solutions of BR analogs were prepared by dissolving 4 or 8 mg in 100 mL of 98% ethanol (v/v) and bringing each solution to a final volume of 10 L with water. The control solution was prepared adding water to 100 mL of 98% ethanol (v/v) until reaching 10 L, and the commercial formulation solution was prepared as specified by the manufacturer. A wetting agent (Break®, BASF, Germany) was added at a rate of 0.2 mL·L^{-1} to all solutions.

Additionally, and using the same methodology described above, at the beginning of veraison of 2015–2016 growing season (January 28, 2016), the same analogs were tested, but only at 0.4 mg·L^{-1}. The commercial formulation at 0.06 mg·L^{-1} was also tested.

Preharvest and Harvest Evaluations

The evolution of berry skin color, equatorial diameter, and soluble solids were measured using 30 berries per plant; berries were sampled from at least 10 clusters. Sampling was performed at 20-day intervals after treatment application until harvest [57 and 61 days after veraison (dav) for 2014–2015 and 2015–2016 seasons, respectively].

TABLE 1 | Detail of plant growth regulator application program in the commercial field in which the study was conducted.

Date	Objective	Commercial product	Active ingredient (ai)	Dose ($g \cdot ha^{-1}$)
06-10-14	Shoot elongation	Splendor®	Thidiazuron, 5.0% (p/v)	2.055
11-11-14	Thinning	Proggib®	Gibberellic acid, 3.2% (p/v)	1.8563
22-11-14	Berry growth*	Splendor®	Thidiazuron, 5.0% (p/v)	0.4
27-11-14	Berry growth*	Proggib®	Gibberellic acid, 3.2% (p/v)	37.4998
		Biozime TF®	Gibberellic acid, 0.036% (p/v)	0.0539
			Indoleacetic acid, 0.036% (p/v)	0.0539
			Zeatin, 0.0094% (p/v)	0.1409
02-12-14	Berry growth*	Proggib®	Gibberellic acid, 3.2% (p/v)	37.4998
22-01-15	Color uniformity**	Ethrel 48®	Ethephon, 48.0% (p/v)	239.928

**Growth regulator applications were performed with a total volume of 1000 $L \cdot ha^{-1}$. **Not applied to the plants used in this study.*

TABLE 2 | Details of the treatments applied in growing season 2014–2015 and/or 2015–2016.

Treatment	Active ingredient	Concentration ($mg \cdot L^{-1}$)
E-0.4*	24-epibrassinolide	0.4
E-0.8		0.8
T-0.4*	3α-22(*S*), 23-trihydroxy-24-nor-5α-cholan-6-one	0.4
T-0.8	(Triol)	0.8
L-0.4*	3α-hydroxy-20-R-B-homo-7-oxa-5α-cholestan-6-one	0.4
L-0.8	(Lactone)	0.8
B-2000*	(25R)-3β-5α-dihydroxy-spirostan-6-one (B-2000)	0.06
Control*		0.0

*E-0.4, 24-epibrassinolide applied at 0.4 $mg \cdot L^{-1}$; E-0.8, 24-epibrassinolide applied at 0.8 $mg \cdot L^{-1}$; T-0.4, Triol applied at 0.4 $mg \cdot L^{-1}$; T-0.8, Triol applied at 0.8 $mg \cdot L^{-1}$; L-0.4, lactone applied at 0.4 $mg \cdot L^{-1}$; L-0.8, lactone applied at 0.8 $mg \cdot L^{-1}$; B-2000, B-2000 applied at 0.06 $mg \cdot L^{-1}$; Control, control treatment. The treatments were applied to cluster at the beginning of veraison at a rate close to 1.2 $L \cdot plant^{-1}$. *Treatments evaluated in a second season (2015–2016).*

Berry skin color was evaluated using the color index for red grapes (CIRG), which is based on the CIELAB parameters L^* (lightness), H (hue angle), and C (chroma) (Carreño et al., 1995) measured with a Chroma Meter CR-400 (Konica Minolta, Japan) and is calculated as $(180 - H)/(L^* + C)$. The CIRG exhibits strong linearity with the visual color of berries and can distinguish between sample groups of different external color (Carreño et al., 1995). The CIRG was measured on 8-mm-diameter circles (50.26 mm^2) on two opposite sides of the equatorial zone of berries. For this evaluation, all berries were cleaned with a cotton cloth to remove dust and prevent irregularities in the measurements. Berry diameter at the equatorial zone was measured with a digital caliper. Finally, the juice of 10 berries was used for determining the soluble solids concentration (°Brix), using a temperature-compensated hand refractometer.

At harvest, four clusters per plant were sampled. Fifty berries were collected from each cluster and were used for the evaluation previously described (30 for berry skin color, diameter, and soluble solids); in addition, total acidity (expressed as tartaric acid), total anthocyanins, and the profiles of anthocyanin groups were evaluated using 10 whole berries pert plant.

Total acidity was measured using the mixed juice of 10 berries; juice was titrated with NaOH (0.1 N) until pH 8.2 was reached using an automatic titrator (HI 901 Tiratatro, Hanna Instrument, United States).

The total concentration [$mg \cdot g^{-1}$ fresh weight (FW)] and total content ($mg \cdot berry^{-1}$) of anthocyanins were evaluated using a spectrophotometric method described by Iland et al. (2004). Briefly, 10 whole berries were ground and macerated with 50% ethanol (v/v) at pH 2.0 for 24 h in darkness at room temperature. Afterward, the samples were centrifuged. The supernatants were recovered, and their absorbance at 520 nm was measured with a UV–visible spectrophotometer (Nanodrop 2000, Thermo Fisher, United States) to determine total anthocyanin concentrations (Iland et al., 2004).

To determine the profiles of the different groups of anthocyanins, the extracts obtained in the previous step were subjected to HPLC analysis in accordance with the methodology described by González et al. (2012). HPLC-diode-array detection (DAD) analysis was performed using a LaChrom Elite® HPLC system with a 1.024 photodiode-array detector (Hitachi LaChrom Elite, Japan). Separation was performed using a Purospher® STAR (Merck, Germany) reverse-phase C18 column (250 mm, 4.6 mm i.d., 5 μm) at 25°C; the detection was carried out at 520 nm. The elution gradient consisted of two solvents: solvent A consisted of 90:10 water:formic acid (v/v) and solvent B consisted of acetonitrile (Fanzone et al., 2010). Aliquots of 200 μL of grape extract were injected after being filtered through a 0.45-μm-pore size membrane. To determine the groups of anthocyanins [dihydroxylated (2-OH), trihydroxylated (3-OH), methylated (Met), and non-methylated (Non-met) groups], standard solutions of delphinidin-3-glucoside, petunidin-3-glucoside, malvidin-3-glucoside, cyanidin-3-glucoside, and peonidin-3-glucoside (Extrasynthese, France) were used as standards.

Experimental Design and Statistical Analysis

A randomized complete block design was used for both growing seasons. Treatments were sorted in five blocks within two rows of plants; this design left one untreated plant as a border between adjacent experimental units (a single plant with all its clusters).

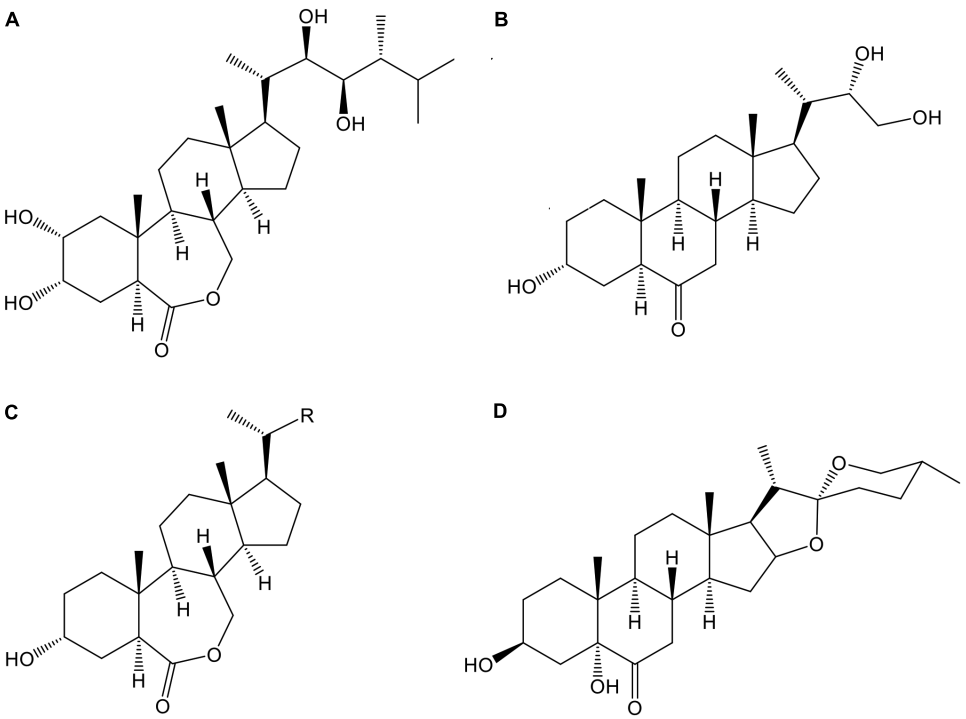


FIGURE 1 | Chemical structure of the different BR analogs used in this study: 24-epibrassinolide **(A)**, 3α-22(*S*), 23-trihydroxy-24-nor-5α-cholan-6-one (**B**, Triol), 3α-hydroxy-20-R-B-homo-7-oxa-5α-cholestan-6-one (**C**, Lactone), and 3β-5α-25(R)-dihydroxy-spirostan-6-one (**D**, commercial formulation, B-2000®).

Differences among treatment means of preharvest and harvest parameters were evaluated by the analysis of variance (ANOVA), and significant differences were subjected to the Tukey–Kramer honestly significant difference (HSD) multiple comparison test ($p \leq 0.05$). In addition, correlation analysis were performed to determine the correlation strength between the different evaluated parameters.

RESULTS

Quality Parameters

At harvest, in both seasons, no significant differences were observed in equatorial diameter, total acidity, or weight of the berries or clusters (Supplementary Tables 1, 2). However, significant differences were found in the soluble solids content of berries (**Figures 2A,B**). On average, compared with the control treatment, BR applications led to higher soluble solids content in berries. In the season 2014–2015 the soluble solids content of berries that received treatments E-0.4, B-2000, T-0.8, E-0.8, and T-0.4 were significantly different from that of the control berries, whereas treatments L-0.4 and L-0.8 did not significantly differ respect to control treatment in this parameter (**Figure 2A**). These results were consistent with those observed in the 2015–2016 season, in which the treatments E-0.4 and B-2000 presented values statistically higher than those of control treatment.

Color of Berries

In both seasons an increase in the values of CIRG was observed from the moment of the application of the treatments until the harvest (data not shown). However, significant differences in CIRG values were observed only at harvest (**Figures 3A–C**).

In the season 2014–2015, ANOVA results and subsequent multiple comparison analyses showed that treatments E-0.4, T-0.4, T-0.8, and B-2000 resulted in significantly higher CIRG values than the control treatment, whereas the remaining treatments resulted in CIRG values that were similar to those of the control treatment (**Figure 3A**). However, analysis of covariance indicated a significant effect of soluble solids content on CIRG value. Nevertheless, and considering the effect of soluble solids content, treatments E-0.4, T-0.8, and L-0.8 still had a statistically significant effect on berry color (**Figure 3B**). On the other hand, the interaction between treatment and soluble solids content with respect to the CIRG was not significant, indicating that effects of treatments on CIRG value did not vary with the level of soluble solids. Although no significant effect of soluble solids content was observed on the CIRG values in the 2015–2016 season, a significant increase in the values of CIRG was observed in treatments E-0.4, T-0.4, and B- 2000 with respect to those of control treatment (**Figure 3C**).

Total Anthocyanins and Anthocyanin Groups

The results of the statistical analysis of total concentration and content of anthocyanins at harvest revealed that in season

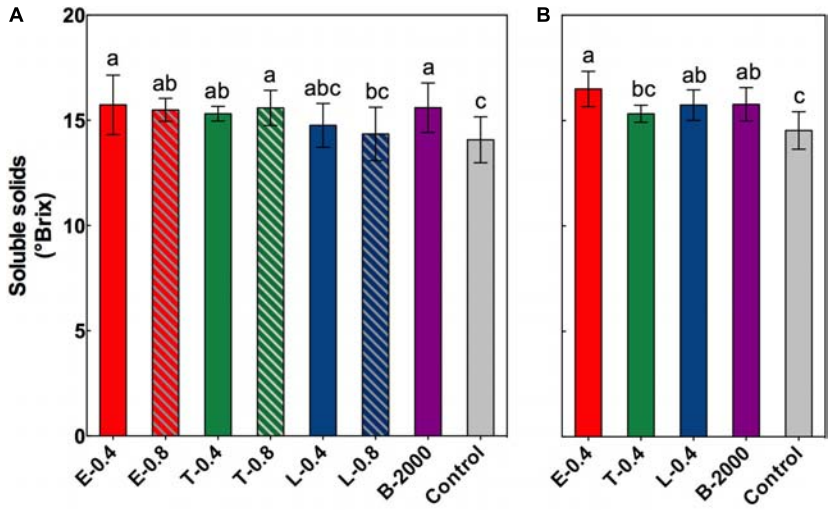


FIGURE 2 | Soluble solids content, expressed as °Brix, at harvest for the growing seasons 2014–2015 **(A)** and 2015–2016 **(B)**. Each bar indicates the mean of five replicates and error lines correspond to standard deviations. Different letters indicate significant differences at $p < 0.05$ according to the Tukey–Kramer HSD test ($n = 5$).

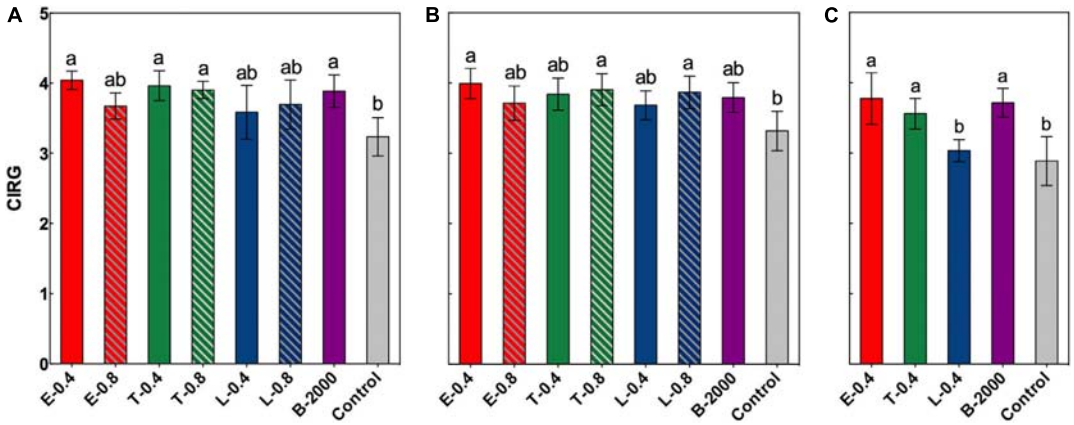


FIGURE 3 | Effect of BRs treatments on CIRG at harvest for the growing seasons 2014–2015 **(A)**, BRs effect in growing season 2014–2015 considering the soluble solids effect **(B)**, and BRs effect on CIRG at harvest in growing season 2015–2016 **(C)**. Each bar indicates the mean of five replicates and error lines correspond to standard deviations. Different letters indicate significant differences at $p < 0.05$ according to the Tukey–Kramer HSD test ($n = 5$).

2014–2015 the anthocyanin in grapes that received treatments B-2000, E-0.4, T-0.4, and L-0.8 were significantly higher than those in grapes that received the control treatment; although the other treatments (E-0.8, T-0.8, and L-0.4) resulted in average values that were higher than those of the control, these differences were not statistically significant (**Figures 4A,C**). Despite the fact that in 2015–2016 season the concentration and content of anthocyanins were lower than in the 2014–2015 season (**Figure 4**), a significant increase in the concentration and anthocyanin content was observed in the treatments E-0.4, T-0.4, and B-2000 with respect to the control treatment (**Figures 4B,D**), which is consistent with the results of the previous season.

The observed changes in total anthocyanin concentration and content were also reflected in the concentration and content of specific groups of anthocyanins (**Tables 3**, **4**). The predominant anthocyanins in all of the samples of both seasons were 2-OH anthocyanins (cyanidin-3-glucoside and peonidin-3-glucoside) rather than 3-OH anthocyanins (delphinidin-3-glucoside, petunidin-3-glucoside, and malvidin-3-glucoside). In the case of 2014–2015 season, ANOVA results revealed that all treatments yielded 2-OH anthocyanins levels that were significantly higher than those of 3-OH anthocyanins, whereas no significant difference in the levels of 2-OH and 3-OH anthocyanins was observed in the control berries (**Table 3**). Compared with the control treatment, treatments B-2000, E-0.4, T-0.4, L-0.8, and T-0.8 resulted in a higher content of 2-OH anthocyanins in berries, whereas the level of 2-OH anthocyanins in berries that received the L-0.4 treatment was the same as in berries that received the control treatment.

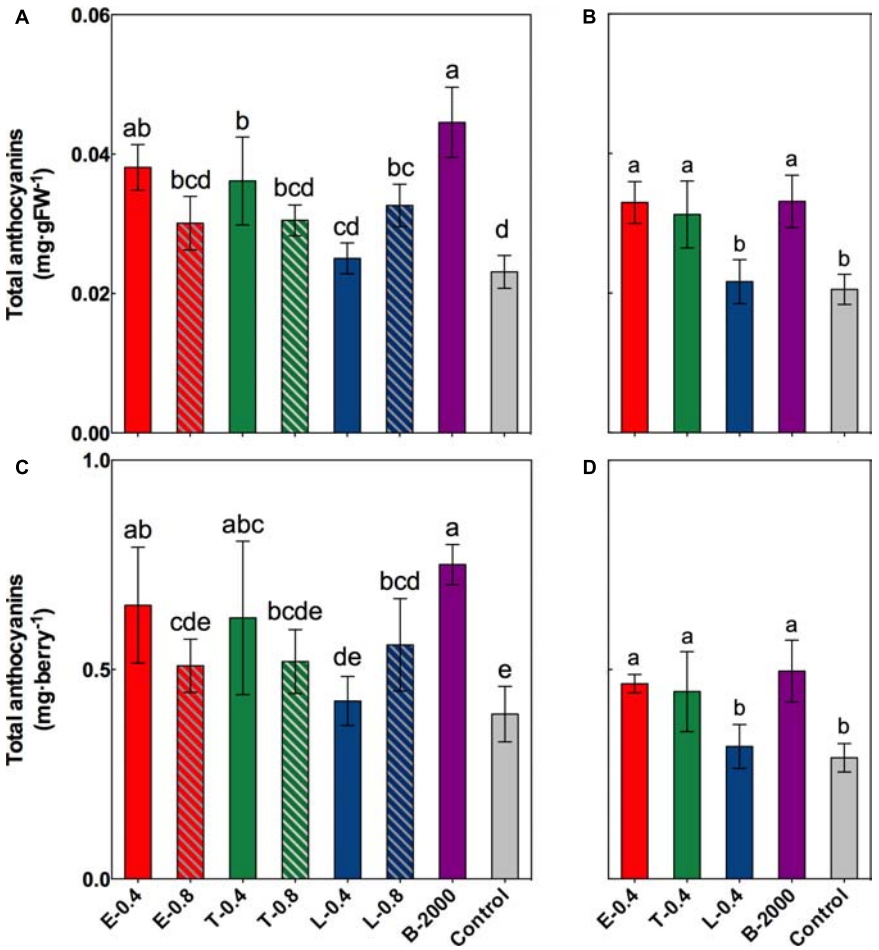


FIGURE 4 | Effect of BR treatments on the total anthocyanin content at harvest, expressed as milligram per gram of fresh weight **(A,B)** or milligram per berry **(C,D)**, for the growing seasons 2014–2015 **(A,C)** and 2015–2016 **(B,D)**. The bars indicate means of five replicates and error lines show their standard deviation. Different letters indicate significant differences at $p < 0.05$ according to the Tukey–Kramer HSD test ($n = 5$).

With respect to 3-OH anthocyanins, only treatments T-0.4, B-2000, and T-0.8 resulted in levels that were significantly higher than those from the control treatment (**Table 3**). On the other hand, in the 2015–2016 season, treatments E-0.4, T-0.4, and B-2000 exhibited statistically higher values of 2-OH anthocyanins than the control treatment; whereas L-0.4 treatment remained not different from the control treatment (**Table 4**).

In both seasons, the analysis of the 2-OH:3-OH anthocyanin ratio (2-OH·3-OH^{-1}) indicated that only treatments B-2000 and E-0.4 yielded a ratio that was significantly greater than that found in control berries (**Tables 3**, **4**).

Compared with the control treatment, in 2014–2015 season, treatments B-2000, E-0.4, T-0.4, and L-0.8 resulted in the highest levels of Met anthocyanins (peonidin-3-glucoside, petunidin-3-glucoside, and malvidin-3-glucoside); the levels resulting from the remaining treatments did not significantly differ from those from the control treatment (**Table 3**). These results were consistent with those observed in the 2015–2016 season (**Table 4**).

In the case of Non-met anthocyanins (cyanidin-3-glucoside and delphinidin-3-glucoside), in season 2014–2015, all treatments, with the exception of L-0.4, produced significantly higher values than the control treatment (**Table 3**). In relation to the ratio between these two groups of anthocyanins, only E-0.4 significantly increased compared to the control treatment, while the rest of treatments remained not different from the control treatment (**Table 3**). While in 2015–2016 season, no significant differences were observed in the content of Non-met anthocyanins, and only the B-2000 treatment presented ratio values of these two groups statistically higher than the control (**Table 4**). Correlation analyzes revealed that berry color (expressed as CIRG) observed in this work (**Figure 5**) are explained by the content and concentration (**Table 5**) of total anthocyanins. On the other hand, the correlation between the content and concentration of the different groups of anthocyanins and CIRG turned out to be significant, especially for the 2-OH anthocyanins expressed as mg·berry^{-1} (**Table 5**), while the correlation between CIRG and anthocyanins 3-OH was significant only when these were expressed as mg·g^{-1} (**Table 5**).

TABLE 3 | Effects of BR treatments on the abundance of dihydroxylated, trihydroxylated, methylated, and non-methylated anthocyanins as well as the dihydroxylated:trihydroxylated anthocyanin and the methylated:non-methylated anthocyanin ratios for season 2014–2015.

Treatments	Anthocyanins (mg·berry^{-1})				2-OH·3-OH^{-1}	Met·Non-met^{-1}
	2-OH	3-OH	Met	Non-met		
E-0.4	0.439 ± 0.1209* ab	0.214 ± 0.0296 abc	0.451 ± 0.1037* ab	0.203 ± 0.0359 b	2.052 ± 0.4687 a	2.205 ± 0.1677 a
E-0.8	0.299 ± 0.0547* cde	0.210 ± 0.0204 abc	0.319 ± 0.0396* cde	0.190 ± 0.0284 b	1.426 ± 0.2572 b	1.698 ± 0.1799 c
T-0.4	0.378 ± 0.0933* bc	0.245 ± 0.0948 ab	0.416 ± 0.1101* abc	0.208 ± 0.0761 b	1.597 ± 0.2381 ab	2.083 ± 0.3377 ab
T-0.8	0.292 ± 0.0456* cde	0.227 ± 0.0337 ab	0.310 ± 0.0377* de	0.209 ± 0.0392 ab	1.294 ± 0.1259 b	1.496 ± 0.1143 c
L-0.4	0.243 ± 0.0475* de	0.182 ± 0.0148 bc	0.267 ± 0.0474* de	0.159 ± 0.0143 bc	1.331 ± 0.1940 b	1.679 ± 0.2036 c
L-0.8	0.333 ± 0.0702* bcd	0.226 ± 0.0410 ab	0.362 ± 0.0656* bcd	0.197 ± 0.0450 b	1.471 ± 0.0913 ab	1.861 ± 0.0949 abc
B-2000	0.507 ± 0.0413* a	0.243 ± 0.0218 a	0.481 ± 0.0379* a	0.270 ± 0.0123 a	2.097 ± 0.2376 a	1.783 ± 0.0933 bc
Control	0.215 ± 0.0381 e	0.179 ± 0.0346 c	0.253 ± 0.0433* e	0.140 ± 0.0258 c	1.217 ± 0.1963 b	1.815 ± 0.1813 bc

2-OH, dihydroxylated anthocyanins; 3-OH, trihydroxylated anthocyanins; Met, methylated anthocyanins; Non-met, non-methylated anthocyanins. Each value represents the mean of five replicates. Different letters indicate significant differences among treatments for the same type of anthocyanins at $p < 0.05$ according to the Tukey–Kramer HSD test (n = 5). Asterisks indicate significant differences within the same treatment for different groups of anthocyanins (2-OH vs. 3-OH or Met vs. Non-met) at $p < 0.05$ according to the Tukey–Kramer HSD test (n = 5).

TABLE 4 | Effects of BR treatments on the abundance of dihydroxylated, trihydroxylated, methylated, and non-methylated anthocyanins as well as the dihydroxylated:trihydroxylated anthocyanin and the methylated:non-methylated anthocyanin ratios for season 2015–2016.

Treatments	Anthocyanins (mg·berry^{-1})				2-OH·3-OH^{-1}	Met·Non-met^{-1}
	2-OH	3-OH	Met	Non-met		
E-0.4	0.280 ± 0.0250* a	0.101 ± 0.0120 abc	0.286 ± 0.0268* ab	0.095 ± 0.0208 a	2.829 ± 0.5982 a	3.169 ± 0.8671 ab
T-0.4	0.236 ± 0.0450* a	0.112 ± 0.0208 ab	0.257 ± 0.0306* b	0.091 ± 0.0333 a	2.139 ± 0.3646 ab	3.099 ± 0.9255 ab
L-0.4	0.145 ± 0.0275* b	0.090 ± 0.0056 bc	0.168 ± 0.0202* c	0.067 ± 0.0132 a	1.616 ± 0.2933 b	2.570 ± 0.3718 b
B-2000	0.293 ± 0.0578* a	0.121 ± 0.0122 a	0.325 ± 0.0442* a	0.088 ± 0.0240 a	2.413 ± 0.3481 a	3.802 ± 0.6181 a
Control	0.131 ± 0.0340* b	0.084 ± 0.0145 c	0.154 ± 0.0265* c	0.065 ± 0.0113 a	1.631 ± 0.4067 b	2.440 ± 0.6471 b

2-OH, dihydroxylated anthocyanins; 3-OH, trihydroxylated anthocyanins; Met, methylated anthocyanins; Non-met, non-methylated anthocyanins. Each value represents the mean of five replicates. Different letters indicate significant differences among treatments for the same type of anthocyanins at $p < 0.05$ according to the Tukey–Kramer HSD test (n = 5). Asterisks indicate significant differences within the same treatment for different groups of anthocyanins (2-OH vs. 3-OH or Met vs. Non-met) at $p < 0.05$ according to the Tukey-Kramer HSD test (n = 5).

DISCUSSION

Exogenous application of growth regulators is an important tool for improving the quality parameters of grape berries, including size, organoleptic characteristics, and color, among others (El-Kereamy et al., 2003; Han and Lee, 2004; Jeong et al., 2004; Symons et al., 2006; Fanzone et al., 2010; González et al., 2012; Xi et al., 2013). BRs compose a class of hormones involved in regulating berry ripening (Symons et al., 2006). However, the effects of exogenous applications of this type of growth regulator to table grapes have never been described. In fact, most studies on BRs as growth regulators have been performed on grapes for wine-make and involve the testing of only one BR analog, 24-epibrassinolide (Symons et al., 2006; Luan et al., 2013; Xi et al., 2013; Xu et al., 2015), thus limiting the understanding of the scope of this potential tool in a productive context in the case of table grapes.

In the current study, although no changes occurred in quality (total acidity, berry size) and yield (berry and cluster weight) parameters at harvest (Supplementary Tables 1, 2), a marked effect of BR analogs on soluble solids content, color of berries, concentration of anthocyanins, and the profiles of different groups of anthocyanins were observed (**Figures 2**–**4** and **Tables 3**, **4**). Compared with the control treatment, treatments E-0.4, B-2000, T-0.8, E-0.8, and T-0.4 were effective at increasing the soluble solids content of berries at harvest (**Figure 2**). Besides, results for treatments E-0.4 and B-2000 were consistent with those observed in the 2015–2016 season, in which these treatments were also statistically higher in soluble solids content than the control treatment. The rest of treatments, although showed average values higher than control treatment, did not present significant differences in soluble solids content. In addition, berries that received those treatments reached Brix values close to 15.5°, which is considerably higher than the 14.0° Brix values observed in berries that received the control treatment. Despite the absence of such differences prior to harvest, this finding suggests a faster increase of °Brix, which in a productive context means earlier harvest. This phenomenon is consistent with the reports by Luan et al. (2013) and Xi et al. (2013), who also observed an increase in the sugar content of berries as a consequence of 24-epibrassinolide applications. More recent studies have shown that this increase can be explained by the overexpression of hexose transporter genes *VvHT2*, *VvHT3*, *VvHT4*, *VvHT5*, and *VvHT6* as a consequence of 24-epibrassinolide application (Xu et al., 2015).

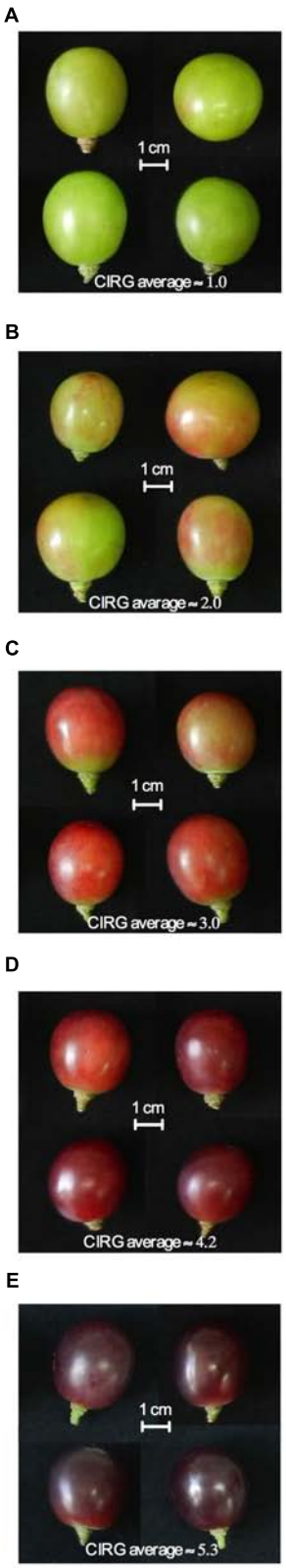


FIGURE 5 | Visual appearance of berries with different mean CIRG values from 1.0 to 5.3 **(A–E)**. In each image, the average CIRG value from the four berries shown is indicated.

Brassinosteroids affect the accumulation of phenolic compounds, including anthocyanins (Symons et al., 2006; Xi et al., 2013); however, the exogenous application of BR analogs has not been previously demonstrated to be associated with the color of berries or with their evolution from veraison to harvest. In our study, compared with the control treatment, treatments E-0.4, T-0.4, T-0.8, and B-2000 effectively increased CIRG at harvest (**Figure 3A**). However, as noted above in the results, in season 2014–2015, the analysis of covariance revealed a significant effect of soluble solids on CIRG values. Despite these, when considering the effect of soluble solids content on CIRG, treatments E-0.4, T-0.8, and L-0.8 showed a significant direct effect in increasing CIRG, while the remains of the treatments did not show significant differences respect to control (**Figure 3B**). If the concentration and content of anthocyanins (**Figure 4**) are considered, the increase in color produced by BRs may occur on at least two levels: one level involves directly a higher levels of anthocyanins (**Figure 4**) and another level involving the effect of the sugar content (expressed as °Brix) on color (**Figure 2A**). In addition, the effects of BR applications on CIRG appear to depend on the analog and concentration used. For example, treatment T-0.4 (application of the Triol at 0.4 $mg{\cdot}L^{-1}$) appears to act by increasing the content of soluble solids, whereas treatment L-0.8 (application of the Lactone at 0.8 $mg{\cdot}L^{-1}$) appears to act by increasing the concentration of anthocyanins. Treatment E-0.4 (application of 24-epibrassinolide at 0.4 $mg{\cdot}L^{-1}$) and treatment T-0.8 (application of the Triol analog at 0.8 $mg{\cdot}L^{-1}$) appear to act on both levels. Although a significant effect of soluble solids content on CIRG values was not observed in the 2015–2016 season, the results for treatments E-0.4, T-0.4, and B-2000 (**Figure 3B**) are consistent with the observed in the previous season (**Figure 3A**), since CIRG values were also statistically higher than those of control treatment.

Aside from the effect of the soluble solids content on the color of the berries or growing season, berries of clusters that received BR treatments exhibited average CIRG values close to 3.9; according to Carreño et al. (1996), these values correspond to colors near red and pink (**Figure 5**), which are very attractive colors for "Redglobe" in the target markets.

Treatments E-0.4, T-0.4, L-0.8, and B-2000 and E-0.4, T-0.4, and B-2000 for seasons 2014–2015 and 2015–2016, respectively, yielded the highest total anthocyanin content and concentration relative to the control values (**Figure 4**). These results are in agreement with those of Symons et al. (2006), Luan et al. (2013), and Xi et al. (2013) regarding the role of this hormone in grape berry ripening, and they suggest a potential role for BR analog growth regulators as a table grape production management tool, especially considering the results obtained regarding berry coloration (**Figures 3**, **5**). In addition, despite that ANOVA results indicate only significant effects of the treatments on content and concentration of anthocyanins, a significant relationship was observed between soluble solids content and anthocyanins (data not shown). This type of effect has been described before, in that a higher sugar content is associated with greater synthesis and accumulation of anthocyanins (Hiratsuka et al., 2001).

TABLE 5 | Details of correlation between the color of the berries (expressed as the CIRG) and the different groups of anthocyanins and the total anthocyanins, expressed as mg·berry^{-1} or mg·g FW^{-1}.

Anthocyanins	mg·berry^{-1}		mg·g FW^{-1}	
	p	*R*	*p*	*R*
2-OH	0.0062*	0.4252	0.0002*	0.5555
3-OH	0.3009	0.1676	0.0079*	0.4138
Met	0.0151*	0.3814	0.0002*	0.5533
Non-met	0.0317*	0.3401	0.0007*	0.5131
Total anthocyanins	0.0153*	0.3804	0.0001*	0.5659

2-OH, dihydroxylated anthocyanins; 3-OH, trihydroxylated anthocyanins; Met, methylated anthocyanins; Non-met, non-methylated anthocyanins. The asterisks denote p-values that indicate statistical significance (n = 40).

The analysis of the effects of different concentrations of BR analogs (with the exception of the commercial formulation) revealed differences in CIRG values and total anthocyanin contents of berries. For example, when treatment E-0.4 (0.4 mg·L^{-1}) was applied, a significant increase in CIRG values was observed relative to those of treatment E-0.8 (0.8 mg·L^{-1}), which produced similar CIRG values to those of the control treatment (**Figures 3**, **4**). A similar behavior was observed in the case of the Triol analog, especially with respect to the effect on anthocyanin content: a concentration of 0.4 mg·L^{-1} (T-0.4) generated values that were significantly higher than in the control, whereas a concentration of 0.8 mg·L^{-1} (T-0.8) resulted in anthocyanin contents that were similar to those of the control. This type of dose–response relationship was already described by Luan et al. (2013) and Xi et al. (2013), who reported that concentrations of 24-epibrassinolide greater than 0.4 mg·L^{-1} produced the same levels of anthocyanins, sugars, and acidity in "Cabernet Sauvignon" plants as those found in untreated plants, suggesting the existence of an optimal concentration for stimulating the development of maturity. Interestingly, the Lactone analog generated a significantly higher CIRG values respect to control (**Figure 3B**) when applied at the highest concentration (L-0.8 treatment) rather than the lowest concentration (L-0.4 treatment). Compared with the control treatment, L-0.8 treatment produced a significantly greater total anthocyanin content, whereas L-0.4 treatment produced values similar to those of control. In this way, and unlike the results of the present study and others that involved 24-epibrassinolide (Luan et al., 2013; Xi et al., 2013), these findings suggest that Lactone analog applied at concentrations greater than 0.4 mg·L^{-1} might be the most effective for increasing CIRG and total anthocyanin content. The differences in the effects among analogs and their concentrations could be explained by differences in the affinity of the receptor or some of its components. In this sense, Clouse (2010) and Codreanu and Russinova (2011) reported that correct binding and/or interaction between BRs (natural or synthetic) and specific portions of the extracellular domain of the receptor is crucial for inducing a response.

A statistically significant correlation was observed between CIRG and total anthocyanin content (mg·berry^{-1}) and concentration (mg·g FW^{-1}) (**Table 5**). The results of a similar analysis but for different groups of anthocyanins revealed a significant correlation between the CIRG and 2-OH, Met and Non-met anthocyanins (**Table 5**). However, when anthocyanins are expressed as mg·g FW^{-1}, the significance of the correlation extends to all 3-OH anthocyanins, with the exception of malvidin-3-glucoside (data not shown). This suggests that the CIRG values observed in this study (**Figures 3**, **5**) are associated with 2-OH anthocyanins. These results agree with those reported by Fernández-López et al. (1998), who associated the presence of 2-OH anthocyanins with grape varieties that have pink or red berries (CIRG $\approx$ 4.0). These authors also associated darker colors of berries (CIRG $\geq$ 6.5) with a greater abundance of 3-OH anthocyanins, particularly malvidin-3-glucoside.

Interestingly, three of the four treatments that resulted in significantly greater CIRG values than those from the control (E-0.4, T-0.4, and B-2000 in both seasons) also resulted in greater values of total anthocyanins and 2-OH anthocyanins, suggesting that the increase in total anthocyanin content occurred differentially toward this group of anthocyanins, i.e., those presenting pink and red colors. In fact, in berries of clusters that received these treatments, the 2-OH:3-OH anthocyanin ratios were close to 2.0 and 2.4 for the seasons 2014–2015 and 2015–2016, respectively (**Tables 3**, **4**). This differential accumulation is supported by the observed differences in the ratio between these two groups of anthocyanins (**Tables 3**, **4**). This phenomenon was particularly clear in berries that received treatments E-0.4 and B-2000 (in both seasons), which significantly differed from the control treatment in their 2-OH:3-OH ratio. The effect of the differential accumulation of anthocyanins could be explained by a varietal effect. As previously indicated, the predominant anthocyanins in "Redglobe" are of the 2-OH type (Carreño et al., 1997; Cantos et al., 2002; Peppi et al., 2007; Ustun et al., 2012), therefore an increase in the total anthocyanin concentration would mean an increase in the predominant type of anthocyanin present in the variety. However, a proportional increase in the amount of non-predominant anthocyanins could also occur. Nevertheless, this phenomenon was not observed in the present study, as indicated by the 2-OH:3-OH anthocyanin ratios (**Table 3**). Additionally, grape clusters treated with ABA exhibit increased total anthocyanin concentrations in berries, without altering the proportion of their different groups respect to the untreated berry clusters (Peppi et al., 2007). This is consistent with the idea that exogenous applications of

BRs, especially treatments E-0.4 and B-2000, cause a differential increase in the amount of 2-OH anthocyanins present in berries. This increment could be caused by altered activity, expression, or post-transcriptional regulation of enzymes involved in the phenylpropanoid pathway, or in transcription factors controlling the expression of those enzymes, particularly flavonoid 3′-hydroxylase (F3'H), which is responsible for generating the precursor of cyanidin-3-glucoside (Bogs et al., 2005; Castellarin et al., 2006); methyltransferase (OMT), which is responsible for the methylation of cyanidin-3-glucoside, giving rise to peonidin-3-glucoside (Mattivi et al., 2006); and/or flavonoid 3′,5′-hydroxylase, that generates the precursor of delphinidin-3-glucoside (Bogs et al., 2005; Castellarin et al., 2006). Similar alterations in the expression and/or activity of the enzymes of the phenylpropanoid pathway occur in "Cabernet Sauvignon" as a consequence of high temperature (Mori et al., 2005), exposure to solar radiation (Matus et al., 2009), and even viral infection (Vega et al., 2011). Notwithstanding, no study has clearly indicated changes in the proportions of different groups of anthocyanins. So far, similar effects in table grapes have not been reported; thus, the present study is the first reporting changes of this type in table grapes and over two growing seasons. Nevertheless, additional studies are needed to determine how treatments E-0.4 and B-2000 give rise to this differential anthocyanin accumulation.

Although no comparisons between BRs and alternative methods for improving the berry color, such as the use of ethephon or ABA, were performed in the present study, the effect of BR treatments is attractive in a productive context given the types of colors they generate and the absence of detrimental effects on quality attributes of the treated clusters (**Figures 3**, **5**).

CONCLUSION

The results of this study demonstrate that exogenous applications of different BRs analogs to "Redglobe" grape clusters result in a significant increase in berry color, in the soluble solids content, and total anthocyanins, altering the distribution of anthocyanin groups. This is the first report describing such effects on table grapes in a productive context.

The increase in the color of berries (expressed as CIRG) induced by treatment with BR analogs could be due to their effect on soluble solids content (treatments T-0.4 and B-2000), concentration of anthocyanins (treatment L-0.8), or the combination of these two effects in the berries (treatments E-0.4 and T-0.8).

The responses of CIRG and total anthocyanin content to the concentrations of the tested BR analogs show that Triols and 24-epibrassinolide have an effective concentration at approximately 0.4 mg·L^{-1}, whereas Lactone appears to have a greater effect at concentrations higher than 0.4 mg·L^{-1}.

This study showed that the activity of BR analogs is not restricted to just 24-epibrassinolide, which has been previously evaluated in other investigations, and that Triol, Lactone, and the commercial formulation (B-2000) were also active. The results demonstrate consistent effects of BRs on grape berries regardless of the analog used in two consecutive growing seasons.

Although several of the treatments performed in this study increased the total concentration of anthocyanins, only treatments E-0.4 and B-2000 resulted in a differential increase in 2-OH anthocyanins in two different growing seasons. This increment could chiefly explain the higher CIRG values observed in this work, which resulted in the expression of red and pink hues in berries, a color feature that is attractive for "Redglobe" markets.

Finally, this work demonstrates the potential of using BRs as a management tool in viticulture, and their use could at least complement the alternatives conventionally employed.

AUTHOR CONTRIBUTIONS

AV and AP-D designed the research. AV performed the research. AV, AP-D, and JA analyzed the data and results. LE, KD, and RC synthesized, characterized, and provided the two novel analogs brassinosteroid. AV wrote the paper.

FUNDING

This work was supported by IDeA Program by Fondef – Conicyt (Grant No. CA13I10239) and Fondo Nacional de Desarrollo Científico y Tecnológico (Grant No. 1160446).

ACKNOWLEDGMENTS

We are grateful to Alejandra Zúñiga from Laboratorio de Enología at Pontificia Universidad Católica de Chile by her continued assistance in the use of HPLC. AV thanks to CONICYT Ph.D. scholarship (21130026).

SUPPLEMENTARY MATERIAL

The Supplementary Material for this article can be found online at: https://www.frontiersin.org/articles/10.3389/fpls.2018.00363/full#supplementary-material

REFERENCES

Bogs, J., McDavid, D., and Robinson, S. P. (2005). Identification of the flavonoid hydroxylases from grapevine and their regulation during fruit development. *Plant Physiol.* 140, 279–291. doi: 10.1104/pp.105.073262

Cameron, I. J. (1994). "Influence of cultural practices on redglobe table grapes," in *Proceedings of the International Symposium on Table Grape Production*, (Anaheim, CA: American Society for Enology and Viticulture), 233–236.

Cantos, E., Espín, J. C., and Tomás-Barberán, F. A. (2002). Varietal differences among the polyphenol profiles of seven table grape cultivars studied by LC-DAD-MS-MS. *J. Agric. Food Chem.* 50, 5691–5696. doi: 10.1021/jf0204102

Carreño, J., Almela, L., Martinez, A., and Fernández-López, J. A. (1997). Chemotaxonomical classification of red table grapes based on anthocyanin profile and external colour. *Food Sci. Technol.* 30, 259–265. doi: 10.1006/fstl.1996.0174

Carreño, J., Martínez, A., Almela, L., and Fernández-López, J. A. (1995). Proposal of an index for the objective evaluation of the colour of red table grapes. *Food Res. Int.* 28, 373–377. doi: 10.1016/0963-9969(95)00008-A

Carreño, J., Martínez, A., Almela, L., and Fernández-López, J. A. (1996). Measuring the color of table grapes. *Color Res. Appl.* 21, 50–54. doi: 10.1002/(SICI)1520-6378(199602)21:1<50::AID-COL5>3.0.CO;2-4

Castellarin, S. D., Di Gaspero, G., Marconi, R., Nonis, A., Peterlunger, E., Paillard, S., et al. (2006). Colour variation in red grapevines (*Vitis vinifera* L.): genomic organisation, expression of flavonoid 3′-hydroxylase, flavonoid 3′,5′-hydroxylase genes and related metabolite profiling of red cyanidin-/blue delphinidin-based anthocyanins in berry skin. *BMC Genomics* 7:12. doi: 10.1186/1471-2164-7-12

Clouse, S. D. (2010). "Brassinosteroid signal transduction and action," in *Plant Hormone: Biosynthesis, Signal, Transduction, Action!*, ed. P. Davis (Dordrecht: Springer), 427–450. doi: 10.1007/978-1-4020-2686-7_20

Clydesdale, F. M. (1993). Color as a factor in food choice. *Crit. Rev. Food Sci. Nutr.* 3, 83–101. doi: 10.1080/10408399309527614

Codreanu, M. C., and Russinova, E. (2011). "Regulatory mechanisms of brassinosteroid signaling in plants," in *Brassinosteroids: A Class of Plant Hormone*, eds S. Hayat and A. Ahmad (Dordrecht: Springer), 29–56. doi: 10.1007/978-94-007-0189-2_2

El-Kereamy, A., Chervin, C., Roustan, J.-P., Cheynier, V., Souquet, J.-M., Moutounet, M., et al. (2003). Exogenous ethylene stimulates the long-term expression of genes related to anthocyanin biosynthesis in grape berries. *Physiol. Plant.* 119, 175–182. doi: 10.1034/j.1399-3054.2003.00165.x

Fanzone, M., Peña-Neira, A., Jofreì, V., Assof, M., and Zamora, F. (2010). Phenolic characterization of malbec wines from mendoza province (Argentina). *J. Agric. Food Chem.* 58, 2388–2397. doi: 10.1021/jf903690v

Fernández-López, J. A., Almela, L., Muñoz, J. A., Hidalgo, V., and Carreño, J. (1998). Dependence between colour and individual anthocyanin content in ripening grapes. *Food Res. Int.* 31, 667–672. doi: 10.1016/S0963-9969(99)00043-5

González, Á. S., Olea, P., Bordeu, E., Alcalde, J. A., and Gény, L. (2012). S-Abscisic acid, 2-chloroethylphosphonic acid and indole-3-acetic acid treatments modify grape (*Vitis vinifera* L. "Cabernet Sauvignon") hormonal balance and wine quality. *Vitis J. Grapevine Res.* 51, 45–52.

Grove, M. D., Spencer, G. F., Rohwedder, W. K., Mandava, N., Worley, J. F., Warthen, J. D., et al. (1979). Brassinolide, a plant growth-promoting steroid isolated from *Brassica napus* pollen. *Nature* 281, 216–217. doi: 10.1038/281216a0

Han, D. H., and Lee, C. H. (2004). The effects of GA3, CPPU and ABA applications on the quality of Kyoho (*Vitis Vinifera* L. x *Labrusca* L.) grape. *Acta Hortic.* 653, 193–197. doi: 10.17660/ActaHortic.2004.653.27

Hiratsuka, S., Onodera, H., and Kawai, Y. (2001). ABA and sugar effects on anthocyanin formation in grape berry cultured in vitro. *Sci. Hortic.* 90, 121–130. doi: 10.1016/S0304-4238(00)00264-8

Iland, P., Bruer, N., Edwards, G., Weeks, S., and Wilkes, E. (2004). *Chemical Analysis of Grapes and Wine: Techniques and Concepts*. Campbelltown: Patrick Iland Wine Promotions, 110.

Jeong, S. T., Goto-Yamamoto, N., Kobayashi, S., and Esaka, M. (2004). Effects of plant hormones and shading on the accumulation of anthocyanins and the expression of anthocyanin biosynthetic genes in grape berry skins. *Plant Sci.* 167, 247–252. doi: 10.1016/j.plantsci.2004.03.021

Kliewer, W. M., and Dokoozlian, N. K. (2005). Leaf area/crop weight ratios of grapevines influence on fruit composition and wine quality. *Am. J. Enol. Vitic.* 2, 170–181.

Kliewer, W. M., and Weaver, R. J. (1971). Effect of crop level and leaf area on growth, composition, and coloration of 'Tokay' grapes. *Am. J. Enol. Vitic.* 22, 172–177.

Kuhn, N., Guan, L., Dai, Z. W., Wu, B. H., Lauvergeat, V., Gomès, E., et al. (2014). Berry ripening: recently heard through the grapevine. *J. Exp. Bot.* 65, 4543–4559. doi: 10.1093/jxb/ert395

Luan, L. Y., Zhang, Z. W., Xi, Z. M., Huo, S. S., and Ma, L. N. (2013). Brassinosteroids regulate anthocyanin biosynthesis in the ripening of grape berries. *S. Afr. J. Enol. Vitic.* 34, 196–203.

Mattivi, F., Guzzon, R., Vrhovsek, U., Stefanini, M., and Velasco, R. (2006). Metabolite profiling of grapes: flavonols and anthocyanins. *J. Agric. Food Chem.* 54, 7692–7702. doi: 10.1021/jf061538c

Matus, J. T., Loyola, R., Vega, A., Peña-Neira, A., Bordeu, E., Arce-Johnson, P., et al. (2009). Post-veraison sunlight exposure induces MYB-mediated transcriptional regulation of anthocyanin and flavonol synthesis in berry skins of *Vitis vinifera*. *J. Exp. Bot.* 60, 853–867. doi: 10.1093/jxb/ern336

Mitchell, J. W., Mandava, N., Worley, J. F., and Plimmer, J. R. (1970). Brassins–a new family of plant hormones from rape pollen. *Nature* 225, 1065–1066. doi: 10.1038/2251065a0

Mori, K., Sugaya, S., and Gemma, H. (2005). Decreased anthocyanin biosynthesis in grape berries grown under elevated night temperature condition. *Sci. Hortic.* 105, 319–330. doi: 10.1016/j.scienta.2005.01.032

Peppi, M. C., and Fidelibus, M. W. (2008). Effects of forchlorfenuron and abscisic acid on the quality of "Flame Seedless" grapes. *HortScience* 43, 173–176.

Peppi, M. C., Fidelibus, M. W., and Dokoozlian, N. (2006). Abscisic acid application timing and concentration affect firmness, pigmentation, and color of Flame Seedless' grapes. *HortScience* 41, 1440–1445.

Peppi, M. C., Fidelibus, M. W., and Dokoozlian, N. K. (2007). Application timing and concentration of abscisic acid affect the quality of "Redglobe" grapes. *J. Hortic. Sci. Biotechnol.* 82, 304–310. doi: 10.1080/14620316.2007.11512233

Schrader, P. L., Luvisi, A. D., Kosareff, J. J., and Dokoozlian, K. N. (1994). "Influence of vine crop load on redglobe table grapes," in *Proceedings of the International Symposium on Table Grape Production*, (Anaheim, CA: American Society for Enology and Viticulture), 211–214.

Spayd, S. E., Tarara, J. M., Mee, D. L., and Ferguson, J. C. (2002). Separation of sunlight and temperature effects on the composition of *Vitis vinifera* cv. Merlot berries. *Am. J. Enol. Vitic.* 53, 171–182.

Symons, G. M., Davies, C., Shavrukv, Y., Dry, I. B., and Rid, J. B. (2006). Grapes on steroids. Brassinosteroids are involved in grape berry ripening. *Plant Physiol.* 140, 150–158. doi: 10.1104/pp.105.070706

Ustun, D., Candir, E., Ozmedir, A. E., Kamiloglu, O., and Soylu, E. M. (2012). Effects of modified atmosphere packaging and ethanol vapor treatment on the chemical composition of "Red Globe" table grapes during storage. *Postharvest Biol. Technol.* 68, 8–15. doi: 10.1016/j.postharvbio.2012.01.006

Vega, A., Gutiérrez, R. A., Peña-Neira, A., Cramer, G. R., and Arce-Johnson, P. (2011). Compatible GLRaV-3 viral infections affect berry ripening decreasing sugar accumulation and anthocyanin biosynthesis in *Vitis vinifera*. *Plant Mol. Biol.* 77, 261–274. doi: 10.1007/s11103-011-9807-8

Wang, Z.-Y., Seto, H., Fujioka, S., Yoshida, S., and Chory, J. (2001). BRI1 is a critical component of a plasma-membrane receptor for plant steroids. *Nature* 410, 380–383. doi: 10.1038/35066597

Xi, Z. M., Zhang, Z. W., Huo, S. S., Luan, L. Y., Gao, X., Maa, L. N., et al. (2013). Regulating the secondary metabolism in grape berry using exogenous 24-epibrassinolide for enhanced phenolics content and antioxidant capacity. *Food Chem.* 141, 3056–3065. doi: 10.1016/j.foodchem.2013.05.137

Xu, F., Xi, Z. M., Zhang, H., Zhang, C. J., and Zhang, Z. W. (2015). Brassinosteroids are involved in controlling sugar unloading in *Vitis vinifera* "Cabernet Sauvignon" berries during véraison. *Plant Physiol. Biochem.* 94, 197–208. doi: 10.1016/j.plaphy.2015.06.005

Conflict of Interest Statement: LE, KD, and RC declare conflict of interest because one of the analogous (Lactone) could be patented and/or protected by copyright.

The other authors declare that the research was conducted in the absence of any commercial or financial relationships that could be construed as a potential conflict of interest.

frontiers in Plant Science

ORIGINAL RESEARCH
published: 06 April 2018
doi: 10.3389/fpls.2018.00428

Evaluation of Seaweed Extracts From *Laminaria* and *Ascophyllum nodosum* spp. as Biostimulants in *Zea mays* L. Using a Combination of Chemical, Biochemical and Morphological Approaches

Andrea Ertani[1], *Ornella Francioso*[2], *Anna Tinti*[3], *Michela Schiavon*[1], *Diego Pizzeghello*[1] *and Serenella Nardi*[1]*

[1] Dipartimento di Agronomia, Animali, Alimenti, Risorse Naturali e Ambiente, Università di Padova, Padova, Italy, [2] Dipartimento di Scienze e Tecnologie Agro-Alimentari, Università di Bologna, Bologna, Italy, [3] Dipartimento di Scienze Biomediche e Neuromotorie, Università di Bologna, Bologna, Italy

OPEN ACCESS

Edited by:
Youssef Rouphael,
University of Naples Federico II, Italy

Reviewed by:
Santiago Sanchez-Cortes,
Instituto de Estructura de la Materia (IEM), Spain
Petronia Carillo,
Università degli Studi della Campania "Luigi Vanvitelli" Caserta, Italy

***Correspondence:**
Serenella Nardi
serenella.nardi@unipd.it

Specialty section:
This article was submitted to Crop and Product Physiology, a section of the journal Frontiers in Plant Science

Received: 06 February 2018
Accepted: 20 March 2018
Published: 06 April 2018

Citation:
Ertani A, Francioso O, Tinti A, Schiavon M, Pizzeghello D and Nardi S (2018) Evaluation of Seaweed Extracts From Laminaria and Ascophyllum nodosum spp. as Biostimulants in Zea mays L. Using a Combination of Chemical, Biochemical and Morphological Approaches. Front. Plant Sci. 9:428. doi: 10.3389/fpls.2018.00428

Seaweed extracts can be employed as biostimulants during crop cultivation owing to their positive effects on plant performance. Therefore, in this study one extract from *Laminaria* (A) and five extracts from *Ascophyllum nodosum* (B–F) were assayed on maize (*Zea mays* L.) plants supplied for 2 days with 0.5 mL L^{-1} of single products to evaluate their capacity to stimulate root growth and morphology, nutrition, and sugars accumulation. Firstly, extracts were chemically characterized via Fourier transform infrared (FT-IR) and FT-Raman spectroscopies, and their content in carbon, nitrogen, phenolic acids and hormones (indole-3-acetic acid, IAA, and Isopentenyladenosine, IPA) was quantified. The auxin like- and gibberellic acid -like activities of all extracts were also determined. FT-IR and FT-Raman spectra provided complementary information depicting distinct spectral pattern for each extract. Bands assigned to alginic and uronic acids were dominant in FT-IR spectra, while those corresponding to polyaromatic rings were evident in FT-Raman spectra. In general, extracts stimulated root growth, nutrition, esterase activity, and sugar content. However, they showed high variation in chemical features, which may explain their different capacity in triggering physiological responses in maize. Among *A. nodosum* extracts for instance, E was the most efficient in promoting root morphology traits, likely because of its elevate content in IAA (32.43 nM), while F extract was the highest in phenol content (1,933 mg L^{-1}) and the most successful in improving plant nutrition. On the other hand, C extract was very effective in stimulating root elongation, but did not influence plant nutrition. B and D extracts induced similar positive effects on plants, although they greatly varied in chemical composition. *Laminaria* extract (A) differed from *A. nodosum* extracts, because of its low content in total phenols and the presence of both IAA- and GA-like activity. We conclude that all seaweed extracts acted as biostimulants in maize, but their chemical properties appeared crucial in predicting the physiological response preferentially elicited by individual seaweed extracts.

Keywords: nutrition, root morphology, spectroscopy, hormones, esterase, maize, phenols

INTRODUCTION

Consumers are increasingly appreciating the production of high quality, healthy fruits and vegetables, especially when they are obtained through a minimal impact on the environment (Spinelli et al., 2010). New approaches have been proposed to grow up the sustainability of agricultural productions and improve the quality of crops and crop-derived products. A promising tool is the use of biostimulants in the form of mixtures of substances and/or microorganisms, which are able to increase the plant nutrient use efficiency and tolerance to abiotic and biotic stresses (Colla et al., 2015; Nardi et al., 2016).

The mechanisms activated by biostimulants in plants are difficult to establish owing to the complex pool of bioactive molecules present in their formulation (Ertani et al., 2011, 2013a, 2014; Guinan et al., 2013). Studying the effects triggered by individual components might produce incorrect results when compared to those determined by the combination, and synergistic action of all constituents in the mixtures may outcome. In addition, the effects of biostimulants are not always consistent among the plant species. This is likely because plants can exhibit different sensitivity thresholds to one or more bioactive molecules (Colla et al., 2015). Also, the extraction method from the source material is critical for the maintenance of the activity of the mixture components, and different extraction procedures from the same matrix may produce more biostimulants with distinct properties and effectiveness (Godlewska et al., 2016; Michalak et al., 2016).

Among biostimulants, a special attention is given to seaweed extracts (Blunden, 1991; Cassan et al., 1992; Calvo et al., 2014). The brown seaweeds *Phaeophyceae* in particular, are employed in sustainable agricultural applications (Goñi et al., 2016), and some of them like *Ascophyllum nodosum, Macrocystis pyrifera*, and *Durvillea potatorum* are widely used in food and industrial applications (Khan et al., 2009). Seaweed extracts elicit an array of positive responses in plants (Prithiviraj, 2008), including the increase in growth, germination rate, chlorophyll synthesis, fruit quality, and post-harvest shelf life (Hong et al., 2007; Khan et al., 2009; Pereira et al., 2009; Calvo et al., 2014; Goñi et al., 2016). They can also induce earlier germination, flowering, and fructification (Mancuso et al., 2006; Sivasankari et al., 2006; Roussos et al., 2009; Ali et al., 2015; Satish et al., 2015), stimulate the proliferation of secondary roots (Mugnai et al., 2008; Pereira et al., 2009; Spinelli et al., 2010) and induce immunity/resistance to pathogens and abiotic stress in plants (Joubert and Lefranc, 2008; Sharma et al., 2014). Recently, the capacity of a commercial extract of *Ascophyllum nodosum* to alleviate drought stress in soybean via changes in physiology and expression of stress-related genes has been reported (Santaniello et al., 2017).

To date, several compounds have been identified in seaweeds as activators of plant defense mechanisms and growth promoters. Among them are: (i) polyphenols, such as phloroglucinol and its derivate eckol (Craigie, 2011; González et al., 2013, 2014; Synytsya et al., 2014; Rengasamy et al., 2015); (ii) polysaccharides, primarily alginate, fucoidan, laminaran, and carrageenans or their derived oligosaccharides (Chandía et al., 2004; Chandía and Matsuhiro, 2008; Khan et al., 2009; Craigie, 2011; González et al., 2014; Rengasamy et al., 2015); (iii) betaines, amino acids, and vitamins (MacKinnon et al., 2010); (iv) substances displaying hormone-like activity. Another valuable active component of seaweed extracts is kahydrin, a derivative of vitamin K1, which favors the secretion of H^+ ions into the apoplast (Lüthje and Böttger, 1995). This process leads to acidification of the rhizosphere, thus changing the redox state of soil and the availability of nutrients to plants.

Despite the chemical constituents of brown seaweed extracts and the physiological effects they can trigger in plants have been widely described by Battacharyya et al. (2015) with a special emphasis on horticultural crops, the comparison between several extracts with respect to their capacity to induce preferential physiological responses in plants based on the characterization of their chemical composition looks relevant in view of predicting seaweed extract specific biostimulatory effects. Therefore, this research was aimed to characterize the chemical properties and the hormone-like activity of different commercial extracts from *Laminaria* and *Ascophyllum nodosum* spp., and to explore their effects on some aspects of maize plants physiology. The main objective was to find a robust relationship between the specific properties of individual biostimulants and the physiological responses preferentially elicited, which may allow the prediction of metabolic targets for other commercial seaweed biostimulants. The choice of commercial seaweed extracts was dictated by the great variety of products that are available in the market of biostimulants, which may differ based on the algal species, and addition of chemicals or bioactive molecules during the manufacturing process.

MATERIALS AND METHODS

Spectroscopic Characterization of Seaweed Extracts

Six commercially available liquid seaweed extracts, one from *Laminaria* spp. and five from *A. nodosum* spp. of North Europe origin, were used in this study. They were produced via extraction with water acidified with sulfuric acid to pH 3–3.5. The mixtures were then centrifuged and the pH adjusted near neutral by addition of potassium hydroxide. Finally, the extracts were sieved through cellulosic membrane filters at 0.8 μm (Membra-Fil® Whatman Brand, Whatman, Milano, Italy). Each extract was classified with different letters: the extract derived from *Laminaria* was named A, while those obtained from *Ascophyllum nodosum* spp. were named from B to F. Extracts were freeze-dried before performing chemical and spectroscopic analyses.

The FT-IR spectra of lyophilized extracts were recorded using an ALPHA FTIR spectrometer (Bruker Optics, Ettlingen, Germany) equipped with an ATR (attenuated total reflectance) sampling device containing diamond crystal. The absorbance spectra were collected between 4,000 cm^{-1} and 400 cm^{-1}, at a spectral resolution of 4 cm^{-1}, with 64 scans co-added and averaged. A background spectrum of air was scanned under the same instrumental conditions before each series of measurements. Spectra were processed with the Grams/386 spectroscopic software (version 6.00, Galactic Industries Corporation, Salem, NH).

Raman spectra of lyophilized extracts were recorded in solid state with a Multiram FT-Raman spectrometer (Bruker Optics, Ettlingen, Germany) equipped with a cooled Ge-diode detector. The spectral resolution was 4 cm^{-1} and 6,000 scans for each spectrum were collected (integration time about 4 h). The excitation source was a Nd^{3+}-YAG laser (1,064 nm, about 30 mW laser power on the sample) in the backscattering (180°) configuration. The low laser power was due to the brown color of the samples which burned out using a higher laser power.

B extract in particular, appeared fluorescent and enriched in mineral content, therefore both its FT-Raman and FT-IR spectra were not comparable to the others (not shown).

Determination of Total Carbon and Nitrogen, Phenols and Hormones (IAA and IPA) in Seaweed Extracts

Carbon (C) and nitrogen (N) contents of seaweed extracts were determined using a dry combustion procedure inside an element analyser (vario MACRO CNS, Hanau, Germany).

Total phenols were quantified via the Folin method according to Arnaldos et al. (2001). Phenols were extracted in water/methanol (1:1 v/v) and filtered at 0.45 μm. The separation of phenols was carried out with HPLC 2,700 (Thermo Finnigan, San Jose, CA, USA) coupled with an 1,806 UV/Vis (Thermo Finnigan, San Jose, CA, USA) detector. The column was a TM-LC 18 (Supelcosil) equipped with pre-column TM-LC 18 (Pelliguard, Supelco). Elution was performed at a flow rate of 1.2 mL min^{-1} using as mobile phase a mixture of water/ n-butanol/ acetic acid (80.5:18:1.5 v/v). The sample injection volume was 20 μL. Detection was performed at 275 nm and the identification of compounds was achieved by comparing their retention time values with those of standards (gallic, protocatetic, vanillic, caffeic, *p*-coumaric, syringic, and p-hydroxybenzoic acids (Sigma-Aldrich). The calibration curve and quantification were performed considering the relationship between peak areas vs. standard concentrations at four concentrations ($n = 4$). A linear fitting with a R squared (R^2) = 0.99 was obtained. All reagents were of analytical grade.

Indole-3-acetic acid (IAA), an auxin, and isopentenyladenosine (IPA), a cytokinin, were determined in extracts using an enzyme-linked immuno-sorbent assay (ELISA) (Phytodetek-IAA and Phytodetek-IPA, respectively, Sigma, St. Louis, MO) as reported in Ertani et al. (2013a,b). The ELISA assay used monoclonal antibodies sensitive in the range between 0.05 and 100 pM. The tracer and standard solutions were prepared following the manufacturer's instructions, and absorbance was read at $\lambda = 405$ nm with a Biorad microplate reader (Hercules, CA). Additional details were previously reported by Schiavon et al. (2010).

Hormone-Like Activities of Seaweed Extracts

The IAA-like activity was estimated by measuring the reduction of watercress (*Lepidium sativum* L.) roots after treatment with either IAA or seaweed extracts, while the gibberellin-like (GA-like) activity was determined by measuring the increase in the epicotyls length of lettuce (*Lactuca sativa* L.) after GA and seaweed extracts application (Audus, 1972). In detail, watercress and lettuce seeds were surface-sterilized by immersion in 8% hydrogen peroxide for 15 min. After rinsing five times with sterile distilled water, 10 seeds were placed on a sterile filter paper in a sterile Petri dish. For watercress, the filter paper was wetted with 1.2 mL of a 1 mM $CaSO_4$ solution (control), or 1.2 mL of 20, 10, 1, and 0.1 mg L^{-1} IAA solution (Sigma, Milan, Italy) for the calibration curve, or 1.2 mL of a serial dilution of seaweed extracts. For lettuce, the experimental design was the same as for watercress except that the sterile filter paper was wetted with 1.4 mL instead of 1.2 mL, and the calibration curve was a progression of 100, 10, 1, and 0.1 mg L^{-1} GA solution (Sigma). The seeds were placed in a germination room in the dark at 25°C. After 48 h for watercress and 72 h for lettuce, seedlings were removed and the root or epicotyl lengths measured with a TESA-CAL IP67 electronic caliper (TESA, Renens, Switzerland) and Data Direct software, version 1 (ArtWare, Asti, Italy). The values obtained were the means of 20 samples and five replications, with the standard errors always 5% of the mean.

Values of hormone-like activity are reported in ppm IAA and ppm GA, expressed respectively as concentration of indoleacetic acid or gibberellic acid of equivalent activity as 1 mg C L^{-1}.

Plant Material and Growth Conditions

Seeds of *Zea mays* L. (var. DK C6286, DeKalb, Padua Italy) were soaked in distilled water overnight and then surface-sterilized in 5% (v/v) sodium hypochlorite for 10 min, while shaking. Seeds were left to germinate for 60 h in the dark, at 25°C, on a filter paper wetted with 1 mM $CaSO_4$ (Ertani et al., 2011). Germinated seedlings were transplanted into 3 L pots containing an aerated complete culture solution, with a density of 12 plants per pot. The nutrient solution was renewed every 48 h and had the following composition (μM): KH_2PO_4 (40), $Ca(NO_3)_2$ (200), KNO_3 (200), $MgSO_4$ (200), FeNaEDTA (10), H_3BO_3 (4.6), $CuCl_2$ (0.036), $MnCl_2$ (0.9), $ZnCl_2$ (0.09), $NaMoO_4$ (0.01). Plants were cultivated for 14 days inside a climatic chamber with a 14 h light/10 h dark cycle, air temperature of 27°C/21°C, relative humidity of 70/85%, and photon flux density of 280 mol m^{-2} s^{-1}. Twelve days after transplant, part of plants were divided in groups and treated for 48 h with 0.5 mL L^{-1} single seaweed extracts. The remaining plants were not supplied with seaweed extracts and served as controls. Plants were randomly harvested from three pots per treatment, carefully washed and dried with blotting paper. A sub-sample of plant material was immediately frozen with liquid nitrogen and kept at −80°C for further analyses of esterase activity. Another sub-sample of plant material was dried in the oven at 65°C and used for element and sugar quantification.

Root Characteristics

Root scanning was performed before the sampling process using an Epson Expression 10000XL 1.0 system (Regent Instruments Company, Canada) as published in Ding et al. (2014). The parameters were recorded with a root image analysis system using the software WinRHIZO: main root length (mm), surface

area (cm^2), average diameter (mm), number of tips, and length of fine roots (cm) ($0<L<0.5$).

Elemental Composition and Soluble Sugars Determination in Maize Leaves

Quantification of elements in leaves was obtained after acid digestion by using a microwave (Milestone Ethos model 1600, Milestone, Shelton, CT). Analytical-grade reagents provided by Merck (Merck, Darmstadt, Germany) were used to prepare all solutions. Water was purified using a Milli-Q system (18.2 MΩ cm, Millipore, Bedford, MA). The digestions were carried out as described in Ertani et al. (2011) inside closed Teflon vessels of 120 mL volume using approximately 500 mg dry leaf material and 10 mL of 30% (v/v) HCl. After digestion, the resulting solution was transferred and diluted with 10 mL ultrapure water. Elements were measured via Inductively Coupled Plasma Atomic Emission Spectroscopy (Spectrum CirosCCD, Kleve, Germany).

For analysis of reducing sugars, samples of leaf material from individual plants were dried for 48 h at 80°C, ground in liquid nitrogen and then 100 mg of each were extracted with 2.5 mL 0.1 N H_2SO_4. Samples were incubated in a heating block for 40 min at 60°C and then centrifuged at 6,000 g for 10 min at 4°C. After filtration (0.2 μm, Membra-Fil®, Whatman, Milan, Italy), the supernatants were analyzed by HPLC coupled to the refractive index detector (RI) (Perkin Elmer 410, Perkin Elmer, Norwalk, CT, USA). The soluble sugars were separated through an Aminex 87 C column (300 × 7.8 mm, BioRad, Segrate, Milan, Italy) using H_2O as eluent at a flow rate of 0.6 mL min^{-1}.

Esterase Enzyme Activity

Esterase activity was performed according to Junge and Klees (1984). Leaves and roots (1 g) were homogenized (1:10, w:v) in liquid N_2 with 0.1 M potassium acetate (pH 4.0) containing 0.1 M phosphate buffer (pH 7.0). The extracts were centrifuged at 15,000 g for 15 min at 2°C and the supernatants used as the enzyme source and expressed as a percentage of the control (0.28 OD min^{-1} fresh wt mg^{-1}).

Statistical Analysis

Data represent the means of measurements on tissue material derived from plants grown in three different pots per treatment. For each analysis, five plants per treatment were used (± std). Analysis of variance (ANOVA) was followed by pair-wise *post-hoc* analyses (Student-Newman-Keuls test) to determine which means differed significantly at $p < 0.05$. To identify the structure of the interdependences of the main parameters studied, joint principal components analysis (PCA) was performed on the following 16 variables: main root length, root surface area, root average diameter, root number of tips, root length of fine roots, leaves elemental composition as calcium, magnesium, sulfur, iron, copper, manganese, molybdenum, zinc, boron, and leaves and roots esterase activity and 46 studied objects. The standardized variables were subjected to PCA; and the rotated orthogonal components (varimax rotation method) were extracted and the relative scores were determined. Only PCs with an eigenvalue >1 were considered for discussion. All statistics were made by SPSS software version 19 (SPSS inc., 1999).

RESULTS

Chemical Characterization and Hormone-Like Activity of Extracts

The main chemical features of the seaweed extracts assayed in this study are listed in **Tables 1**, **2**. Carbon (C) content ranged from 3.7 to 12.2% (g/100 mL), while nitrogen (N) content varied from 0.1 to 7.2% (g/100 mL) (**Table 1**). Maximum value of IPA was measured in C extract (8.45 nM), while E extract was particularly enriched in IAA (32.43 nM). D extract was the least in both hormones (2.72 and 9.70 nM for IPA and IAA, respectively).

The amount of total phenols (TP) was extremely variable in the extracts (**Table 2**). In particular, *A. nodosum*–derived extracts were more enriched in phenols, especially E (1,589.4 mg L^{-1}) and F (1,933.8 mg L^{-1}), compared to the extract from *Laminaria* (A, 211.5 mg L^{-1}). Among phenolic acids (gallic acid, protocatechuic acid, vanillic acid, caffeic acid, *p*-coumaric acid, syringic acid, *p*-hydroxybenzoic), *p*-hydrozybenzoic acid was dominant in all extracts, with values between 22.72 mg L^{-1} (E) and 81.11 mg L^{-1} (F). This phenolic compound was the unique determined in C extract. Syringic acid was exclusively identified in F, while *p*-coumaric acid was present in both A and B extracts. Gallic acid was measured in all samples except in C, and its content was maximum in E extract (33.16 mg L^{-1}). A and E extracts also contained significant levels of protocatechuic, vanillic and caffeic acids.

The hormone-like activity of the extracts was evaluated via Audus test (**Table 3**). Only A and B extracts displayed GA-like activity (1.09E-07 ppm IAA and 1.34E-06 ppm GA). However, the IAA-like activity in these extracts was not detectable (B) or negligible (A). C extract was the highest in IAA-like activity (0.23 IAA ppm), followed by extracts F (0.11 IAA ppm), E (0.10 IAA ppm), and D (0.06 IAA ppm).

Spectroscopic Characterization of Seaweed Extracts Via FT-IR and FT-Raman

The FT-IR spectra of different seaweed extracts are shown in **Figure 1**. Overall, the region included within 2,500–4,000 cm^{-1} is not particularly discriminant, except for A extract, because it displayed very similar features: a strong –OH at about 3,400 cm^{-1} and weak –CH stretching bands at about 2,950 cm^{-1}. In particular, A extract differed from the other extracts because the –CH stretching bands were not observed; moreover, three components accounting for the mineral part of this sample

TABLE 1 | Content of total carbon (C), total nitrogen (N), IPA and IAA in extracts obtained from seaweeds *Laminaria* (A) and *Ascophyllum nodosum* spp. (B–F).

Seaweed extract	C tot (%)	N tot (%)	IPA (nMol)	IAA (nMol)
A	4.20	0.40	4.12	14.6
B	3.70	7.20	4.91	11.61
C	4.00	3.50	8.45	10.39
D	12.20	0.30	2.72	9.70
E	5.60	0.10	5.79	32.43
F	3.70	0.10	3.74	17.79

TABLE 2 | Content of total phenols and individual phenolic acids in extracts obtained from the seaweeds *Laminaria* (A) and *Ascophyllum nodosum* spp. (B–F).

Seaweed extract	Tot. phenols	Gallic	Protocatechuic	Vanillic	Caffeic	p-Coumaric	Syringic	p-Hydroxybenzoic
				mg L^{-1}				
A	211.5	3.47	11.36	5.38	11.42	12.36	n.d.	38.81
B	555.9	6.19	13.25	15.24	n.d.	21.75	n.d.	50.28
C	900.4	n.d.	n.d.	n.d.	n.d.	n.d.	n.d.	58.36
D	1,244.9	4.97	n.d.	18.45	n.d.	n.d.	n.d.	50.15
E	1,589.4	33.16	16.11	8.16	5.99	n.d.	n.d.	22.72
F	1,933.8	2.90	1.97	n.d.	3.14	n.d.	4.94	81.11

TABLE 3 | Hormone-like activity of extracts obtained from the seaweeds *Laminaria* (A) and *Ascophyllum nodosum* spp. (B–F).

Seaweed extract	IAA-like activity	GA-like activity
	(ppm IAA)	(ppm GA)
A	0.01	1.09E-07
B	–	1.34E-06
C	0.23	–
D	0.06	–
E	0.10	–
F	0.11	–

IAA- and GA-like activities are expressed as ppm IAA and ppm GA, which correspond to the concentration of either indoleacetic acid or gibberellic acid of equivalent activity as 1 mg C L^{-1}.

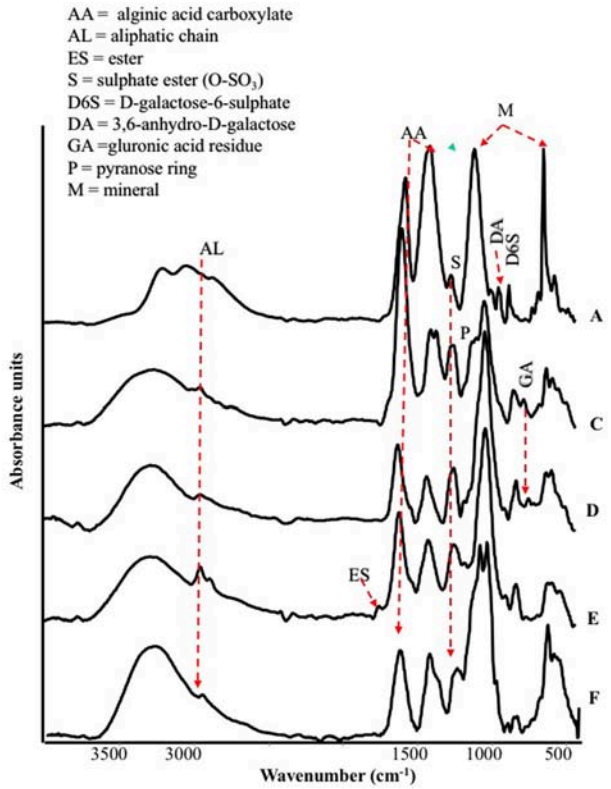


FIGURE 1 | ATR/FT-IR spectra of extracts derived from *Laminaria* (A) and from *Ascophyllum nodosum* spp. (C–F).

(see below) were observed in the region 3,500–2,500 cm^{-1}. The bands detected in the region between 1,800 and 600 cm^{-1} in particular, were the most characteristics for each extract, therefore they deserved a detailed description. The spectra of all extracts revealed a strong band at 1,612–1,547 cm^{-1} likely due to the asymmetric stretching of carboxylate vibration in alginic acid (Gómez-Ordó-ez and Rupérez, 2011), while the strong band within 1,412–1,380 cm^{-1} could be ascribed to the symmetric stretching vibration of the same carboxylate groups (Mathlouthi and Koenig, 1986), with the contribution of –CH and C-OH deformation vibration. The extracts also exhibited a broad band around 1,230 cm^{-1} corresponding to sulfate ester groups (S = O), which is characteristic of functional groups observed in fucoidans (Gómez-Ordó-ez and Rupérez, 2011). The bands around 1,081–1,026 cm^{-1} could be assigned to C–C–H and O–C–H deformation, C–O stretching, and C–O and C–C stretching vibrations of pyranose rings, with the contribution of mineral compounds, especially in the spectrum of sample A, whose wavenumber lies at 1,080 cm^{-1}. On the other side, the spectra of the other samples show bands at lower wavenumbers (1,025–1,030 cm^{-1}) due to carbohydrates and a shoulder at 1,080 cm^{-1}. The band in the anomeric region between 950 and 750 cm^{-1} was typical of carbohydrates (Pereira et al., 2009, 2013). In particular, the band at 960 cm^{-1}, well visible in A and F extracts, could be assigned to the C–O stretching vibration of uronic acid residues, the one observed at 814 cm^{-1}, evident with different intensity in all samples, likely corresponded to C1–H deformation vibration of mannuronic acid residues, while the one recorded at 732 cm^{-1} (D and F extracts, in particular) was characteristic of glucuronic acid residues. Going into more details, E extract was also characterized by a high content in lipids, as revealed by two sets of strong C-H vibration at 2,930 cm^{-1} and 2,854 cm^{-1}, which are the most intense in the spectra depicted in **Figure 1**, and the stretching vibration of carboxylic ester groups, evidenced by the band pinpointed at 1,734 cm^{-1} (Leal et al., 2008). Finally, the shoulder at 1,510 cm^{-1} was typical of aromatic rings vibration, consistently with the highest content of gallic and protocatechuic acids determined in E extract and of p-hydroxybenzoic acid in F extract (**Table 2**).

The spectral features of extracts were also supported by FT-Raman spectra. In particular, in **Figure 2** are shown, as an example, the FT-Raman spectra of two extracts (E and F). FT-Raman spectra confirmed the presence of carboxylate vibration

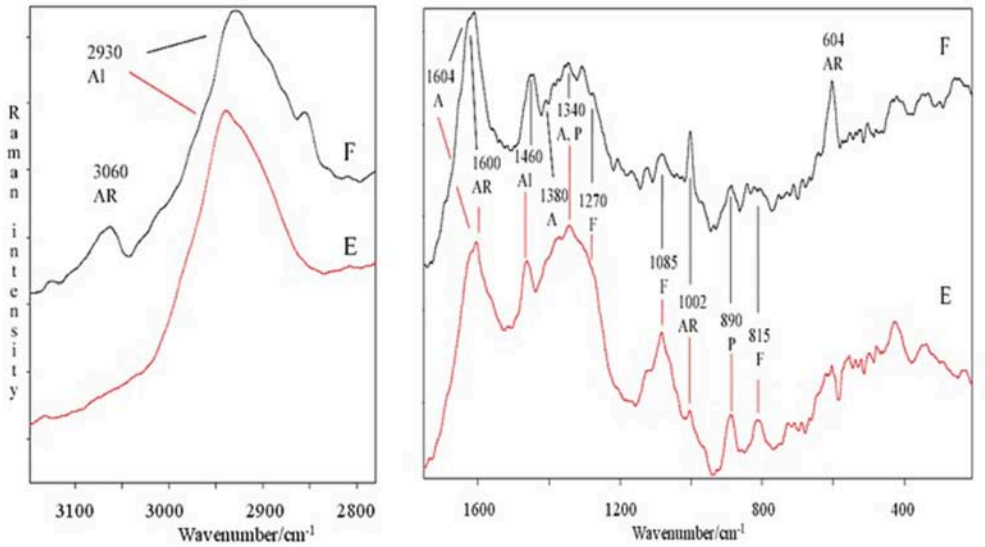


FIGURE 2 | Comparison of FT-Raman spectra of E and F extracts derived from *Ascophyllum nodosum* in the 3,100–2,800 and 1,700–200 cm^{-1} regions. Abbreviations as in **Figure 1**; in addition, F, fucoidans.

in alginic acid at 1,604 and 1,380–1,390 cm^{-1} (shoulder) (Gómez-Ordó-ez and Rupérez, 2011). Others bands at about 2,930 and 1,460 cm^{-1} are due to the stretching and bending vibrations of aliphatic groups. These last bands were common in E and F extracts, while only in F the contemporaneous presence of the bands at 3,060, about 1,600 and 1,002 cm^{-1} indicated a moderate/high presence of aromatic groups, due to polysubstituted aromatic compounds, as the phenolic acids reported in **Table 2**. The bands at 1,002 cm^{-1} (the most intense normally observed in the spectra of aromatic compounds) and about 1,600 cm^{-1} are present also in the spectrum of E extract, indicating a lower presence of total aromatics/polyaromatics, as compared to F extract (**Table 2**). The E extract exhibited also very intense bands at 1,085 and at about 1,270 cm^{-1} (shoulder) (Synytsya et al., 2014; Marinval et al., 2016) which are typical of symmetric and asymmetric stretching of sulfate groups in fucoidans, respectively, together with the band at about 815 cm^{-1}, attributable to bending of primary C6-O-S (Campos-Vallette et al., 2010; Synytsya et al., 2014). These three bands were present also in the other spectra, but with a lower intensity. Raman bands, common to both spectra, at 1,340–1,346 cm^{-1} were assigned to in-plane CCH, COH and OCH deformations in pyranoid rings with contribution of CH_2 wagging and CH_3 symmetric bending of galactose and fucose, respectively (Synytsya et al., 2014). Finally, the band at about 890 cm^{-1} can be generically attributed to β-glycosidic bond (Marinval et al., 2016) or to β-D-terminal in mannose or glucose containing carbohydrates (Yang and Zhang, 2009).

Root Growth in Response to Seaweed Extracts

In general, root size and architecture of maize plants was positively influenced by the addition of seaweed extracts from *Laminaria* and *A. nodosum* spp. (**Table 4**). E extract was the most successful in stimulating root elongation (plus 81%), despite values were not significantly different from those measured for plants treated with B, C, and D extracts. Conversely, A and F extracts were the least efficient in promoting root development. In general, root surface was greater in plants supplied with seaweed extracts than in untreated plants, with maximum values measured in plants added either with C or E extract. Furthermore, plants treated with seaweed extracts, produced a higher number of root tips (plus 58 and 88% for B and E, respectively) and showed a more pronounced length of thin roots (about plus 80% for both C and E) than the controls. An example of image comparison between roots of untreated plants (control) and plants treated with a seaweed extract is depicted in **Figure 3**.

Effect of Seaweed Extracts on the Content of Soluble Sugars (Glucose and Fructose) in Plants

The leaf glucose content showed great variation in response to seaweed extract application to maize plants, as shown in **Table 5**. Precisely, plants accumulated more glucose when treated with A, B, C, or F extracts, while a reduction in content of this sugar was evident in plants supplied with D or E (minus 38–40%). In roots, the level of glucose consistently decreased in plants treated with extracts derived from *A. nodosum*. Conversely, it did not change in plants supplied with A extract. A different trend in fructose content was observed, being the accumulation of this sugar generally higher in both leaves and roots of plants treated with the seaweed extracts (**Table 5**). Only plants treated with C extract accumulated less fructose than control plants (minus 38 and 24% in leaves and roots, respectively).

TABLE 4 | Root growth-associated parameters of control maize plants (untreated) and plants supplied for 48 h with extracts obtained from the seaweeds *Laminaria* (A) and *Ascophyllum nodosum* (B–F).

Treatment	Root length (cm)	Surface (cm^2)	Diameter (mm)	Tip number	Fine root length (cm)
Control	884 ± 151c	103 ± 20d	0.37 ± 0.02b	1642 ± 272d	696 ± 129d
A	1017 ± 64c	121 ± 7cd	0.38 ± 0.01b	2204 ± 197a	835 ± 58c
B	1432 ± 123ab	151 ± 13bc	0.34 ± 0.01c	2603 ± 142ab	1183 ± 110ab
C	1542 ± 68a	175 ± 8ab	0.36 ± 0.01b	2318 ± 86bc	1245 ± 57a
D	1422 ± 133ab	155 ± 18bc	0.34 ± 0.02c	2473 ± 228ab	1167 ± 107ab
E	1602 ± 140a	208 ± 21a	0.41 ± 0.01a	3092 ± 297a	1266 ± 102a
F	1115 ± 112bc	115 ± 13cd	0.32 ± 0.01c	1845 ± 238c	944 ± 88b

Data represent the means of five measurements per treatment (± std). Different letters along the same column indicate significant differences between treatments ($p < 0.05$) according to Student-Newman-Keuls test.

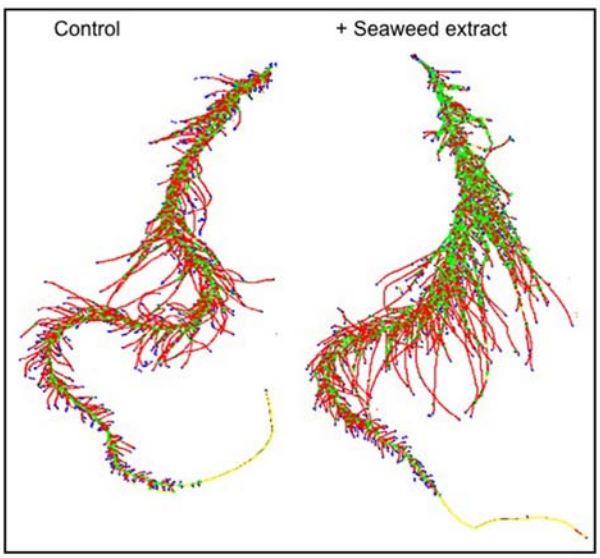


FIGURE 3 | Image comparison between roots of untreated maize plants (control) and plants treated with a seaweed extract. Image was obtained using an Epson Expression 10000XL 1.0 system (Regent Instruments Company, Canada), equipped with a root image analysis system using the software WinRHIZO.

Elemental Composition in Maize Leaves

A number of elements (Ca, S, Mg, Fe, Cu, Mn, Mo, Zn, B) have been quantified in leaves of plants treated or not with the seaweed extracts (**Table 6**). Generally, the concentration of Ca, Mg, S, and Mo increased in leaves after application of extracts to plants. In particular, Ca concentration was enhanced by 3 fold in plants treated with D and F extracts, while Mg, S, and Mo concentration increased twice in plants supplied with F extract. For the remaining elements analyzed, higher accumulation was observed in leaves of plants treated with certain seaweed extracts. Boron, as an example, was more accumulated in leaves of maize only after addition of E and F extracts.

Plants added with C extract did not shown any remarkable increase in nutrient content with the exception of Cu, while F extract was the most efficient in promoting plant nutrition.

TABLE 5 | Glucose and fructose content in leaves and roots of control maize plants (untreated) and plants supplied for 48 h with extracts obtained from the seaweeds *Laminaria* (A) and *Ascophyllum nodosum* spp. (B–F).

Treatment	Glucose		Fructose	
	mg g^{-1} dwt.			
	Leaves	Roots	Leaves	Roots
Control	9.8 ± 0.5c	29.5 ± 1.1ab	7.8 ± 1.0d	9.7 ± 1.2bc
A	12.2 ± 0.8b	32.1 ± 1.3a	11.3 ± 0.8c	9.2 ± 1.1bc
B	11.0 ± 0.4b	24.5 ± 1.5b	13.5 ± 0.5b	10.9 ± 0.4b
C	16.1 ± 0.8a	27.7 ± 1.6b	4.8 ± 0.5e	7.4 ± 1.0c
D	5.9 ± 1.3d	16.7 ± 1.3d	12.6 ± 0.7bc	10.8 ± 0.7b
E	6.1 ± 0.8d	26.5 ± 1.6b	10.7 ± 0.8c	11.8 ± 0.7ab
F	14.3 ± 1.3a	21.6 ± 1.8c	17.0 ± 0.4a	13.2 ± 1.1a

Data represent the means of five measurements per treatment (± std). Different letters along the same column indicate significant differences between treatments ($p < 0.05$) according to Student-Newman-Keuls test.

Effects of Extracts on Esterase Enzyme Activity

The activity of esterase in leaves and roots of maize plants was increased by seaweed extracts (**Figures 4A,B**). In particular, *Laminaria*'s extract (A) determined a more pronounced increment of esterase activity in foliar tissues than in roots (plus 38 and 78%, respectively). Among extracts derived from *A. nodosum*, F extract appeared the most effective in promoting esterase activity (plus 112% than the controls). In roots, extracts from *A. nodosum* stimulated esterase activity more than *Laminaria*'s extract. Maximum values of percent increases in activity were measured in plants treated with B (plus 154%) and F (plus 198%) extracts.

Principal Components Analysis

Three factors accounted for 78.27% of the total variance. Factor 1 (PC1) explained 42.21% of the variance and was positively correlated with Mn, Fe, Mo, Ca, B, Zn (**Table 7**). Factor 2 explained 20.88% and was positively correlated with the esterase activity in leaves and roots and the concentration of Mg, Cu and S (**Table 7**). Factor 3 explained the remaining 15.18% and was positively correlated with root parameters such as root surface,

TABLE 6 | Leaf elemental composition of control maize plants (untreated) and plants supplied for 48 h with extracts obtained from the seaweeds *Laminaria* (A) and *Ascophyllum nodosum* spp. (B–F).

Treatment	Ca	Mg	S	Fe	Cu	Mn	Mo	Zn	B
					mg g^{-1} dwt.				
Control	8.25 ± 0.12e	5.29 ± 0.09d	3.59 ± 0.22de	0.191 ± 0.005c	0.0332 ± 0.007c	0.0369 ± 0.002b	0.0017 ± 0.0001c	0.0794 ± 0.0059c	0.0198 ± 0.015b
A	13.97 ± 0.22d	7.73 ± 0.18c	3.34 ± 0.12e	0.190 ± 0.005c	0.0281 ± 0.010c	0.0343 ± 0.001b	0.0026 ± 0.0001b	0.1410 ± 0.022ab	0.0129 ± 0.010c
B	18.94 ± 0.49c	8.48 ± 0.41b	4.44 ± 0.21c	0.264 ± 0.016b	0.0302 ± 0.015c	0.0471 ± 0.004a	0.0025 ± 0.0001b	0.1604 ± 0.017a	0.0207 ± 0.007b
C	8.48 ± 0.16e	5.27 ± 0.13d	2.29 ± 0.11f	0.093 ± 0.001d	0.0439 ± 0.018a	0.0158 ± 0.001c	0.0013 ± 0.0001d	0.0778 ± 0.005c	0.0124 ± 0.012c
D	27.47 ± 0.36a	8.86 ± 0.10b	3.97 ± 0.08d	0.255 ± 0.003b	0.0475 ± 0.023a	0.0472 ± 0.001a	0.0024 ± 0.0001b	0.1040 ± 0.007bc	0.0199 ± 0.005b
E	19.70 ± 0.50c	8.75 ± 0.11b	5.32 ± 0.07b	0.275 ± 0.004b	0.0358 ± 0.005b	0.0380 ± 0.001b	0.0027 ± 0.0001b	0.0707 ± 0.001c	0.0260 ± 0.027a
F	25.72 ± 0.57b	11.07 ± 0.28a	7.51 ± 0.17a	0.330 ± 0.004a	0.0382 ± 0.008b	0.0490 ± 0.001a	0.0034 ± 0.0001a	0.0997 ± 0.004c	0.0220 ± 0.010a

Values are expressed (mg g^{-1} d.wt.) for macroelements and Fe, while for microelements in ppm (mg kg^{-1} d.wt.). Data represent the means of five measurements per treatment (± std). Different letters along the same column indicate significant differences between treatments ($p < 0.05$) according to Student-Newman-Keuls test.

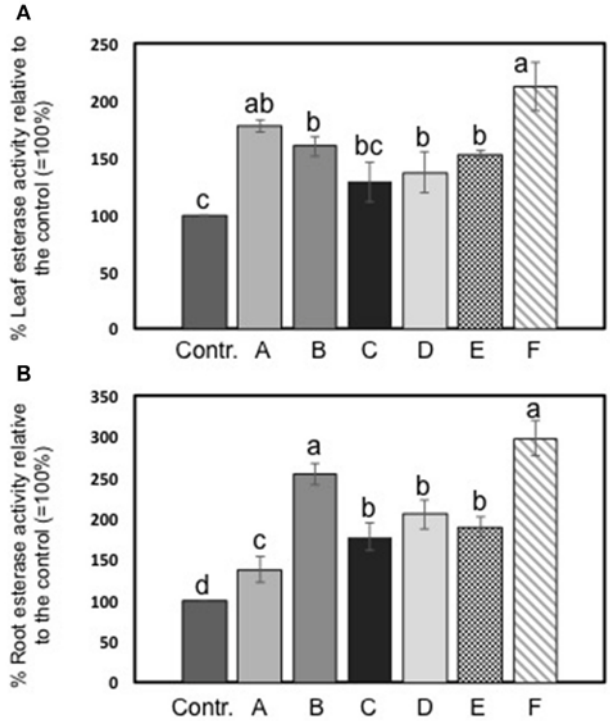


FIGURE 4 | Esterase activity in leaves **(A)** and roots **(B)** of maize untreated plants and plants added for 2 days with seaweed extracts from *Laminaria* spp. (A) and *Ascophyllum nodosum* spp. (B–F). Values are expressed as percent of the control (untreated plants). Data represent the means of five measurements per treatment (± std). Different letters above bars indicate significant differences between treatments ($p < 0.05$) according to Student-Newman-Keuls test.

root length, fine roots, number of tips, and diameter (**Table 7**). Plotting data according to PC1 and PC2 (**Figures 5A,B**) allowed three clusters to be identified corresponding to maize plant treated with C extract (=3 in **Figures 5**, **6**) and with seaweed F extract (=6 in **Figures 5**, **6**) (top), while control plants (=7 in **Figures 5**, **6**) and plants treated with extracts A, B, D, E (=1, 2, 4, 5, respectively, in **Figures 5**, **6**) scattered around the origin. In particular, plants treated with F extract (=6 in the **Figures 5**, **6**) were characterized by high values of esterase activity and Mg, whereas plants treated with C extract (=3 in **Figures 5**, **6**) had low values in Fe, Mn, Mo, and Ca. From the projection of PC1 with PC3 (**Figures 6A,B**) it can be noted that untreated plants (=7 in **Figures 5**, **6**) and plants treated with extracts A, B, D, E, F (=1, 2, 4, 5, 6, respectively, in **Figures 5**, **6**) where distributed along a line, evidencing decreasing values in root surface, root length and fine roots from the upper (seaweed extracts B and E, = 2 and 5, respectively, in **Figures 5**, **6**) to lower (extract A and untreated, =1 and 7, respectively, in **Figures 5**, **6**) samples of the cluster.

DISCUSSION

Extracts from seaweeds are rich in several bioactive compounds that might act in plants inducing an array of positive

TABLE 7 | Loadings values of some morphometric and chemical variables on the axes identified by principal components analysis for control (untreated) maize plants and plants supplied for 48 h with extracts obtained from the seaweeds *Laminaria* (A) and *Ascophyllum nodosum* spp. (B–F).

Variable	PC1	PC2	PC3
Mn	0.940	0.091	−0.175
Fe	0.916	0.317	−0.101
Mo	0.868	0.240	−0.027
Ca	0.826	0.373	0.244
B	0.634	0.403	0.150
Zn	0.550	−0.099	−0.087
EstL	0.115	0.928	−0.195
EstR	0.190	0.885	−0.169
Mg	0.427	0.832	0.054
S	0.600	0.756	−0.153
Cu	−0.334	0.558	0.343
Surface	−0.122	−0.152	0.948
Length	−0.046	−0.063	0.946
Fine	−0.122	0.091	0.903
Tips	0.153	−0.154	0.820
Diameter	−0.076	−0.449	0.520

physiological responses, such as improved biomass production, amelioration of nutrition and resistance to stress (Engel et al., 2006). For this reason, they can be employed in agricultural practices to promote the health status of crops.

In this research, extracts from *Laminaria* and *A. nodosum* spp. were tested in maize plants to assay their biostimulant properties. Overall, they enhanced root system development and plant nutrition. The increased root growth by extracts is well evidenced by the PCA analysis, which highlighted a gradient where the untreated plants displayed the lowest values. Though, extracts revealed distinct chemical properties, which could explain their different efficacy in eliciting physiological responses related to plant growth and increased capacity to absorb nutrients (**Figure S1**). Differences in chemical composition of extracts could be ascribed to the algal species and, within the species *A. nodosum*, to different locations where the seaweeds were collected. Also, the amount of biologically active extracted compounds could vary depending on the sampling season and environmental conditions. Specifically, we found several functional groups attributable to polysaccharides (i.e., alginate or fucoidan), for which a number of biological activities have been recognized, including the stimulation of natural defense responses in plants (Hernández-Herrera et al., 2016). Spectroscopic analyses also revealed the presence of functional groups corresponding to lipids and phenols in seaweed extracts. Each type of biomolecule displayed a characteristic signal in FT-IR and Raman spectra. Even though Raman spectroscopy is an emerging tool for the study of bio-macromolecules, in our study only E and F extracts showed an acceptable signal to noise ratio, while for other extracts a strong fluorescence covered completely the weak Raman signals. Diagnostic bands of polysaccharides, mainly agars and carrageenans, were well identified by using FT-IR.

Differences in chemical composition between two *A. nodosum* commercial biostimulants, especially in terms of phenolic compounds, were reported by Goñi et al. (2016) as well. In this case, heterogeneity was evidenced between these biostimulants based on their impacts on the *Arabidopsis thaliana* transcriptome.

All extracts exerted a positive effect on the activity of esterase enzyme, which is considered a marker of plant developmental processes, being involved in organogenesis and functioning as an early indicator of somatic embryogenesis (Chibbar et al., 1988; Pedersen and Andersen, 1993; Krsnik-Rasol et al., 1999; Balen et al., 2003). Esterase belongs to a group of enzymes that hydrolyse ester bonds, and occurs in several isoforms in plant and animal cells. The increase of esterase enzymes associated with wall fractions, for instance, has been proposed to be involved in the turnover of phenolic acids that are cross-linked to wall polysaccharides (Thaker et al., 1986). Therefore, higher esterase activity in maize plants treated with *Laminaria* and *A. nodosum* spp. extracts was indicative of their stimulatory effect on plant biomass production.

Among extracts from *A. nodosum*, E was the most effective in promoting root associated parameters (root length, surface, tip number, and fine roots), as confirmed by the PCA analysis (**Figure 6**) as well. This was likely because of its higher content in hormones such as auxin (IAA = 32.43 nM) and cytokinin (IPA = 5.79 nM), and intermediate values of IAA-like activity. In this respect, several studies have shown that auxins and/or substances endowed with auxin-like activity contained in seaweed extracts and other biostimulants can induce positive effects on lateral root and hair formation (Mugnai et al., 2008; Pereira et al., 2009; Schiavon et al., 2010; Spinelli et al., 2010; Ertani et al., 2013a,b).

Stimulation of root development by seaweed extracts was likely responsible of increased accumulation of several macro- and micro-elements in plants, with a more pronounced effect associated to extracts derived from *A. nodosum* compared to *Laminaria*, like suggested by PCA analysis (**Figures 6**, **7**). The increase of minerals' accumulation in plants treated with algal extracts was previously reported and explained as a result of the up-regulation of genes coding for nitrate, sulfate, and iron transporters, and/or stimulation of root cell division and lateral root/hair development (Mugnai et al., 2008; Pereira et al., 2009; Spinelli et al., 2010).

In our study, F extract was the most successful in enhancing the plant capacity to absorb and accumulate nutrients. This extract displayed similar intermediate values of IAA-like activity as E extract, but was not efficient in improving all root parameters. We hypothesize that the remarkable effect caused by F extract on plant nutrition was primarily due to enhanced transcription and/or activity of plant membrane nutrient transporters rather than to an increase in root absorption surface. It is noteworthy that F extract was the highest in phenol content (1,933 mg L^{-1}). Phenols are important signaling molecules and in adequate amounts exert several positive effects in plants, even when they are exogenously applied or present in biostimulant formulations (Ertani et al., 2011). However, if their concentration is too high in the plant or in the surrounding environment, phenol compounds might become toxic to some extent, thus

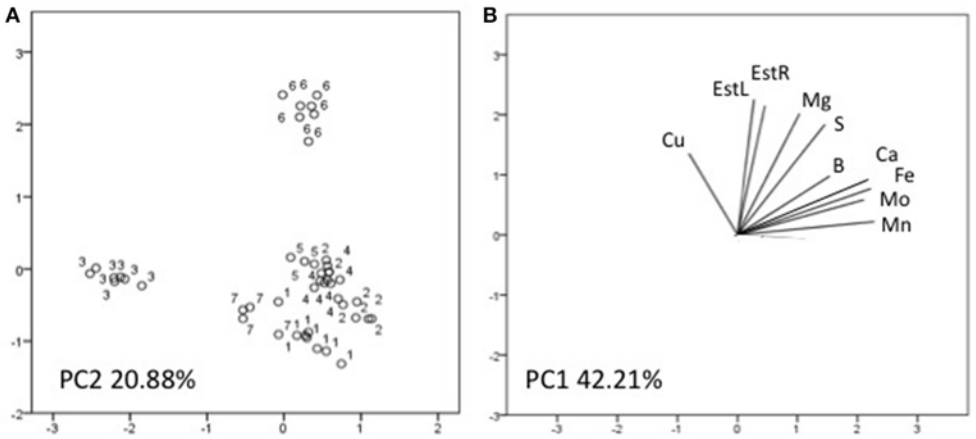


FIGURE 5 | Position of the samples representative of maize plants untreated and treated with seaweed extracts **(A)** and of the variables **(B)** projected in the plane determined by the first two principal axes of the PCA. Seaweed extracts correspond to the circle number A = 1, B = 2, C = 3, D = 4, E = 5, F = 6, and untreated plants = 7. EstL and EstR: esterase activity in leaves and in roots, respectively.

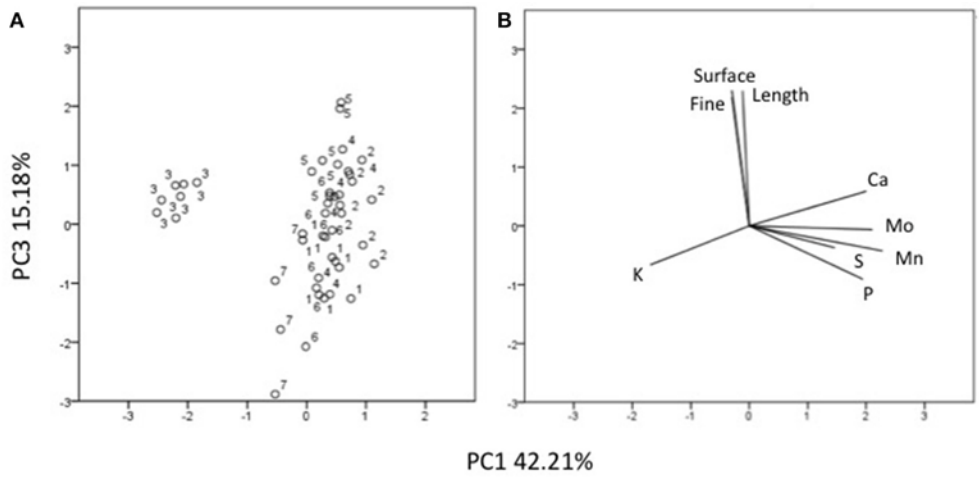


FIGURE 6 | Position of the samples representative of maize plants untreated and treated with seaweed extracts **(A)** and of the variables **(B)** projected in the plane determined by the axis 1 with axis 3 of the PCA. Seaweed extracts correspond to the circle number A = 1, B = 2, C = 3, D = 4, E = 5, F = 6, and untreated plants = 7. Length = root length, surface = root surface area, fine = root fine length.

overcoming the positive effects on root growth normally exerted by substances endowed with auxin-like activity (Muscolo et al., 2013). On the other hand, extracts that are very low in total phenol content may be unable to significantly increase root development as well, regardless if they display IAA-like activity. This may be the case of A extract from *Laminaria*, which was the lowest in total phenols, while displaying GA-like activity (Nardi et al., 2000).

B and D extracts determined equivalent increases in root growth, despite they greatly varied in chemical composition, especially with respect to N content, type of hormone-like activity and phenol content. Perhaps, their similar contents in IPA and IAA could have overcome differences in the type of hormone-like activity. It is also possible that nitrogen in B extract was not all promptly available for plant absorption whether in the form of long peptides, despite some of them may function as signaling molecules (Ertani et al., 2013a,b), or other organic N compounds. B and D extracts also caused a similar accumulation of numerous of elements, with the exception of Ca, Cu (for which D extract promoted higher accumulation) and Zn (more accumulated in plants treated with E), thus suggesting that active molecules in B and D extracts acted on both mineral nutrient transporters and growth signaling pathways, as it can be hypothesized for E extract.

C extract induced similar increases in root growth as B and D extracts, likely by virtue of its high content in IPA (8.45 nM) and IAA-like activity. Nevertheless, it was significantly different from all other extracts, as revealed from PCA analysis (**Figures 5**, **6**): despite the elevate IAA-like activity and its capacity to stimulate root development, apparently it did not exert any appreciable effect on plant nutrition. Even when the total content of individual nutrients in plants treated with extract C was

calculated, values were lower compared to those in plants treated with the other extracts (data not shown). Therefore, while for F extract we hypothesized that its composition was very effective in promoting plant nutrition but not root development, in this case it is possible that the formulation of C extract was more successful in eliciting responses related to root growth.

C extract also caused a decrease of fructose content in plants. Fructose seems to be related to secondary metabolites production, in particular of erythrose-4-P, which acts as a substrate for lignin and phenolic compounds synthesis (Caretto et al., 2015). The other extracts determined a general increase of fructose content, especially in leaves, which may suggest their capacity to trigger the phenlylpropanoid pathway. This metabolic route has been established as one of the main target of biostimulant action in plants (Schiavon et al., 2010; Colla et al., 2015; Ertani et al., 2017) and is critical for plant development and stress conditions overcoming. On the other side, glucose content was found to increase in leaves of plants treated with the majority of extracts, while it was reduced or unaffected in roots. Glucose in plants either acts as a substrate for cellular respiration or osmolyte to maintain cell homeostasis (Rosa et al., 2009). The reduction of this sugar, especially in the roots, may be ascribed to higher consumption because of the respiration process in order to produce more ATP for active nutrient transport.

CONCLUSIONS

This study underlines a close relationship between the chemical properties of commercial seaweed extracts from *Laminaria* and *A. nodosum* spp. and select plant physiological responses they are able to positively induce. The results obtained strongly support the utility a robust chemical characterization of commercial seaweed extracts based on different approaches in predicting the metabolic targets of biostimulants in plants and could be extended to other seaweed biostimulants available in the market.

AUTHOR CONTRIBUTIONS

AE analyzed the chemical characteristics of seaweed extracts and plants, and wrote part of the manuscript, OF and AT performed the spectroscopic analyses and wrote the relative part in the manuscript, DP performed the statistical analysis, root measurements and esterase activity assay, MS wrote part of the manuscript and edited it, SN supervisioned the study.

ACKNOWLEDGMENTS

We would like to thank Dr. Piergiorgio Stevanato for kindly providing us the Epson Expression 10000XL 1.0 system for root morphology analysis, and Federica Zanellato for the precious help in performing lab experiments.

SUPPLEMENTARY MATERIAL

The Supplementary Material for this article can be found online at: https://www.frontiersin.org/articles/10.3389/fpls.2018.00428/full#supplementary-material

Figure S1 | Heat map of plant-associated parameters influenced by individual seaweed extracts. Different colors indicate different levels of induction (+, ++, + + +), repression (–) or no effect (=).

REFERENCES

Ali, N., Farrell, A., Ramsubhag, A., and Jayaraman, J. (2015). The effect of *Ascophyllum nodosum* extract on the growth, yield and fruit quality of tomato grown under tropical conditions. *J. Appl. Phycol.* 28, 1353–1362. doi: 10.1007/s10811-015-0608-3

Arnaldos, T. L., Mu-oz, R., Ferrer, M. A., and Calderón, A. A. (2001). Changes in phenol content during strawberry (Fragaria x ananassa, cv. Chandler) callus culture. *Physiol. Plant*. 113, 315–322. doi: 10.1034/j.1399-3054.2001.1130303.x

Audus, L. J. (1972). *Plant Growth Substances. Chemistry and Physiology*, Vol. 1. London: Leonard Hill Books.

Battacharyya, D., Babgohari, M. Z., Rathor, P., and Prithiviraj, B. (2015). Seaweed extracts as biostimulants in horticulture. *Sci. Hortic.* 196, 39–48. doi: 10.1016/j.scienta.2015.09.012

Balen, B., Krsnik-Rasol, M., and Simeon-Rudolf, V. (2003). Isoenzymes of peroxidase and esterase related to morphogenesis in *Mammillaria gracillis* Pfeiff. tissue culture. *J. Plant Physiol.* 160, 1401–1406. doi: 10.1078/0176-1617-00935

Blunden, G. (1991). "Agricultural uses of seaweeds and seaweed extracts," in *Seaweed Resources in Europe: Uses and Potential*, eds M. D. Guiry and G. Blunden (Chichester: Wiley), 65–81.

Calvo, P., Nelson, L., and Kloepper, J. W. (2014). Agricultural uses of plant biostimulants. *Plant Soil*. 383, 3–41. doi: 10.1007/s11104-014-2131-8

Campos-Vallette, M. M., Chandía, N. P., Clavijo, E., Leal, D., Matsuhiro, B., Osorio-Roman, I. O., et al. (2010). Characterization of sodium alginate and its block fractions by surface-enhanced Raman spectroscopy. *J. Raman Spectrosc.* 41, 758–763. doi: 10.1002/jrs.2517

Caretto, S., Linsalata, V., Colella, G., Mita, G., and Lattanzio, V. (2015). Carbon fluxes between primary metabolism and phenolic pathway in plant tissues under stress. *Int. J. Mol. Sci.* 16, 26378–26394. doi: 10.3390/ijms161125967

Cassan, L., Jean, I., Lamaze, J., and Morotgaudry, J. F. (1992). The effect of the *Ascophylum nodosum* extract Geomer GA14 on the growth of spinach. *Bot. Mar.* 35, 437–439. doi: 10.1515/botm.1992.35.5.437

Chandía, N. P., and Matsuhiro, B. (2008). Characterization of a fucoidan from *Lessonia vadosa* (Phaeophyta) and its anticoagulant and elicitor properties. *Int. J. Biol. Macromol.* 42, 235–240. doi: 10.1016/j.ijbiomac.2007.10.023

Chandía, N. P., Matsuhiro, B., Mejías, E., and Moenne, A. (2004). Alginic acids in Lessonia vadosa: partial hydrolysis and elicitor properties of the polymannuronic acid fraction. *J. Appl. Phycol.* 16, 127–133. doi: 10.1023/B:JAPH.0000044778.44193.a8

Chibbar, R. N., Schyluk, J., Georges, F., Mallard, C. S., and Constabel, F. (1988). Esterase isozymes as markers of somatic embryogenesis in cul- tured carrot cells. *J. Plant Physiol.* 133, 367– 370 doi: 10.1016/S0176-1617(88)80218-9

Colla, G., Nardi, S., Cardarelli, M., Ertani, A., Lucini, L., Canaguier, R., et al. (2015). Protein hydrolysates as biostimulants in horticulture. *Sci. Hortic.* 196, 28–38. doi: 10.1016/j.scienta.2015.08.037

Craigie, J. S. (2011). Seaweed extract stimuli in plant science and agriculture. *J. Appl. Phycol.* 23, 371–393. doi: 10.1007/s10811-010-9560-4

Ding, Y., Feng, R., Wang, R., Guo, J., and Zheng, A. (2014). Dual effect of Se on Cd toxicity: evidence from plant growth, root morphology and responses of the antioxidative systems of paddy rice. *Plant Soil* 375, 289–301. doi: 10.1007/s11104-013-1966-8

Ertani, A., Schiavon, M., Altissimo, A., Franceschi, C., and Nardi, S. (2011). Phenol-containing organic substances stimulate phenylpropanoid metabolism in *Zea mays*. *J. Plant Nutr. Soil Sci.* 174, 496–503. doi: 10.1002/jpln.201000075

Ertani, A., Pizzeghello, D., Altissimo, A., and Nardi, S. (2013a). Use of meat hydrolysate derived from tanning residues as plant biostimulant. *J. Plant Nutr. Soil Sci*. 176, 287–295. doi: 10.1002/jpln.201200020

Ertani, A., Pizzeghello, D., Baglieri, A., Cadili, V., Tambone, F., Gennari, M., et al. (2013b). Humic-like substances from agro-industrial residues affect growth and nitrogen assimilation in maize (*Zea mays* L.) plantlets. *J. Geochem. Exp*. 129, 103–111. doi: 10.1016/j.gexplo.2012.10.001

Ertani, A., Pizzeghello, D., Francioso, O., Sambo, P., Sanchez-Cortes, S., and Nardi, S. (2014). *Capsicum chinensis* L. growth and nutraceutical properties are enhanced by biostimulants in a long-term period: chemical and metabolomic approaches. *Front. Plant Sci*. 5:375. doi: 10.3389/fpls.2014.00375

Ertani, A., Schiavon, M., and Nardi, S. (2017). Transcriptome-wide identification of differentially expressed genes in *Solanum lycopersicon* L. in response to an alfalfa-protein hydrolysate using microarrays. *Front. Plant Sci*. 8:1159. doi: 10.3389/fpls.2017.01159

Engel, S., Puglisi, M. P., Jensen, P. R., and Fenical, W. (2006). Antimicrobial activities of extracts from tropical Atlantic marine plants against marine pathogens and saprophytes. *Mar. Biol*. 149, 991–1002. doi: 10.1007/s00227-006-0264-x

Godlewska, K., Michalak, I., Tuhy, Ł., and Chojnacka, K. (2016). Plant growth biostimulants based on different methods of seaweed extraction with water. *Biomed. Res. Int*. 2016:5973760. doi: 10.1155/2016/5973760

Gómez-Ordó-ez, E., and Rupérez, P. (2011). FTIR-ATR spectroscopy as a tool for polysaccharide identification in edible brown and red seaweeds. *Food Hydrocoll*. 25, 1514–1520. doi: 10.1016/j.foodhyd.2011.02.009

Goñi, O., Fort, A., Quille, P., McKeown, P. C., Spillane, C., and O'Connell, S. (2016). Comparative transcriptome analysis of two *Ascophyllum nodosum* extract biostimulants: same seaweed but different. *J. Agric. Food Chem*. 64, 2980–2989. doi: 10.1021/acs.jafc.6b00621

González, A., Castro, J., Vera, J., and Moenne, A. (2013). Seaweed oligosaccharides stimulate plant growth by enhancing carbon and nitrogen assimilation, basal metabolism, and cell division. *J. Plant Growth Regul*. 32, 443–448. doi: 10.1007/s00344-012-9309-1

González, A., Contreras, R. A., Zú-iga, G., and Moenne, A. (2014). Oligo-carrageenan kappa-induced reducing redox status and activation of TRR/TRX system increase the level of indole-3-acetic Acid, gibberellin A3 and trans-zeatin in *Eucalyptus globulus* trees. *Molecules* 19, 12690–12698. doi: 10.3390/molecules190812690

Guinan, K. J., Sujeeth, N., Copeland, R. B., Jones, P. W., O'Brien, N. M., Sharma, H. S. S., et al. (2013). Discrete roles for extracts of *Ascophyllum nodosum* in enhancing plant growth and tolerance to abiotic and biotic stresses. *Acta Hortic*. 1009, 127–135. doi: 10.17660/ActaHortic.2013.1009.15

Hernández-Herrera, R. M., Santacruz-Ruvalcaba, F., Za-udo-Hernández, J., and Hernández-Carmona, G. (2016). Activity of seaweed extracts and polysaccharide-enriched extracts from *Ulva lactuca* and *Padina gymnospora* as growth promoters of tomato and mung bean plants. *J. Appl. Phycol*. 28, 2549–2560. doi: 10.1007/s10811-015-0781-4

Hong, D. D., Hien, H. M., and Son, P. N. (2007). Seaweeds from Vietnam used for functional food, medicine and fertilizer. *J. Appl. Phycol*. 19, 817–826 doi: 10.1007/s10811-007-9228-x

Joubert, J. M., and Lefranc, G. (2008). Seaweed biostimulants in agriculture: recent studies on mode of action two types of products from alga: growth and nutrition stimulants and stimulants of plant Demence reactions. Book of abstracts: Biostimulants in modern agriculture. Warsaw, 16.

Junge, W., and Klees, H. (1984). "Peroxidases," in *Methods of Enzymatic Analysis IV*, ed H. V. Bergmeyer, *3rd Edn* (New York, NY: Academic Press), 8–14.

Khan, W., Rayirath, U. P., Subramanian, S., Jithesh, M. N., Hodges, D. M., Critchley, A. T., et al. (2009). Seaweed extracts as biostimulants of plant growth and development. *Plant Growth Regul*. 28, 386–399. doi: 10.1007/s00344-009-9103-x

Krsnik-Rasol, M., Cipcic, H., and Hagege, D. (1999). Isoesterases related to cell differentiation in plant tissue culture. *Chemico-Biol. Interact*. 120, 587–592. doi: 10.1016/S0009-2797(99)00073-3

Leal, D., Matsuhiro, B., Rossi, M., and Caruso, F. (2008). FT-IR spectra of alginic acid block fractions in three species of brown seaweeds. *Carbohydr. Res*. 343, 308–316. doi: 10.1016/j.carres.2007.10.016

Lüthje, S., and Böttger, M. (1995). On the function of a K-type vitamin in plasma membranes of maize (*Zea mays* L.) roots. *Mitt. Inst. Allg. Bot. Hamburg* 25, 5–13.

MacKinnon, S. L., Hiltz, D., Ugarte, R., and Craft, C. A. (2010). Improved methods of analysis for betaines in *Ascophyllum nodosum* and its commercial seaweed extracts. *Appl. Phycol*. 22, 489–494. doi: 10.1007/s10811-009-9483-0

Mathlouthi, M., and Koenig, J. L. (1986). Vibrational spectra of carbohydrates. *Adv. Carbohydr. Chem. Biochem*. 44, 7–66. doi: 10.1016/S0065-2318(08)60077-3

Mancuso, S., Azzarello, E., Mugnai, S., and Briand, X. (2006). Marine bioactive substances (IPA extract) improve foliar ion uptake and water stress tolerance in potted *Vitis vinifera* plants. *Adv. Hortic. Sci*. 20, 156–161.

Marinval, N., Sabourel, P., Haddad, O., Maire, M., Bassand, K., Geinguenaud, F., et al. (2016). Identification of a pro-angiogenic potential and cellular uptake mechanism of a LMW highly sulfated fraction of fucoidan from *Ascophyllum nodosum*. *Mar. Drugs* 14:185. doi: 10.3390/md14100185

Michalak, I., Chojnacka, K., Dmytryk, A., Wilk, R., Gramza, M., and Rój, E. (2016). Evaluation of supercritical extracts of algae as biostimulants of plant growth in field trials. *Front. Plant Sci*. 7:1591. doi: 10.3389/fpls.2016.01591

Mugnai, S., Azzarello, E., Pandolfi, C., Salamagne, S., Briand, X., and Mancuso, S. (2008). Enhancement of ammonium and potassium root influxes by the application of marine bioactive substances positively affects *Vitis vinifera* plant growth. *J. Appl. Phycol*. 20, 177–182. doi: 10.1007/s10811-007-9203-6

Muscolo, A., Sidari, M., and da Silva, J. A. T. (2013). Biological effects of water-soluble soil phenol and soil humic extracts on plant systems. *Acta Physiol. Plant* 35, 309–320. doi: 10.1007/s11738-012-1065-0

Nardi, S., Pizzeghello, D., Gessa, C., Ferrarese, L., Trainotti, L., and Casadoro, A. (2000). Low molecular weight humic fraction on nitrate protein synthesis in maize seedlings. *Soil. Biol. Biochem*. 32, 415–419. doi: 10.1016/S0038-0717(99)00168-6

Nardi, S., Pizzeghello, D., Schiavon, M., and Ertani, A. (2016). Plant biostimulants: physiological responses induced by protein hydrolyzed-based products and humic substances in plant metabolism. *Sci. Agric*. 73, 18–23. doi: 10.1590/0103-9016-2015-0006

Pedersen, S., and Andersen, S. B. (1993). Developmental expression of isoenzymes during embryiogenesis in barley anther culture. *Plant Sci*. 91, 75–86 doi: 10.1016/0168-9452(93)90190-B

Pereira, L., Amado, A. M., Critchley, A. T., van de Velde, F., and Ribeiro-Claro, P. J. A. (2009). Identification of selected seaweed polysaccharides (phycocolloids) by vibrational spectroscopy (FTIR-ATR and FT-Raman). *Food Hydrocoll*. 23, 1903–1909. doi: 10.1016/j.foodhyd.2008.11.014

Pereira, L., Gheda, S. F., and Ribeiro-Claro, P. J. A. (2013). Analysis by vibrational spectroscopy of seaweed polysaccharides with potential use in food, pharmaceutical, and cosmetic industries. *Int. J. Carbohydr. Chem*. 2013:7. doi: 10.1155/2013/537202

Prithiviraj, B. (2008). Rapid bioassays to evaluate the plant growth promoting activity of *Ascophyllum nodosum* (L.) Le Jol. using a model plant, *Arabidopsis thaliana (L.)* Heynh. *J. Appl. Phycol*. 20, 423–429 doi: 10.1007/s10811-007-9280-6

Rengasamy, K. R. R., Kulkarni, M. G., Stirk, W. A., and Van Staden, J. (2015). A new plant growth stimulant from the brown seaweed *Ecklonia maxima*. *J. Appl. Phycol*. 27, 581–587. doi: 10.1007/s10811-014-0337-z

Rosa, M., Prado, C., Podazza, G., Interdonato, R., González, J. A., Hilal, M., et al. (2009). Soluble sugars—metabolism, sensing and abiotic stress A complex network in the life of plants. *Plant Signal Behav*. 4, 388–393. doi: 10.4161/psb.4.5.8294

Roussos, P. A., Denaxa, N. K., and Damvakaris, T. (2009). Strawberry fruit quality attributes after application of plant growth stimulating compounds. *Sci. Hortic*. 119, 138–146. doi: 10.1016/j.scienta.2008.07.021

Satish, L., Ceasar, S. A., Shilpha, J., Rency, S. A., Rathinapriya, P., and Ramesh, M. (2015). Direct plant regeneration from *in vitro*-derived shoot apical meristems of finger millet (*Eleusine coracana* (L.) Gaertn.). *In Vitro Cell Dev. Biol. Plant*. 51, 192–200. doi: 10.1007/s11627-015-9672-2

Schiavon, M., Pizzeghello, D., Muscolo, A., Vaccaro, S., Francioso, O., and Nardi, S. (2010). High molecular size humic substances enhance phenylpropanoid metabolism in Maize (*Zea mays* L.) *J. Chem. Ecol*. 36, 662–669. doi: 10.1007/s10886-010-9790-6

Santaniello, A., Scartazza, A., Gresta, F., Loreti, E., Biasone, A., Di Tommaso, D., et al. (2017). *Ascophyllum nodosum* seaweed extract alleviates drought stress in Arabidopsis by affecting photosynthetic performance and related gene expression. *Front. Plant Sci.* 8:1362. doi: 10.3389/fpls.2017.01362

Sharma, H. S. S., Fleming, C., Selby, C., Rao, J. R., and Martin, T. (2014). Plant biostimulants: a review on the processing of macroalgae and use of extracts for crop management to reduce abiotic and biotic stresses. *J. Appl. Phycol.* 26, 465–490. doi: 10.1007/s10811-013-0101-9

Sivasankari, S., Venkatesalu, V., Anantharaj, M., and Chandrasekaran, M. (2006). Effect of seaweed extracts on the growth and biochemical constituents of Vigna sinensis. *Bioresour. Technol.* 97, 1745–1751. doi: 10.1016/j.biortech.2005.06.016

Spinelli, F., Fiori, G., Noferini, M., Sprocatti, M., and Costa, G. (2010). A novel type of seaweed extract as a natural alternative to the use of iron chelates in strawberry production. *Sci Hortic.* 125, 263–269. doi: 10.1016/j.scienta.2010.03.011

Synytsya, A., Bleha, R., Synytsya, A., Pohl, R., Hayashi, K., Yoshinaga, K., et al. (2014). Mekabu fucoidan: structural complexity and defensive effects against avian influenza A viruses. *Carbohydr. Polym.* 111, 633–644. doi: 10.1016/j.carbpol.2014.05.032

Thaker, V. S., Saroop, S., Vaishnav, P. P., and Singh, Y. D. (1986). Role of peroxidase and esterase activities during cotton fiber development. *J. Plant Growth Regul.* 5, 17–27. doi: 10.1007/BF02027383

Yang, L., and Zhang, L. M. (2009). Chemical structural and chain conformational characterization of some bioactive polysaccharides isolated from natural sources. *Carbohydr. Polym.* 76, 349–361 doi: 10.1016/j.carbpol.2008.12.015

Conflict of Interest Statement: The authors declare that the research was conducted in the absence of any commercial or financial relationships that could be construed as a potential conflict of interest.

frontiers in Plant Science

ORIGINAL RESEARCH
published: 10 April 2018
doi: 10.3389/fpls.2018.00472

A Vegetal Biopolymer-Based Biostimulant Promoted Root Growth in Melon While Triggering Brassinosteroids and Stress-Related Compounds

*Luigi Lucini[1], Youssef Rouphael[2], Mariateresa Cardarelli[3], Paolo Bonini[4], Claudio Baffi[1] and Giuseppe Colla[5]**

[1] Department for Sustainable Food Process, Università Cattolica del Sacro Cuore, Piacenza, Italy, [2] Department of Agricultural Sciences, University of Naples Federico II, Portici, Italy, [3] Consiglio per la Ricerca in Agricoltura e l'Analisi dell'Economia Agraria, Centro di Ricerca Orticoltura e Florovivaismo, Pontecagnano Faiano, Italy, [4] NGA Laboratory, Tarragona, Spain, [5] Department of Agricultural and Forestry Sciences, University of Tuscia, Viterbo, Italy

OPEN ACCESS

Edited by:
Barbara De Lucia, Università degli Studi di Bari Aldo Moro, Italy

Reviewed by:
Georgios Soteriou, Agricultural Research Institute, Cyprus
Vincenzo Candido, University of Basilicata, Italy

****Correspondence:***
Giuseppe Colla
giucolla@unitus.it

Specialty section:
This article was submitted to Plant Breeding, a section of the journal Frontiers in Plant Science

Received: *03 March 2018*
Accepted: *26 March 2018*
Published: *10 April 2018*

Citation:
Lucini L, Rouphael Y, Cardarelli M, Bonini P, Baffi C and Colla G (2018) A Vegetal Biopolymer-Based Biostimulant Promoted Root Growth in Melon While Triggering Brassinosteroids and Stress-Related Compounds. Front. Plant Sci. 9:472. doi: 10.3389/fpls.2018.00472

Plant biostimulants are receiving great interest for boosting root growth during the first phenological stages of vegetable crops. The present study aimed at elucidating the morphological, physiological, and metabolomic changes occurring in greenhouse melon treated with the biopolymer-based biostimulant Quik-link, containing lateral root promoting peptides, and lignosulphonates. The vegetal-based biopolymer was applied at five rates (0, 0.06, 0.12, 0.24, or 0.48 mL plant^{-1}) as substrate drench. The application of biopolymer-based biostimulant at 0.12 and 0.24 mL plant^{-1} enhanced dry weight of melon leaves and total biomass by 30.5 and 27.7%, respectively, compared to biopolymer applications at 0.06 mL plant^{-1} and untreated plants. The root dry biomass, total root length, and surface in biostimulant-treated plants were significantly higher at 0.24 mL plant^{-1} and to a lesser extent at 0.12 and 0.48 mL plant^{-1}, in comparison to 0.06 mL plant^{-1} and untreated melon plants. A convoluted biochemical response to the biostimulant treatment was highlighted through UHPLC/QTOF-MS metabolomics, in which brassinosteroids and their interaction with other hormones appeared to play a pivotal role. Root metabolic profile was more markedly altered than leaves, following application of the biopolymer-based biostimulant. Brassinosteroids triggered in roots could have been involved in changes of root development observed after biostimulant application. These hormones, once transported to shoots, could have caused an hormonal imbalance. Indeed, the involvement of abscisic acid, cytokinins, and gibberellin related compounds was observed in leaves following root application of the biopolymer-based biostimulant. Nonetheless, the treatment triggered an accumulation of several metabolites involved in defense mechanisms against biotic and abiotic stresses, such as flavonoids, carotenoids, and glucosinolates, thus potentially improving resistance toward plant stresses.

Keywords: biostimulants, *Cucumis melo* L., hormone-like activity, lignosulfonates, metabolomics, peptides

INTRODUCTION

A well-developed root system is an important agronomic trait of horticultural crops, with implications on crop productivity, abiotic stress tolerance as well as nutrient uptake and assimilation (Koevoets et al., 2016). In fruiting vegetables (i.e., tomato, eggplant, pepper, melon, and watermelon), the most intense root growth development is usually concentrated during the first phenological stages and decreases over time due to the limited translocation of photosynthates (i.e., soluble sugars) resulting from the strong sink demand for reproductive growth (Heuvelink, 2005). Therefore, a vigorous and well-developed root system during early phenological stages able to explore the soil volume is a primary requisite for securing yield stability under both optimal and suboptimal conditions (Jung and McCouch, 2013).

Breeding and genetic engineering efforts to improve crop productivity are practically focused on shoot traits, whereas the root traits are still not fully exploited source of crop improvement (Wachsman et al., 2015; Koevoets et al., 2016). More recently, a variety of biostimulant substances (i.e., humic and fulvic acids, protein hydrolysates, and seaweed extracts) and microbial inoculants (i.e., mycorrhizal fungi and plant growth promoting rhizobacteria-PGPR) has been introduced as an efficient, safe, and sustainable tool to optimize root system thus boosting crop performance, and nutrient use efficiency as well as enhancing tolerance to environmental stressors (du Jardin, 2015; Colla et al., 2015a, 2017a; Rouphael et al., 2015, 2017b,c). The stimulation of biomass production in response to biostimulant application has been usually associated to the action of specific *signaling molecules* on plant metabolism and physiology (Colla et al., 2015b, 2017a).

Over the past decade, vegetal-based biopolymers like peptides and lignosulphonates have gained prominence worldwide as biostimulant molecules in vegetable cropping systems (Ertani et al., 2011; Matsumiya and Kubo, 2011; Colla et al., 2013, 2014, 2017b). Matsumiya and Kubo (2011) have shown that bioactive peptides isolated from soybean seeds had phytohormone-like activities since they able to promote the root hair formation (root hair length and numbers) of annual baby's-breath, cabbage, Italian clover, and lettuce. Furthermore, Colla et al. (2014) also demonstrated a root stimulation effect induced by a commercial legume seeds-derived protein hydrolysate Trainer. The mode of action through which Trainer improves the absorption and transport of nutrients may be at least partially associated to changes in root growth with lateral root formation through an auxin-signaling mediated pathway (Colla et al., 2014, 2015a, 2017a). In addition, also lignosulphonates have been shown to elicit both auxin and gibberellin-like activities incurring a significant increase in maize biomass (Ertani et al., 2011). In their study Ertani et al. (2011) demonstrated that lignosulphonates treatment improved N assimilation in maize plants through the stimulation of key enzymes (glutamate-synthase and glutamine-synthetase) and also by promoting photosynthetic activity through the stimulation of both rubisco enzyme activity as well as chlorophyll biosynthesis. Besides the biostimulant-mediated enhancement of root growth, vegetal-based biopolymers can also upregulate the expression of genes encoding for plasma membrane H^+-ATPases and nitrate transporters, resulting in greater efficiency in nitrate uptake and assimilation (Tavares et al., 2017).

Despite the above described effects of biopolymers in plants, conclusive evidences regarding their molecular targets are far from being elucidated. In this perspective, untargeted profiling approaches by metabolomics have proven to be a powerful tool for shedding light on the mode of action of vegetal-based biopolymers (Lucini et al., 2015, 2016; Ntatsi et al., 2017). Indeed, metabolomics allows identifying those compounds altered by treatment, thus driving optimal target uses of biopolymers-based products in agriculture. In turn, this information on the mechanisms through which biostimulants act in plant, can drive a better use of these products by highlighting those scenarios where they provide a real and essential contribution to crop production.

The elucidation of fundamental plant physiological and biochemical responses to vegetal-based biopolymer application can be instrumental to understand the mechanisms behind the well-developed root system; hence the objective of the current study was to assess the morphological, physiological, and metabolomic changes in response to vegetal-based biopolymer application on greenhouse melon. Melon plants treated with vegetal-based biopolymer by substrate drench were compared to untreated plants in terms of biomass production and partitioning, root morphology, SPAD index, chlorophyll fluorescence as well as metabolic profiling.

MATERIALS AND METHODS

Plant Material, Growth Conditions, Biostimulant Treatments, and Experimental Design

The greenhouse trial was carried out in the spring 2016 growing season in a 300 m^2 polyethylene greenhouse at the Experimental Farm of Tuscia University, central Italy (latitude 42°25′N, longitude 12°08′E, altitude 310 m). Transplants of melon (*Cucumis melo* L. - cv. Giorillo commercialized by Seminis, Milan, Italy) grown in polystyrene plug trays (84 holes) were planted at the second true-leaf stage on 4th May in black plastic pots (15 cm diameter; one plant per pot) containing 1.8 L of sandy soil. Fertilization was performed prior planting by mixing for each kilogram of soil 2 g of a slow mineral fertilizer containing (g kg^{-1}) 150 N, 39.2 P, 124.5 K, 12.1 Mg, 80.0 S, 3.0 Fe, 0.1 B, 0.1 Mn, 0.02 Cu, and 0.02 Zn. Plants were grown under natural light conditions. The greenhouse was maintained at daily temperatures between 18 and 28°C, and day/night relative humidity of 60/85%. Just after transplanting, plants were irrigated with 200 mL of water per pot.

The five biostimulant application treatments were four rates of the commercial Quik-Link product: 0, 0.06, 0.12, 0.24, or 0.48 mL $plant^{-1}$ (= 0, 0.3, 0.6, 1.2, or 2.4 L ha^{-1}, respectively; the rates per hectare were calculated considering a localized biostimulant placement near the transplants with a plant

density of 5,000 plants ha^{-1}). Quik-link is a biopolymer-based biostimulant manufactured by Italpollina S.p.A., Rivoli Veronese, Italy; the product contains Lateral root promoting peptides (LRPP) and other biopolymers (e.g., lignosulphonates) with high biological activity on plants, and micronutrients (10 g kg^{-1} Fe, 7 g kg^{-1} Mn, 3 g kg^{-1} Zn, 1 g kg^{-1} Cu, 0.2 g kg^{-1} Mo). Quik-link is allowed in organic agriculture according to the Council Regulation (EC) No. 834/2007 of 28 June 2007.

Treatments were arranged in a randomized block design with four replicates. Each experimental unit consisted of 10 plants. Fifty milliliters of a water solution containing Quik-link product was applied manually after 2 days from transplanting around the collar level of each plant. During the experiment, plants were irrigated as needed with a drip irrigation system.

Growth Measurements and Root Characteristics

At the end of the greenhouse experiment (16th May; 12 days after transplanting-DAT) melon plants were separated into leaves, stems, and roots. All plant tissues were dried at 80°C for 72 h until they reached a constant weight which corresponded to their dry biomasses. Shoot dry weight was equal to the sum of the aerial vegetative parts (leaves + stems), and the root-to-shoot ratio was also calculated. Two plants per experimental plot were used for the root morphology determination. Root system collection and sample preparation were performed following the protocol described previously by Rouphael et al. (2017a). Briefly, the melon root were gently washed with fresh water, until the roots were free from any sandy particles. The determination of the root system architecture components was done using a WinRHIZO Pro (Regent Instruments Inc., Canada), connected to a STD4800 scanner. The following root morphology characteristics were recorded: total root length, mean root diameter, and total root surface area (Colla et al., 2014).

Soil Plant Analysis Development Index and Chlorophyll Fluorescence

Soil Plant Analysis Development (SPAD) index and the fluorescence measurements of melon leaves were also recorded at the end of the experiment. The relative leaf chlorophyll concentration expressed as a rational unit was measured using a portable chlorophyll meter SPAD-502 (Minolta Corporation, Ltd., Osaka, Japan). The SPAD measurements were made on the fully expanded leaves which correspond to the third leaf starting from the apical shoot, as described by Colla et al. (2011). Twenty random readings per experimental unit were taken and averaged to a single SPAD value for each biostimulant application treatment.

On the same date, the chlorophyll fluorescence was measured every 20 min on dark-adapted leaves (two measurements per plant) by means of a chlorophyll fluorometer Handy PEA (Hansatech Instruments Ltd., King's Lynn, United Kingdom) with excitation source intensity higher than 3,000 μmol m^{-2} s^{-1} at the sample surface as described previously by Borgognone et al. (2016). Briefly, the minimal fluorescence intensity (F_0) in a dark-adapted state was measured in the presence of a background weak light signal (about 2–3 μmol photons m^{-2} s^{-1}). The maximal fluorescence level in the dark-adapted state (F_m) was induced by 0.8 s saturating light pulse (3,000 μmol photons m^{-2} s^{-1}). The maximum quantum yield of open photosystem II (PSII) (F_v/F_m) was calculated as $(F_m–F_0)/F_m$, as also described by Borgognone et al. (2016).

Collection of Samples and Metabolomic Analysis

The first fully expanded leaf (the second leaf from the growing tip) and root (terminal roots) samples were collected from two plants per experimental plot treated with 0 or 0.24 mL $plant^{-1}$ of the biostimulant Quik-link. Leaf and root tissues were then quenched in liquid nitrogen and grounded into a fine powder using mortar and pestle, thereafter leaf and root samples were stored at −80°C for successive metabolomic analysis.

Samples (1.0 g) of five replicates per treatment were extracted by homogenization in 15 mL of 80% methanol added with 0.1% HCOOH, using an Ultra-Turrax (Ika T-25, Staufen, Germany). Extracts were centrifuged (9000 × *g*), filtered through a 0.22 μm cellulose syringe filter and then transferred to an amber vial for analysis. Metabolomic analysis was done using a 1290 UHPLC liquid chromatography system coupled to a G6550 quadrupole-time-of-flight mass spectrometer (UHPLC-ESI/QTOF-MS – Agilent Technologies, Santa Clara, CA, United States). The mass spectrometer was equipped with a JetStream dual Electrospray ionization source.

Instrumental conditions were taken from previous experiments (Pretali et al., 2016). Briefly, UHPLC separation was achieved on an Agilent Zorbax Eclipse-plus column (75 mm × 2.1 mm i.d., 1.8 μm) with a mobile phase consisting of water (A) and methanol (B), and a flow of 220 μL min^{-1} at 35°C. The gradient was operated from 5 to 90% B in 35 min, whereas the QTOF mass spectrometer was set in positive polarity, scan acquisition (100–1200 m/z^+) and extended dynamic range mode. Nebulizer pressure was 60 psig, sheath gas was nitrogen at 10 L min^{-1} (350°C), drying gas was nitrogen at 10 L min^{-1} (280°C) and capillary voltage was 4 kV.

Features deconvolution, as well as the following mass and retention time alignment, were done in Profinder B.05 (from Agilent Technologies). Annotation was achieved using accurate mass, isotope accurate spacing and isotope ratio against the database PlantCyc 9.5 (Plant Metabolic Network[1]; released November 2014). Hence, a Level 2 of identification (i.e., putatively annotated compounds) was achieved, with reference to COSMOS Metabolomics Standards Initiative[2]. Compounds were finally filtered by frequency, retaining only those compounds that were present in 100% of replications within at least one treatment, then exported for statistics.

Statistical Analysis of Experimental Data

Analysis of variance (ANOVA) of the experimental data was made using the SPSS software package (IBM SPSS Statistics version 20.0.0). Orthogonal contrasts (Gomez and Gomez, 1983)

[1]http://www.plantcyc.org

[2]http://cosmos-fp7.eu/msi

were used to compare the biostimulant concentration effects on morphological and physiological parameters. Duncan test was also performed at $P = 0.05$ on each of the significant variables measured.

Regarding metabolomics, the dataset was interpreted in Agilent Mass Profiler Professional B.12.06 (from Agilent Technologies) as previously reported (Lucini et al., 2017). Compounds abundance was normalized at the 75th percentile and baselined to the median of control following the adoption of a threshold of 10000 counts. Pairwise comparisons were done in Volcano Plot analysis, by combining analysis of variance ($P < 0.05$, Bonferroni multiple testing correction) and fold-change analysis (cut-off = 5). The dataset was next exported into SIMCA 13 (Umetrics, Malmo, Sweden), pareto-scaled and elaborated for Principal Component Analysis. Thereafter, Orthogonal Projections to Latent Structures Discriminant Analysis (OPLS-DA) modeling was carried out for leaves and roots, separately. OPLS-DA supervised multivariate analysis targeted separating variation between the groups into predictive and orthogonal (i.e., ascribable to technical and biological variation) components. Outliers were excluded according to Hotelling's T2 and, adopting 95 and 99% confidence limits for suspect and strong outliers, respectively. The OPLS-DA model was validated through cross validation CV-ANOVA ($p < 0.01$) and overfitting was excluded by permutation testing ($n = 100$). OPLS-DA goodness-of-fit R^2Y and goodness-of-prediction Q^2Y were recorded and finally variables importance in projection (VIP analysis) was used to select those having the highest discrimination potential (VIP score > 1.46).

RESULTS

Morphological and Physiological Parameters

In the current study, no significant differences on dry weight of stem, SPAD index and the maximum quantum use efficiency of PSII in dark-adapted state (F_v/F_m) were observed between biostimulant application rates. The mean dry weight of stem, SPAD index and F_v/F_m were 32.9 mg plant^{-1}, 51.2 and 0.82, respectively (**Table 1**).

The growth parameters in particular the dry weight of leaves as well as the total dry biomass were influenced by the vegetal-based biopolymer applications (**Table 1**). For instance, increasing the concentration of the biopolymer-based biostimulant from 0.0 to 0.48 mL plant^{-1} incurred a quadratic behavior of both leaves and total shoot biomass (**Figure 1** and **Table 1**). Our experimental data indicated that biopolymer-based biostimulant at 0.12 and 0.24 mL plant^{-1} enhanced dry weight of melon leaves and total biomass by 30.5 and 27.7%, respectively, compared to biopolymer applications at 0.06 mL plant^{-1} and untreated plants, with no significant difference found between 0.12 and 0.24 mL plant^{-1} rates (**Table 1**).

Root Morphological Characteristics

The root-to-shoot ratio as well as the average root diameter were not affected by the vegetal-based biopolymer applications. The mean root-to-shoot ratio and root diameter of melon plants recorded in the current study were 0.25 and 0.36 mm, respectively (**Table 2**). Similarly, to the effects on plant growth parameters (dry weight of leaves and total biomass), the root dry biomass and the root morphology characteristics (total root length and surface) in biostimulant-treated plants were significantly higher at 0.24 mL plant^{-1} and to a lesser extent at 0.12 and 0.48 mL plant^{-1}, in comparison to untreated melon plants (**Table 2**). In fact, the root application of the commercial biopolymer biostimulant Quik-link at a rate of 0.24 mL plant^{-1} elicited significant increase in 12-days melon transplants root biomass, total root length, and surface amounting to 34.1, 32.0, and 32.6%, respectively, compared to biostimulant applications at 0.06 mL plant^{-1} and untreated plants (**Figure 1** and **Table 2**).

Metabolic Profiling of Melon Leaves and Roots

The changes in metabolic profile of roots and leaves of melon, following treatment with the biostimulant Quik-link at the rate of 0.24 mL plant^{-1}, was investigated through UHPLC-ESI/QTOF-MS untargeted metabolomics. Overall, more than 2,300 compounds were annotated and passed the filter thresholds adopted. The following unsupervised Principal Component Analysis carried out from metabolomic profiles allowed to identify two main clusters, one per matrix (**Figure 2**). Even if matrix type was, as expected, the main classification factor, two clearly distinct sub-clusters could be evidenced for root profiles, whereas leaf profiles were partially overlapped. This indicated that the effect of Quik-link was more evident in roots, and suggested the need of more focused multivariate analyses to better point out those compounds being responsible of differences across treatments. The supervised OPLS-DA model, carried out separately for roots and leaves (**Figure 3**), provided with an excellent separation of control and Quik-link treated samples. In more detail, goodness-of-fit R^2Y was 0.62 and 0.65 and prediction ability Q^2Y was 0.68 and 0.66, for leaves and roots respectively. Therefore, prediction ability was above the acceptability threshold of 0.5 (Rombouts et al., 2017) and was considered as acceptable. Coherently, cross-validation ANOVA resulted in in a Fischer's probability of 0.004 for both OPLS-DA models. Furthermore, overfitting was excluded by permutation testing and no outliers could be pointed out by Hotelling's T2 (**Supplementary Material**). Provided that differences between treatments were represented in the dataset, Volcano analysis ($P < 0.05$, fold-change > 5) allowed exporting differential compounds for roots and leaves separately (**Tables 3** and **4**, respectively). These compounds were ascribed into classes by taking into count their biochemical function and physiological roles.

Regarding roots, most of the compounds were related to stress response (i.e., flavonoids, carotenoids, and glucosinolates) rather than hormone network (mainly brassinosteroids and cytokinins), followed by membrane lipids and other compounds. Although a well-defined trend cannot be observed for membrane lipids, flavonoids and carotenoids, glucosinolates were down-accumulated (excepting

TABLE 1 | Effect of biopolymer-based biostimulant applications on dry weight of leaves, stems, total biomass, SPAD index, and maximum quantum use efficiency of photosystem II in dark-adapted state (F_v/F_m) of melon plants grown under greenhouse conditions.

Biostimulant (mL plant^{-1})	Dry biomass (mg plant^{-1})			SPAD index	F_v/F_m
	Leaves	Stems	Total		
0.00	570.3 c	32.8	603.1 b	50.1	0.82
0.06	569.2 c	37.1	606.3 b	53.8	0.83
0.12	787.0 a	31.6	814.6 a	51.2	0.82
0.24	700.7 ab	28.9	729.6 ab	52.3	0.82
0.48	657.0 bc	33.9	690.9 bc	48.7	0.82
Significance	Q**	ns	Q**	ns	ns
R^2	0.53	–	0.53	–	–

*Q, quadratic; NS, **Non-significant or significant at $P \leq 0.01$, respectively. R^2 = coefficient of determination for quadratic regression model. Different letters within each column indicate significant differences according to Duncan's multiple range test (P = 0.05).*

FIGURE 1 | Twelve-day-old plants of melon treated with 0 **(A)**, 0.06 **(B)**, 0.12 **(C)**, 0.24 **(D)**, or 0.48 **(E)** mL plant^{-1} of biopolymer-based biostimulant.

TABLE 2 | Effect of biopolymer-based biostimulant applications on dry weight of roots, root-to-shoot ratio, total root length, average root diameter, and total root surface of melon plants grown under greenhouse conditions.

Biostimulant (mL plant^{-1})	Root dry biomass (mg plant^{-1})	Root-to-shoot ratio	Total root length (cm plant^{-1})	Root diameter (mm)	Total root surface (cm^2 plant^{-1})
0.00	128.3 c	0.22	709.5 c	0.35	86.9 c
0.06	161.7bc	0.26	882.1bc	0.36	106.3bc
0.12	185.6 ab	0.23	1006.0 ab	0.37	121.5 ab
0.24	194.4 a	0.27	1050.4 a	0.36	128.1 a
0.48	180.3 ab	0.26	979.6 ab	0.36	120.3 ab
Significance	Q**	ns	Q**	ns	Q*
R^2	0.95	–	0.95	–	0.96

*Q, quadratic; NS, *, **Non-significant or significant at $P \leq 0.05$ and $P \leq 0.01$, respectively. R^2 = coefficient of determination for quadratic regression model. Different letters within each column indicate significant differences according to Duncan's multiple range test (P = 0.05).*

for 2/3-(5′-methylthio)pentylmalate). Cytokinins were also down-accumulated whereas brassinosteroids were generally up-accumulated (**Table 3**). VIP analysis from OPLS-DA supervised modeling (**Supplementary Material**) confirmed the involvement of brassinosteroids, being 6-alpha-hydroxycampestanol (isobaric with 3-epi-6-deoxocathasterone and 6-deoxocathasterone), campest-4-en-3beta-ol and campesterol among the compounds with the highest VIP score. Notably, other top-ranking OPLS-DA VIP score compounds such as the carotenoids zeaxanthin and 15,15′-dihydroxy-beta-carotene, were in common with Volcano plot analysis.

However, Volcano Plot analysis highlighted that the changes in leaves metabolic profile following application of Quik-link was distinct from response in roots (**Table 4**). A complex and wide alteration of hormone profile, involving among other brassinosteroids, auxin, abscisic acid (ABA), cytokinins, and gibberellins, was observed. The most clear trends were related to the increase of ABA intermediates (excepting the ABA glucose ester), brassinosteroids (excepting the precursor campesterol), and cytokinins. Furthermore, several stress-related compounds were selected by Volcano Plot, namely the oxidative stress-related 3-hexenal and hydroxycaprate, few alkaloids, carotenoids (all of them up-accumulated excepting for bixin) and flavonoids (also found to be up-accumulated). Membrane lipids and sterols were also altered by the treatment, together with compounds from other classes. Among these latter, pyrrole-related (delta1-pyrroline-2-carboxylate and 4-(1-methyl-2-pyrrolidinyl)-3-oxobutanoate methyl ester) and photosynthesis-related (2-methoxy-6-*all trans*-octaprenyl-2-methoxy-1,4-benzoquinol and a

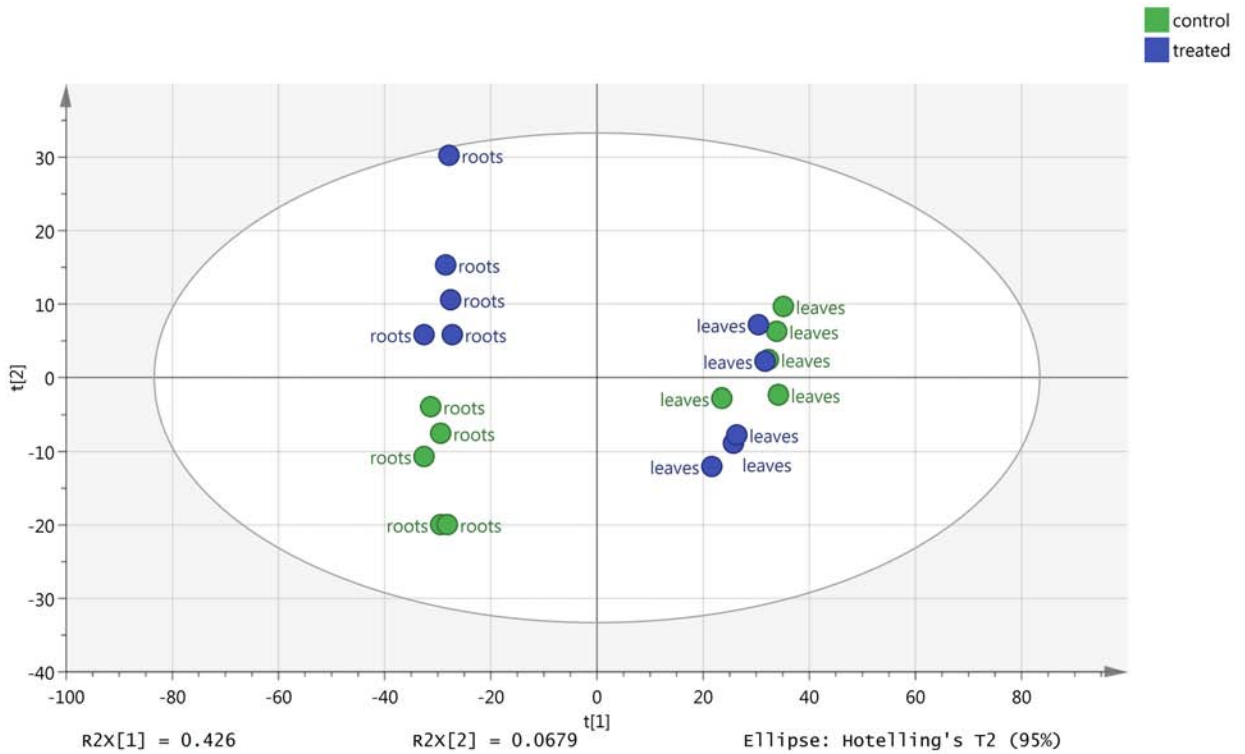


FIGURE 2 | Principal Component Analysis (PCA)-based unsupervised analysis of melon roots and leaves metabolomic profile following root application of 0 (control) or 0.24 mL plant^{-1} of the biopolymer-based biostimulant.

primary fluorescent chlorophyll catabolite) metabolites were pointed out. Discriminating compounds gained by VIP analysis following OPLS-DA confirmed the involvement of brassinosteroids (26-hydroxybrassinolide), membrane lipids (a lysophosphatidylcholine and a digalactosylglycerol), and photosynthesis-related compounds (haematoporphyrin IX, menaquinol-8, and dimethyl phylloquinone) in response to Quik-link treatment (**Supplementary Material**).

DISCUSSION

The use of biopolymer-based biostimulant in vegetable cropping systems can promote root growth leading to a better transplant establishment, and higher crop productivity. The current study demonstrated that substrate drench of a biopolymer-based biostimulant elicit dose-dependent (especially at 0.12 and 0.24 mL plant^{-1}) increases of biomass production of melon transplants. A presumed mechanism behind the stimulation of biomass production in response to root application of vegetal-based biopolymers could be the presence of *signaling compounds* in particular bioactive peptides (e.g., LRPP) as well as lignosulfonates. The former *signaling molecules* in the Quik-link formulation which is easily perceived by root organ may have triggered a signal transduction pathway through modulation of various endogenous phytohormone biosynthesis (Matsumiya and Kubo, 2011; Colla et al., 2017a). Our findings are in agreement with the results of several research teams (Ertani et al., 2011; Matsumiya and Kubo, 2011; Colla et al., 2013, 2014) who showed that foliar of root applications of small peptides and lignosulfonate-humate elicited hormone-like activities on a wide range of agronomic and horticultural crops (baby's-breath, cabbage, Italian clover, and lettuce, maize and tomato), thus boosting plant growth and yield.

Another putative mechanism behind the enhancement of plant growth parameters (dry weight of leaves and total biomass) induced by Quik-link application could be attributed to the stimulation of root morphology characteristics in particular the total root length and surface area (at 0.24 mL plant^{-1} and to a lesser extent at 0.12 and 0.48 mL plant^{-1}), which may improve nutrient uptake and utilization efficiency with beneficial effect on biomass production (Matsumiya and Kubo, 2011). Our results are in line with previous studies assessing the stimulation action of a plant-derived protein hydrolysate containing bioactive peptides and commercial lignosulfonate-humate on root biomass and morphology (Ertani et al., 2011; Colla et al., 2014). For instance, Ertani et al. (2011) demonstrated that the application of lignosulfonate-humates to the nutrient solution (applied at 0.0, 0.5, or 1.0 mg L^{-1}) elicited a dose-dependent increase of root dry weight (from 17 to 24%) in hydroponically-maize compared to the untreated control. Similarly, Colla et al. (2014) showed that the root application of 6 mL L^{-1} of a plant-derived protein hydrolysate increased root dry weight, length, and surface area of tomato cuttings by 24–35% in comparison to untreated plants.

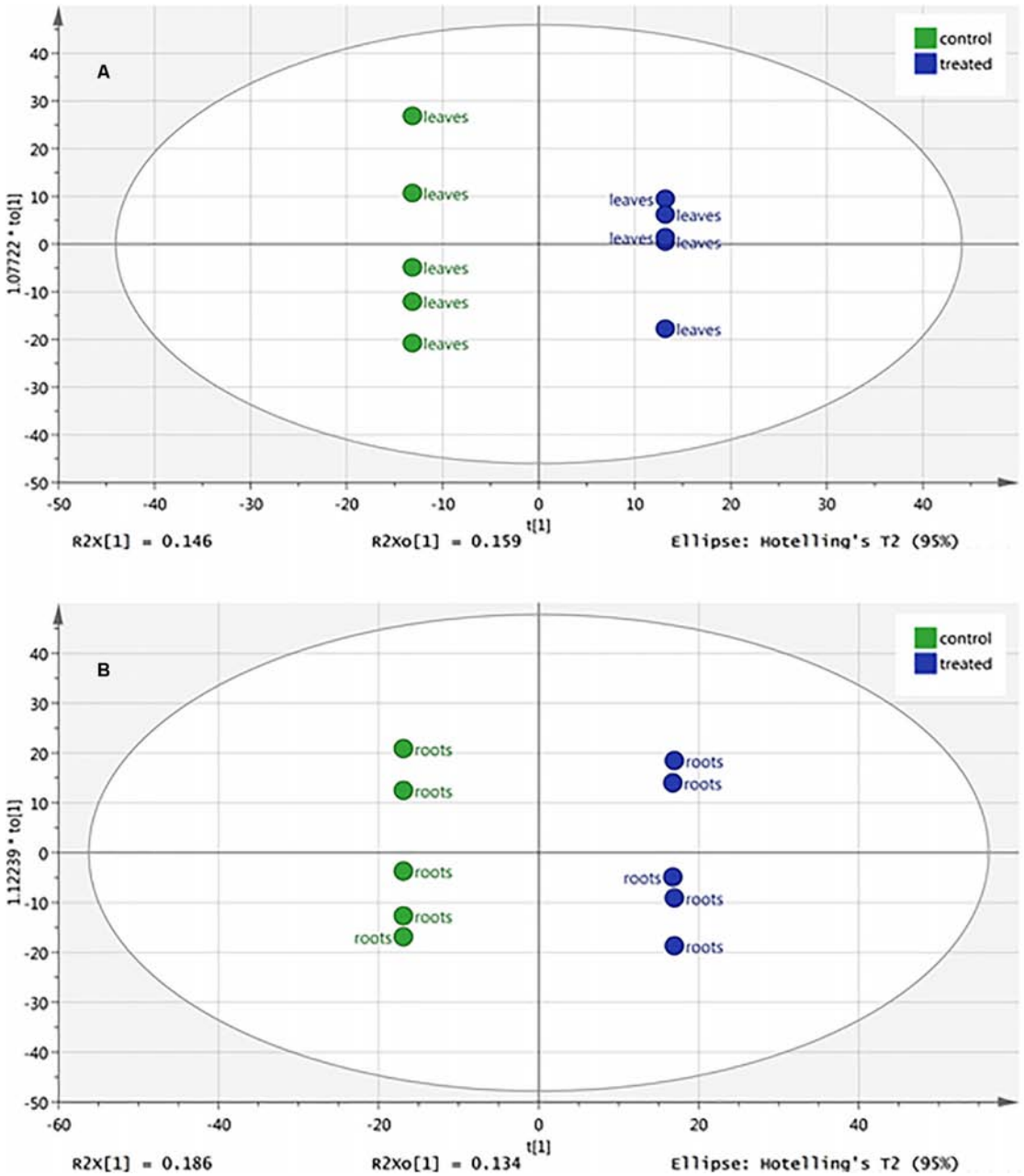


FIGURE 3 | Orthogonal Projections to Latent Structures Discriminant Analysis (OPLS-DA) of melon leaf **(A)** and root **(B)** metabolomic profile following root application of 0 (control) or 0.24 mL plant^{-1} of the biopolymer-based biostimulant. Ellipses denote Hotelling's T2 (95%).

Quik-link application resulted in a metabolic reprogramming, with roots undergoing a stronger change in biochemical profile. Indeed, despite Volcano Plot analysis highlighted a higher number of differential compounds in leaves, PCA unsupervised multivariate statistics showed that the metabolic profile of roots was markedly different in treated samples, as compared to control. Metabolomics was used to better understand the changes induced by the treatment and to highlight the processes underlying the morphological changes observed. Roots response to Quik-link application involved compounds falling into two main processes, namely stress response and hormone profile.

Among the latter, brassinosteroids appeared to play a pivotal role, with several compounds pointed out both by Volcano Plot analysis (most of them being up-accumulated) and by OPLS-DA VIP analysis. Brassinosteroids are polyhydroxy steroid lactone hormones that are essential for normal plant growth, having effect on various developmental processes of plants (Li and Chory, 1999; Zhu et al., 2013). They are produced

TABLE 3 | Differential metabolites changing in melon root after applying the biopolymer-based biostimulant, as identified through Volcano Plot analysis ($P < 0.05$, Bonferroni multiple testing correction, and fold-change cut-off = 5).

Class		Metabolite annotated	*P*-value	Fold-change	Regulation
Hormone activity	Cytokinin	Kinetin-9-*N*-glucoside/kinetin-7-*N*-glucoside	0	16	Down
		N6-(Delta2-isopentenyl)-adenosine 5′-monophosphate	0	16	Down
	Brassinosteroid	Typhasterol	0	79	Down
		6alpha-hydroxycampestanol	1.04E−13	1.89E+06	Up
		Teasterone/campesterol	0.44	8.27E+04	Up
	Auxin	Indole-3-acetyl-glutamate	0.38	9.46E+03	Down
	Gibberellin	Gibberellin A9	0	2.62E+02	Up
Stress response	Flavonoid	Quercetagetin-7-*O*-glucoside	0	1.61E+02	Up
		A 4′-hydroxyflavanone	0	16	Down
	Carotenoid	Canthaxanthin	0	16	Down
		15,15′-dihydroxy-beta-carotene	2.96E−10	9.87E+04	Up
		Zeaxanthin	0	28	Down
	Glucosinolate	2 / 3-(5′-methylthio)pentylmalate	0	16	Up
		5-methylthiopentylhydroximoyl-cysteinylglycine	0	1.21E+05	Down
		7-methylthioheptylhydroximoyl-glutathione	0	16	Down
		8-methylthiooctyl glucosinolate	0	5.96E+04	Down
Lipids	Membrane lipid	sphingosine 1-phosphate	4.81E−10	5.26E+05	Down
		1-18:1-2-16:2-monogalactosyldiacylglycerol	0	16	Up
		1-18:3-2-18:1-phosphatidylcholine	0	16	Down
Others		Salicyl alcohol	0.56	2.50E+02	Up
		4alpha-formyl-4beta-methyl-5alpha-cholesta-8,24-dien-3beta-ol	0	16	Up
		D-sedoheptulose-1,7-bisphosphate	0	6.88E+02	Down

According to Mass Profiler Professional, P-values of 0 denote very high significance, whereas fold-change = 16 identify very high fold-changes.

from plant sterols, via teasterone, typhasterol, and castasterone, by an isoprenoid biosynthetic pathway that includes acetyl CoA (Ali, 2017). Brassinosteroids signaling pathway leaded to the identification of a putative brassinosteroid receptor as well as brassinosteroid-responsive genes (Li and Chory, 1999; Lin et al., 2017). These hormones are implicated in a wide range of physiological and biochemical responses in plants, including seed germination, cell division and elongation, vascular differentiation, photomorphogenesis, photosynthesis, and senescence (Ali, 2017). They have also been found to protect plants from both abiotic and biotic stress factors, such as salinity, drought, heavy metals, and pathogens (Ali, 2017). Interestingly, beside promoting crop yield, they are reported to regulate plant architecture (Lin et al., 2017), to promote root growth in a dose-dependent manner (Mussig, 2003) and to have an important role in directing epidermal cell fate in roots, regulating differentiation into hair or non-hair cells (Zhu et al., 2013). Therefore, the changes of brassinosteroids in plant tissues can help to explain the increased root development following Quik-link application. Indeed, these hormones display direct interaction with gibberellins signaling pathway, promoting mitotic activity and expression of cyclins in the root meristem (Zhu et al., 2013). In general, brassinosteroids are reported to have a wide interplay with several hormones including an antagonistic role toward ABA (Clouse, 2016) and ethylene biosynthesis (Zhu et al., 2015).

It is described that exogenous brassinosteroids are taken up through the roots to be translocated, unchanged, to the shoot (Symons et al., 2008). However, the same authors reported that, when applied directly to shoot tissues, exogenous brassinosteroids are relatively immobile. Coherently, brassinosteroids were found among differential compounds also in leaves both following Volcano Plot analysis (with 6-alpha-hydroxy-castasterone, cathasterone, and 22alpha-hydroxy-campest-4-en-3-one being up-accumulated) and OPLS-DA VIP analysis (26-hydroxybrassinolide). Several other hormones were found among differential compounds in leaves; the ethylene precursor 1-aminocyclopropane-1-carboxylate and the jasmonate precursor 13-hydroperoxylinoleate were both down-accumulated. Moreover, ABA precursors were up-accumulated, and its storage form beta-D-glucopyranosyl abscisate was down-accumulated. This might suggest that ABA biosynthesis was hampered by brassinosteroids. Furthermore, cytokinins and the most of gibberellins were both up-accumulated, whereas a clear trend could not be observed for auxins. Generally, brassinosteroids induced response appeared to affect the complex interplay and cross-talk between plant hormones, in agreement with previous literature (Li and Chory, 1999; Zhu et al., 2015; Clouse, 2016; Lin et al., 2017).

A correlation between net photosynthesis and brassinosteroids action has been also reported (Ali, 2017); indeed, a benzoquinol, a chlorophyll catabolite, and two pyrrole-related intermediates were up-accumulated in leaves following application of Quik-link. Similarly, hematoporphyrin IX, menaquinol-8, and demethylphylloquinone were among the compounds with the highest VIP score in OPLS-DA.

In a previous study focused on the effect of brassinosteroids on chill injury of fruits and vegetables during post-harvest,

TABLE 4 | Differential metabolites changing in melon leaves after applying the biopolymer-based biostimulant, as identified through Volcano Plot analysis ($P < 0.05$, Bonferroni multiple testing correction, and fold-change cut-off = 5).

Class		Metabolite annotated	*P*-value	Fold-change	Regulation
Hormones	Abscisic acid	Abscisic aldehyde	0	16	Up
		Beta-D-glucopyranosyl abscisate	0.02	3.09E+02	Down
		Antheraxanthin	0.03	3.11E+02	Up
		Zeinoxanthin	0.03	0.98E+02	Up
		3,4,3′,4′-tetradehydroisozeaxanthin	0	16	Up
	Auxin	Phenylacetate	0.04	1.02E+02	Up
		Indole acetaldehyde	0.05	6.78E+02	Down
		Indole-3-acetyl-leucine	0.05	1.08E+03	Up
	Brassinosteroid	Campesterol	0.05	0.37E+02	Down
		6-alpha-hydroxy-castasterone	0.05	1.42E+02	Up
		cathasterone	0.05	4.15E+02	Up
		(22-alpha)-hydroxy-campest-4-en-3-one	0.05	4.34E+03	Up
	Cytokinin	CPPU	0.03	0.16E+02	Up
		N6-dimethylallyladenine	0.05	0.25E+02	Up
		Zeatin-7-*N*-glucoside	0	16	Up
	Ethylene	1-aminocyclopropane-1-carboxylate	0.05	1.99E+02	Down
	Gibberellin	Gibberellin A25	0.05	1.96E+02	Down
		Ent-kaurene	0.03	0.10E+02	Up
		Ent-7-alpha-Hydroxykaurenoate	0.03	0.15E+02	Up
		Gibberellin A9 methyl ester / gibberellin A12	0.05	1.55E+03	Up
	Jasmonate	(S)-13-Hydroperoxylinolenate	0.05	0.41E+02	Down
Stress response	Oxidative stress	3-hexenal	0	16	Up
		Hydroxycaprate	0	16	Down
	Alkaloid	(S)-corytuberine	0	16	Up
		N-formyldemecolcine	0	16	Down
	Carotenoid	Bixin	1.14E−07	7.65E+04	Down
		3,5-dihydroxy-6,7-didehydro-5,6-dihydro-12'-apo-beta-caroten-12'-al	0	16	Up
		4-methylocta-2,4,6-trienedial	0	16	Up
		3-hydroxy-beta-ionone	0	16	Up
		Zeaxanthin	0.05	1.11E+04	Up
	Flavonoid	Sakuranin	2.22E−12	9.86E+04	Up
		6a-hydroxymaackiain	0	16	Up
		2,5,7-trihydroxy-4'-methoxyisoflavanone	0	16	Up
		Delphinidin-3-*O*-beta-D-glucoside	0	16	Down
		Cyanidin-3-*O*-rutinoside-5-*O*-beta-D-glucoside	0	16	Up
		2′,3,4,4′,6′-pentahydroxychalcone 4′-*O*-beta-D-glucoside	0	16	Up
Lipids	Membrane lipid	1-18:3-2-16:0-monogalactosyldiacylglycerol	0	16	Up
		Acetylcholine	1.95E−11	1.48E+05	Up
	Sterol	4-alpha-carboxy-4-beta-methyl-5-alpha-cholesta-8-en-3-beta-ol	0	16	Up
		Stigmasterol	0	16	Up
		Delta24-25-sitosterol	0	16	Down
		11-alpha-hydroxy-9,15-dioxoprost-13-enoate	0	16	Up
Others		5-deoxystrigol	0	2.58E+04	Down
		18-episcalar-17(25)-en-19-ol	0	16	Up
		2-methoxy-6-*all trans*-octaprenyl-2-methoxy-1,4-benzoquinol	0	16	Up
		Primary fluorescent chlorophyll catabolite	0	16	Up
		Delta1-pyrroline-2-carboxylate	0	16	Up
		4-(1-methyl-2-pyrrolidinyl)-3-oxobutanoate methyl ester	0	16	Up
		4-methylthiobutylhydroximoyl-cysteinylglycine	0	16	Down

According to Mass Profiler Professional, P-values of 0 denote very high significance, whereas fold-change = 16 identify very high fold-changes.

Soleimani Aghdam et al. (2016) reported: (i) enhanced membrane integrity, (ii) enhancing antioxidant capacity, and (iii) alteration in phenylalanine ammonia-lyase (PAL) and polyphenol oxidase (i.e., two key enzymes in phenolics biosynthesis and degradation) activities. Similarly, carotenoids were induced in tomato fruits following application of brassinosteroids (Zhu et al., 2015). This might explain the involvement of flavonoids and carotenoids in roots and, more markedly, in leaves (where both were up-accumulated). Nonetheless, these responses related to flavonoids and carotenoids, together with the changes in alkaloids and glucosinolates (two additional classes of metabolites displaying a pivotal role in stress response) are likely connected, at least in part, into the brassinosteroids-dependent response to biotic and abiotic stresses.

CONCLUSION

The application of the commercial vegetal-based biopolymer Quik-link as substrate drench improved the melon growth parameters in a dose-dependent manner. The improve in plant biomass production was associated to the stimulation of the root growth and morphology traits, thus inducing a '*nutrient acquisition response*' that favors nutrient uptake and utilization efficiency. Metabolomics allowed to point out a wide and complex biochemical response to the treatment, in which hormone profile, and brassinosteroids in particular, appeared to play a pivotal role. Root metabolic profile was markedly altered following application of the test product. In roots, brassinosteroids could have been responsible of changes in root development. These hormones are known to be transported to shoots, where they might have interfered with other hormones through a complex and wide cross talk that resulted in the hormonal imbalance. Besides potentially shaping plant structure, brassinosteroids were likely responsible of the changes in other secondary metabolites such as phenolics and carotenoids, as well as the modulation of photosynthetic activity. Regardless the eventual direct role of brassinosteroids, the treatment triggered an up-accumulation of several metabolites involved in defense mechanisms against biotic and abiotic stresses, such as flavonoids, carotenoids, and glucosinolates. Although more focused and dedicated experiments are needed in this direction, this could result in an improved resistance toward plant stress.

AUTHOR CONTRIBUTIONS

LL performed the metabolomic study, analyzed the data, and wrote many parts of the manuscript. YR performed the agronomic study, analyzed the data, and wrote many parts of the manuscript. MC performed the root growth measurements and implemented the manuscript. PB gave support in the data analysis and interpretation. CB collaborated in the metabolomic study. GC provided intellectual inputs for defining the experimental design, data analysis and interpretation, and improved the manuscript.

SUPPLEMENTARY MATERIAL

The Supplementary Material for this article can be found online at: https://www.frontiersin.org/articles/10.3389/fpls.2018.00472/full#supplementary-material

FIGURE S1 | Hotelling's T2 investigation on outliers as carried out in UHPLC/QTOF-MS metabolomic investigations on root and leaf metabolic profile following root application of the biopolymer-based biostimulant.

FIGURE S2 | Visual representation of the VIP-selected discriminating compounds from the OPLS-DA modeling on roots **(A)** and leaves **(B)** metabolic profile, following root application of the biopolymer-based biostimulant.

TABLE S1 | VIP-selected discriminating compounds from OPLS-DA modeling on root and leaf metabolic profile, following root application of the biopolymer-based biostimulant.

TABLE S2 | Whole dataset gained from UHPLC/QTOF-MS metabolomic investigations on melon metabolic profile following root application of the biopolymer-based biostimulant. Compounds abundance, together with retention time and composite spectra (mass-abundance pairwise) are provided.

REFERENCES

Ali, B. (2017). Practical applications of brassinosteroids in horticulture - Some field perspectives. *Sci. Hort.* 225, 15–21. doi: 10.1016/j.scienta.2017.06.051

Borgognone, D., Rouphael, Y., Cardarelli, M., Lucini, L., and Colla, G. (2016). Changes in biomass, mineral composition, and quality of cardoon in response to NO-3:Cl- ratio and nitrate deprivation from the nutrient solution. *Front. Plant Sci.* 7:978. doi: 10.3389/fpls.2016.00978

Clouse, S. D. (2016). Brassinosteroid/abscisic acid antagonism in balancing growth and stress. *Dev. Cell* 38, 118–120. doi: 10.1016/j.devcel.2016.07.005

Colla, G., Hoagland, L., Ruzzi, M., Cardarelli, M., Bonini, P., Canaguier, R., et al. (2017a). Biostimulant action of protein hydrolysates: unraveling their effects on plant physiology and microbiome. *Front. Plant Sci.* 8:2202. doi: 10.3389/fpls.2017.02202

Colla, G., Cardarelli, M., Bonini, P., and Rouphael, Y. (2017b). Foliar applications of protein hydrolysate, plant and seaweed extracts increase yield but differentially modulate fruit quality of greenhouse tomato. *HortScience* 52, 1214–1220. doi: 10.21273/hortsci12200-17

Colla, G., Nardi, S., Cardarelli, M., Ertani, A., Lucini, L., Canaguier, R., et al. (2015a). Protein hydrolysates as biostimulants in horticulture. *Sci. Hortic.* 196, 28–38. doi: 10.1016/j.scienta.2015.08.037

Colla, G., Rouphael, Y., Di Mattia, E., El-Nakhel, C., and Cardarelli, M. (2015b). Co-inoculation of *Glomus intraradices* and *Trichoderma atroviride* acts as a biostimulant to promote growth, yield and nutrient uptake of vegetable crops. *J. Sci. Food. Agric.* 95, 1706–1715. doi: 10.1002/jsfa.6875

Colla, G., Rouphael, Y., Canaguier, R., Svecova, E., and Cardarelli, M. (2014). Biostimulant action of a plant-derived protein hydrolysate produced through enzymatic hydrolysis. *Front. Plant Sci.* 5:448. doi: 10.3389/fpls.2014.00448

Colla, G., Rouphael, Y., Mirabelli, C., and Cardarelli, M. (2011). Nitrogen-use efficiency traits of mini-watermelon in response to grafting and nitrogen-fertilization doses. *J. Plant Nutr. Soil Sci.* 174, 933–994. doi: 10.1002/jpln.201000325

Colla, G., Svecova, E., Rouphael, Y., Cardarelli, M., Reynaud, H., Canaguier, R., et al. (2013). Effectiveness of a plant –derived protein hydrolysate to improve crop performances under different growing conditions. *Acta Hortic.* 1009, 175–179. doi: 10.17660/actahortic.2013.1009.21

du Jardin, P. (2015). Plant biostimulants: definition, concept, main categories and regulation. *Sci. Hortic.* 196, 3–14. doi: 10.1016/j.scienta.2015.09.021

Ertani, A., Francioso, O., Tugnoli, V., Righi, V., and Nardi, S. (2011). Effect of commercial lignosulfonate-humate on *Zea mays* L. metabolism. *J. Agric. Food Chem.* 59, 11940–11948. doi: 10.1021/jf202473e

Gomez, K. A., and Gomez, A. A. (1983). "Comparison between treatment means," in *Statistical Procedures for Agricultural Research*, 2nd Edn, eds K. A. Gomez and A. A. Gomez (Hoboken NJ: John Wiley & Sons), 187–240.

Heuvelink, E. (2005). *Tomatoes*, 1st Edn. Oxfordshire: CAB International. doi: 10.1079/9780851993966.0000

Jung, J. K., and McCouch, S. (2013). Getting to the roots of it: genetic and hormonal control of root architecture. *Front. Plant Sci.* 4:186. doi: 10.3389/fpls.2013.00186

Koevoets, I. T., Venema, J. H., Elzenga, J. T. M., and Testerink, C. (2016). Roots withstanding their environment: exploiting root system architecture responses to abiotic stress to improve crop tolerance. *Front. Plant Sci.* 7:1335. doi: 10.3389/fpls.2016.01335

Li, J., and Chory, J. (1999). Brassinosteroid actions in plants. *J. Exp. Bot.* 50, 275–282. doi: 10.1093/jxb/50.332.275

Lin, Y., Zhao, Z., Zhou, S., Liu, L., Kong, W., Chen, H., et al. (2017). Top Bending Panicle1 is involved in brassinosteroid signaling and regulates the plant architecture in rice. *Plant Physiol. Biochem.* 121, 1–13. doi: 10.1016/j.plaphy.2017.10.001

Lucini, L., Borgognone, D., Rouphael, Y., Cardarelli, M., Bernardi, J., and Colla, G. (2016). Mild potassium chloride stress alters the mineral composition, hormone network, and phenolic profile in artichoke leaves. *Front. Plant Sci.* 7:948. doi: 10.3389/fpls.2016.00948

Lucini, L., Rocchetti, G., Kane, D., and Trevisan, M. (2017). Phenolic fingerprint allows discriminating processed tomato products and tracing different processing sites. *Food Control* 73, 696–703. doi: 10.1016/j.foodcont.2016.09.020

Lucini, L., Rouphael, Y., Cardarelli, M., Canguier, R., Kumar, P., and Colla, G. (2015). The effect of a plant-derived biostimulant on metabolic profiling and crop performance of lettuce grown under saline conditions. *Sci. Hortic.* 182, 124–133. doi: 10.1016/j.scienta.2014.11.022

Matsumiya, Y., and Kubo, M. (2011). "Soybean peptide: novel plant growth promoting peptide from soybean," in *Soybean and Nutrition*, ed. H. El-Shemy (Rijeka: In Tech Europe Publisher), 215–230.

Mussig, C. (2003). Brassinosteroids promote root growth in Arabidopsis. *Plant Phys.* 133, 1261–1271. doi: 10.1104/pp.103.028662

Ntatsi, G., Aliferis, K. A., Rouphael, Y., Napolitano, F., Makris, K., Kalala, G., et al. (2017). Salinity source alters mineral composition and metabolism of *Cichorium spinosum*. *Environ. Exp. Bot.* 141, 113–123. doi: 10.1016/j.envexpbot.2017.07.002

Pretali, L., Bernardo, L., Butterfield, T. S., Trevisan, M., and Lucini, L. (2016). Botanical and biological pesticides elicit a similar induced systemic response in tomato (*Solanum lycopersicum*) secondary metabolism. *Phytochemistry* 130, 56–63. doi: 10.1016/j.phytochem.2016.04.002

Rombouts, C., Hemeryck, L. Y., Van Hecke, T., De Smet, S., De Vos, W. H., and Vanhaecke, L. (2017). Untargeted metabolomics of colonic digests reveals kynurenine pathway metabolites, dityrosine and 3-dehydroxycarnitine as red versus white meat discriminating metabolites. *Sci. Rep.* 7:42514. doi: 10.1038/srep42514

Rouphael, Y., Cardarelli, M., Bonini, P., and Colla, G. (2017a). Synergistic action of a microbial-based biostimulant and a plant derived-protein hydrolysate enhances lettuce tolerance to alkalinity and salinity. *Front. Plant Sci.* 8:131. doi: 10.3389/fpls.2017.00131

Rouphael, Y., Colla, G., Giordano, M., El-Nakhel, C., Kyriacou, M. C., and De Pascale, S. (2017b). Foliar applications of a legume-derived protein hydrolysate elicit dose dependent increases of growth, leaf mineral composition, yield and fruit quality in two greenhouse tomato cultivars. *Sci. Hortic.* 22, 353–360. doi: 10.1016/j.scienta.2017.09.007

Rouphael, Y., De Micco, V., Arena, C., Raimondi, G., Colla, G., and De Pascale, S. (2017c). Effect of *Ecklonia maxima* seaweed extract on yield, mineral composition, gas exchange and leaf anatomy of zucchini squash grown under saline conditions. *J. Appl. Phycol.* 29, 459–470. doi: 10.1007/s10811-016-0937-x

Rouphael, Y., Franken, P., Schneider, C., Schwarz, D., Giovannetti, M., Agnolucci, M., et al. (2015). Arbuscular mycorrhizal fungi act as biostimulants in horticultural crops. *Sci. Hortic.* 196, 91–108. doi: 10.1016/j.scienta.2015.09.002

Soleimani Aghdam, M., Babalar, M., and Askari Sarcheshmeh, M. A. (2016). "Impact of brassinosteroids on postharvest physiology of fruits and vegetables," in *Eco-Friendly Technology for Postharvest Produce Quality*, ed. M. W. Siddiqui (Amsterdam: Elsevier Science), 203–218. doi: 10.1016/B978-0-12-804313-4.00006-2

Symons, G. M., Ross, J. J., Jager, C. E., and Reid, J. B. (2008). Brassinosteroid transport. *J. Exp. Bot.* 59, 17–24. doi: 10.1093/jxb/erm098

Tavares, O. C. H., Santos, L. A., Ferreira, L. M., Sperandio, M. V. L., da Rocha, J. G., Garcia, A. C., et al. (2017). You have full text access to this content Humic acid differentially improves nitrate kinetics under low- and high-affinity systems and alters the expression of plasma membrane H+-ATPases and nitrate transporters in rice. *Ann. Appl. Biol.* 170, 89–103. doi: 10.1111/aab.12317

Wachsman, G., Sparks, E. E., and Benfey, P. N. (2015). Genes and networks regulating root anatomy and architecture. *New Phytol.* 208, 26–38. doi: 10.1111/nph.13469

Zhu, J. Y., Sae-Seaw, J., and Wang, Z. Y. (2013). Brassinosteroid signalling. *Development* 140, 1615–1620. doi: 10.1242/dev.060590

Zhu, T., Tan, W. R., Deng, X. G., Zheng, T., Zhang, D. W., and Lin, H. H. (2015). Effects of brassinosteroids on quality attributes and ethylene synthesis in postharvest tomato fruit. *Postharvest Biol. Technol.* 100, 196–204. doi: 10.1016/j.postharvbio.2014.09.016

Conflict of Interest Statement: The authors declare that the research was conducted in the absence of any commercial or financial relationships that could be construed as a potential conflict of interest.

frontiers in Plant Science

ORIGINAL RESEARCH
published: 17 April 2018
doi: 10.3389/fpls.2018.00427

Low-Molecular-Weightt Polysaccharides From *Pyropia yezoensis* Enhance Tolerance of Wheat Seedlings (*Triticum aestivum L.)* to Salt Stress

Ping Zou[1], Xueli Lu[1], Changliang Jing[1], Yuan Yuan[1], Yi Lu[2], Chengsheng Zhang[1], Lei Meng[3], Hongtao Zhao[3] and Yiqiang Li[1]**

[1] *Marine Agriculture Research Center, Tobacco Research Institute of Chinese Academy of Agricultural Sciences, Qingdao, China,* [2] *College of Agriculture, Qingdao Agricultural University, Qingdao, China,* [3] *State Key Laboratory of Bioactive Seaweed Substances, Qingdao, China*

OPEN ACCESS

Edited by:
Giuseppe Colla, Università degli Studi della Tuscia, Italy

Reviewed by:
Daniele Massa, Consiglio per la Ricerca in Agricoltura e l'Analisi dell'Economia Agraria (CREA), Italy
Suprasanna Penna, Bhabha Atomic Research Centre, India

***Correspondence:**
Hongtao Zhao
zht@bmscn.com
Yiqiang Li
liyiqiang@caas.cn

Specialty section:
This article was submitted to Crop and Product Physiology, a section of the journal Frontiers in Plant Science

Received: *18 November 2017*
Accepted: *20 March 2018*
Published: *17 April 2018*

Citation:
Zou P, Lu X, Jing C, Yuan Y, Lu Y, Zhang C, Meng L, Zhao H and Li Y (2018) Low-Molecular-Weightt Polysaccharides From Pyropia yezoensis Enhance Tolerance of Wheat Seedlings (Triticum aestivum L.) to Salt Stress. Front. Plant Sci. 9:427. doi: 10.3389/fpls.2018.00427

Soil salinity is one of the major issues worldwide that affects plant growth and reduces agricultural productivity. Seaweed polysaccharides have been shown to promote crop growth and improve the resistance of plant to abiotic stresses. *Pyropia yezoensis* is a commercially important edible red alga in Southeast Asia. However, there is little research on the application of polysaccharides from *P. yezoensis* in agriculture. The molecular weight (MW) of polysaccharides influences their properties. Therefore, in this study, four representative polysaccharides from *P. yezoensis* (PP) with different MWs (MW: 3.2, 10.5, 29.0, and 48.8 kDa) were prepared by microwave-assisted acid hydrolysis. The relationship between the degradation of polysaccharides from *P. yezoensis* (DPP) and their effects on plant salt tolerance was investigated. The results showed that exogenous PP and DPPs increased wheat seedling shoot and root lengths, and fresh and dry weights, alleviated membrane lipid peroxidation, increased the chlorophyll content and enhanced antioxidant activities. The expression level examination analysis of several Na^+/K^+ transporter genes suggested that DPPs could protect plants from the damage of salt stress by coordinating the efflux and compartmentation of Na^+. The results demonstrated that polysaccharides could regulate antioxidant enzyme activities and modulate intracellular ion concentration, thereby to protect plants from salt stress damage. Furthermore, there was a significant correlation between the tolerance of wheat seedlings to salt stress and MW of polysaccharides. The results suggested that the lower-MW samples (DPP1, 3.2 kDa) most effectively protect wheat seedlings against salt stress.

Keywords: ***Pyropia yezoensis*, polysaccharides, molecular weight, salt stress, antioxidant enzyme activities**

INTRODUCTION

Soil salinization is a major concern in agriculture. It is estimated that by 2050, salinization will degrade up to 30% of all cultivated land (Van Oosten et al., 2017). High salinity is a major abiotic stress factor that restricts crop growth and yield worldwide. Salinity reduces photosynthesis, plant growth and development in plants by causing osmotic stress, ion injury or oxidative stress (Rivero et al., 2014). Plants have developed several comprehensive mechanisms to tolerate or overcome the effects of salinity (Bose et al., 2014). The application of exogenous biostimulators may effectively protect plants against salinity.

Pyropia yezoensis is a commercially important edible red alga in Southeast Asia. It is cultivated and consumed in China, Japan, and South Korea. The chemical structure of *P. yezoensis* polysaccharides was investigated by Zhou et al. (2012). It has a backbone of alternating (1 → 4) -3,6-anhydro-α-L-galactopyranose units and (1 → 3) - linked β-D-galactose or (1 → 4)-linked α-L-galactose 6-sulfate units. In recent years, *P. yezoensis* polysaccharides have attracted attention as biomedical material because of their and antioxidants (Zhao et al., 2006; Zhou et al., 2012), anticancer (Yu et al., 2015), hypolipidemic (Qian et al., 2014), immunoregulatory (Zhang et al., 2003), the neurotrophic and neuroprotective activities (Mohibbullah et al., 2015). Moreover, it has been demonstrated that polysaccharides carrageenans and their oligosaccharides can trigger defense systems against plant pathogens including bacteria, virus and fungi (Stadnik and de Freitas, 2014). Sangha et al. (2010) found that λ-carrageenan can activate defense mechanisms of *Arabidopsis thaliana* (L.) Heynhold against *Sclerotinia sclerotiorum*(Libert) de Baryvia an increase in oxalate oxidase activity and the genes expression associated with jasmonic acid signaling. Vera et al. (2012) demonstrated the application of κ-, ι-, and λ-oligo-carrageenans resulted in a durable protection against tobacco mosaic virus (TMV), *Botrytis cinerea* Persoon and *Pectobacterium carotovorum* (Jones) Waldee.

The activities of polysaccharides depend on the molecular weight (MW), the number of sulfate groups and the molar ratio of sulfate/total sugar. The MW of polysaccharides influences their physicochemical behavior. It may be difficult for high-MW crude polysaccharides to permeate the basement membrane and exert its effects *in vivo*. In contrast, a low-MW polysaccharide prepared from *Laminaria japonica* has effective scavenging activities on superoxide radical, hydroxyl radical, and hypochlorous acid *in vitro* (Zha et al., 2016). Zhou's work indicated that the ultrasonic degraded polysaccharide from *Porphyra yezoensis* exhibited stronger antioxidant activity than that of the natural polysaccharide (Zhou et al., 2012). These results indicate that polysaccharide antioxidant activity may be inversely proportional to MW. The aim of this paper was to study the relationship between MW of polysaccharides from *P. yezoensis* and their activities of improving plant's tolerance to salt stress. Therefore, the polysaccharides were partially degraded in the aqueous phase with the assistance of microwaves. Then the effects of four degraded and natural polysaccharides on wheat seedlings under salt stress were investigated. These polysaccharides may represent a potentially simple, efficacious, and sustainable approach for inhibiting the harm to commercially important crops caused by abiotic stress.

MATERIALS AND METHODS

Materials and Equipments

In February 2017, *P. yezoensis* individuals were collected from a farming raft in Rizhao, Shandong Province, China. These *P. yezoensis* individuals were washed twice with fresh water, flash-frozen, and stored at −20°C. The frozen samples were lyophilised and ground in a mill. The dried sample powder was stored at 4°C.

Standard sugars, Nitro blue terazolium (NBT), hydrogen peroxide (H_2O_2), trichloroacetic acid (TCA) were purchased from Sigma Chemicals Co. All other reagents were of analytical grade. Microwave synthesis/extraction reaction station (Type: MAS-II) was purchased from Shanghai SINEO Microwave Chemistry Technology Co., Ltd. (China).

Preparation of Polysaccharide Fraction From *P. yezoensis*

Dry algae powder (100 g) was extracted with distilled water (4 L) at 100°C for 4 h. The slurry was filtered through gauze and the liquid supernatant was filtered using siliceous earth. The filtrate was dialyzed against distilled water for 48 h then the solution was concentrated to approximately ¼ of its original volume under reduced pressure. The polysaccharide was precipitated using four times volume of ethanol and then lyophilized to yield white powdered products and referred as PP.

To prepare the polysaccharide fraction, a reaction solution (2% w/v) was prepared by dissolving PP (2.0 g) in distilled water (100 mL). Different volumes of hydrogen chloride (HCl) were added to the solution at various temperatures and time intervals (Li et al., 2013). The mixtures were exposed to microwave irradiation with constant magnetic stirring and temperature monitoring with an infrared thermometer in a laboratory microwave reaction station. The reaction mixture was then removed and subjected to gel permeation chromatography. Four degraded polysaccharides, namely, DPP1, DPP2, DPP3, and DPP4, were prepared according to the conditions listed in **Supplementary Table S1**. After the reaction was complete, the solution was precipitated with ethanol then washed with it twice.

Chemical Analysis of Four Degraded and Natural Polysaccharides

Polysaccharide MW were determined using an Agilent 1260 gel permeation chromatograph (Agilent Technologies, United States) fitted with a refractive index detector. The chromatography was run on a TSK G4000-PWxl column with 0.05 M aqueous $NaNO_3$ as the mobile phase. The flow rate was 0.5 mL/min and the column temperature was 30°C. The standards used to calibrate the column were dextrans MW 1000,

5000, 12,000, 25,000, 50,000, 80,000, 270,000, and 670,000 Da (Sigma, United States).

The total sugar content was determined by the phenol-sulphuric acid method using D-galactose as the reference standard. The sulfate content was determined by the barium chloride ($BaCl_2$) method (Zhao et al., 2006). The protein content was measured by the Bradford method using bovine serum albumin (BSA) as the reference standard (Mishra et al., 2013). Uronic acid was estimated from a modified carbazole method using glucuronic acid as the reference standard (Hou et al., 2012). Fourier transform infrared (FT-IR) spectra of the polysaccharides were plotted with a Thermo Fisher Scientific Nicolet iS10 FT-IR spectrometer in potassium bromide (KBr) disks.

Effect of Polysaccharides on Wheat Seedlings Under Salt Stress

Plant Material and Treatments

Wheat (*Triticum aestivum* L. Jimai 22) seeds were surface-sterilized with a 1% (v/v) sodium hypochlorite solution for 10 min and thoroughly rinsed with distilled water. Seeds were germinated for 24 h at 25°C and then sown on Petri dishes. Germinated seeds were sown on Petri dishes containing nylon mesh and Hoagland solution. The plates were placed in a growth incubator under the following controlled environmental conditions: a 14 h/10 h photoperiod, a 25°C daytime/20°C night time cycle, a 65% relative humidity, and a photosynthetic photon flux intensity of 800 μmol m^{-2} s^{-1}. When the second leaves were fully expanded, the wheat seedlings were randomly divided into seven groups. Seven treatments included a control check (neither PP nor NaCl), 100 mM NaCl alone as a negative control, 5 different PP-NaCl mixtures [0.01% (w/v) DPP of 3.2 kDa, 10.5 kDa, 29.0 kDa, 48.8 kDa, and PP of 370.5 kDa). There were 40 plants in every petri dishes and each group contained three petri dishes. The nutrient solutions were renewed every 1 day.

Growth Parameters

After 10 days of salt stress, three samples were randomly selected from each group and their physiological indexes were determined. After that, 30 plants were randomly chosen and harvested to measure their shoot lengths, root lengths, and fresh weight. Samples were then dried at 105°C for 2 h to determine the dry weights.

Determination of Lipid Peroxidation and Membrane Permeability

Membrane permeability was assessed by measuring the relative electric leakage (REL) of the second fully expanded leaf (Li et al., 2017). Leaves (1.0 g) were cut into 0.5-cm pieces and placed in a 50 mL test tube containing 30 mL distilled water. Afterward, the leaf samples in test tubes were vacuumed for 30 min, immersed and vibrated for 20 min, and then measured the conductivity of the solution (EC1) (DDSJ-308A, Shanghai Instrument and Electrical Scientific Instrument Ltd. Shanghai, China). Samples were then boiled for 30 min. When the solution was cooled to room temperature, the conductivity (EC2) was measured again. REL was calculated as EC1/EC2 × 100%.

The malondialdehyde (MDA) content in plants indicates the level of lipid peroxidation. MDA levels were determined using a thiobarbituric acid (TBA) reaction (Zhang et al., 2016). After 10 days NaCl treatment, 0.5 g leaf samples were homogenized in 10% (w/v) trichloroacetic acid (TCA). The homogenates were then centrifuged at 4,000 × *g* for 10 min. Two milliliters of 0.6% (w/v) TBA was added to 2 mL of the supernatant. The mixture was heated in boiling water for 15 min and cooled immediately afterward. The mixture was centrifuged at 10,000 × *g* for 15 min. Absorbances (optical densities) were read at 450, 532, and 600 nm. The MDA content was recorded as μg MDA/g FW.

Chlorophyll Contents and Photosynthetic Characters

After a 10-days NaCl treatment, the chlorophyll a (Chl a), chlorophyll b (Chl b) and total chlorophyll (Chla+b) content in the seedlings were measured in 95% ethanol (Ma et al., 2017). The chlorophyll content was measured in a spectrophotometer at 665 and 649 nm. The entire procedure was performed under subdued light to avoid chlorophyll degradation. Transpiration rate (E), photosynthetic rate (Pn), stomatal conductance (gs) and intercellular CO_2 concentration (Ci) were determined with a portable photosynthesis system (L.MAN-LCPro-SD, BioScientific Ltd., United Kingdom). Atmospheric conditions were as follows: 25 ± 2°C, gas flow rate of 200 μmol s^{-1}, photosynthetic photon flux density of 800 μmol $m^{-2}s^{-1}$ and CO_2 concentration of 400 ± 5 μmol $m^{-2}s^{-1}$.

Soluble Sugar Content and Proline Content

The soluble sugar was measured as follows: 0.5-g leaf samples were chopped and heated at 100°C in 5 mL distilled water for 30 min. The extract was diluted 5×. Extract aliquots of 500 μL, 1 mL of 5% (v/v) phenol, and 5 mL of sulphuric acid were mixed and left to stand 5 min. The absorbance was then read at 485 nm. The soluble sugar concentration was quantified by comparison against a glucose standard curve.

The proline content was determined by grinding 0.2-g leaf samples in liquid nitrogen and homogenizing them in 5 mL of 3% (w/v) sulphosalicylic acid (Huang et al., 2015). After heating at 100°C for 10 min, the homogenate was cooled to room temperature and centrifuged at 5,000 × *g* for 4 min. The proline content in the supernatant was determined spectrophotometrically at 520 nm.

Antioxidant Enzyme Activities

After a 10-days salt stress, 0.5-g samples of the second fully expanded leaves were used to extract enzymes. The samples were homogenized in liquid nitrogen and brought up to a volume of 5 mL with cold sodium phosphate buffer solution (pH 7.8). The homogenates were centrifuged at 12,000 × *g* and 4°C for 15 min. The supernatants were immediately used in the determination of enzyme activities.

The total soluble protein was determined by the Bradford method (Huang et al., 2015). A 100-μL aliquot of supernatant and 5 mL of Coomassie Brilliant Blue G-250 were mixed, and the absorbance was read at 595 nm. The protein concentration was quantified by comparison with a standard curve using BSA.

The superoxide dismutase (SOD) activity was assayed by the extent to which it inhibited the photochemical reduction of β-nitro blue tetrazolium chloride (NBT) (Qiu et al., 2014). The 3 mL reaction mixture consisted of 0.1 mL enzyme extract, 50 mM phosphate buffer (pH7.8), 0.1 m MEDTA,130 mM methionine,0.75 mM NBT, and 0.02 mM riboflavin. Their action was initiated by placing the tubes under two 40W fluorescent lamps. After 10 min, the reaction tubes were removed from the light source. Non-illuminated and illuminated reactions without supernatant served as calibration standards. One unit of SOD was defined as the amount of enzyme needed to inhibit NBT reduction by 50% at 560 nm.

The activity of CAT was determined from the rate of disappearance of H_2O_2 as measured by the decline in the absorbance at 240 nm (Zou et al., 2016). CAT activity was assayed in a 3-mL reaction mixture containing 0.1 mL of enzyme extract, 50 mM phosphate buffer (pH7.8) and 0.2 percent H_2O_2. The decomposition of H_2O_2 was measured by following the decline in absorbance at 240 nm for 3 min and the catalase (CAT) activity was expressed as H_2O_2 reduced min^{-1} mg^{-1} protein.

The peroxidase (POD) activity was determined via the method described by Rasool et al. (2012). The reaction mixture contained 0.05 mL enzyme extract, 0.95 mL guaiacol solution and 2 mL 0.2% H_2O_2 solution in phosphate buffer solution. The increase in absorbance was recorded at 470 nm for 3 min. The POD activity was calculated from the rate of the formation of the guaiacol dehydrogenation product and was expressed as μmol GDHP min^{-1} mg^{-1} protein.

Measurement of Na^+ and K^+ Concentration

The measurement of Na^+ and K^+ concentration has been described previously (Zhang et al., 2018). The plant tissues, leaves, sheaths, and roots were dried at 60°C overnight. The dry samples (0.5 g) were incinerated in a muffle furnace at 500°C for 6 h. The ashes were dissolved in 5 ml concentrated nitric acid and volumed with distilled water at 500 ml. The Na^+ and K^+ concentrations were determined using an Atomic Absorption Spectrometer 900T (PerkinElmer, United States).

Expression Analysis of Genes Encoding Na^+/K^+ Transporter

Total RNA was extracted from the leaves, sheaths, and roots of the wheat seedlings using Plant RNA Extraction Kit (Takara, Dalian, China). Total RNA was quantified by UV spectrophotometer. First-strand cDNA was synthesized by PrimeScript™ RT Reagent Kit with gDNA Eraser (Takara, Dalian, China). qRT-PCR was performed in an ABI 7500 [Life Tech (Applied Biosystems), United States] using the TB Green™ *Premix Ex Taq*™ (Takara, Dalian, China). The PCR products were amplified and detected via a melting curve analysis. The expression level of gene was analyzed using comparative threshold cycle method ($2^{-\Delta\Delta Ct}$) with β-actin as the control. The sequences of the primers used were listed in **Supplementary Table S2**.

Statistical Analysis

Each test was performed randomly in triplicate, each of the data points was expressed as the average ± SD of three independent replicates. Data were subjected to ANOVA analysis by SPSS (version 19.0) and Duncan's test ($P < 0.05$) to compare the mean value of different treatments.

RESULTS AND DISCUSSION

Chemical Analysis of the Four Degraded and Natural Polysaccharides From *P. yezoensis*

The results of the chemical analyses are shown in **Table 1** and **Supplementary Figure S1**. Four degraded products had sulfate and protein contents similar to that of natural PP. The results indicated that free radical degradation could not cause desulphation. Though the total sugar and uronic acid contents of degraded products were lower than that of natural PP, there were no significant differences among the four degraded polysaccharides.

The FT-IR spectra for natural and degraded *P. yezoensis* polysaccharides are shown in **Figure 1**. Typical polysaccharides absorptions at 3272, 2930, 1659, 1205, 1021, 870, and 769 cm^{-1} were evident in all the samples. Absorption at 1,205 cm^{-1} represented a sulfate moiety from the stretching of the S = O bond. The signal at 769 cm^{-1} indicated a sulfate attached to a primary hydroxyl group (Zhao et al., 2006). The weak peak at 870 cm^{-1} was due to the 3,6-anhydro-galactose unites in the polysaccharide. The peak at 1659 cm^{-1} appeared was assigned to the carboxylic group. These results suggested that no major functional group transformations occurred in the degradation process.

Microwave irradiation has been recently considered for the degradation of polymer compounds because it reduces the MW simply by breaking the weakest chemical bonds without altering

TABLE 1 | Properties of four degraded (DPP1,2,3,4) and natural polysaccharides (PP) from *P. yezoensis*.

Sample	MW (kDa)	Total sugar (%)	Uronic acid (%)	Protein (%)	Sulfate (%)
PP	370.5	77.7 ± 1.5^a	9.1 ± 0.1^a	3.6 ± 0.1	19.8 ± 1.4
DPP1	3.2	57.4 ± 1.8^b	4.1 ± 0.1^b	3.3 ± 0.1	21.2 ± 2.3
DPP2	10.5	58.0 ± 6.7^b	4.5 ± 0.2^b	3.3 ± 0.1	20.9 ± 2.4
DPP3	29.0	56.4 ± 2.9^b	4.8 ± 0.3^b	3.4 ± 0.3	21.3 ± 2.1
DPP4	48.8	57.6 ± 3.2^b	4.7 ± 0.6^b	3.5 ± 0.1	21.9 ± 2.3

Values are mean ± SD of three replicates. Different letters indicate significant differences at $P < 0.05$.

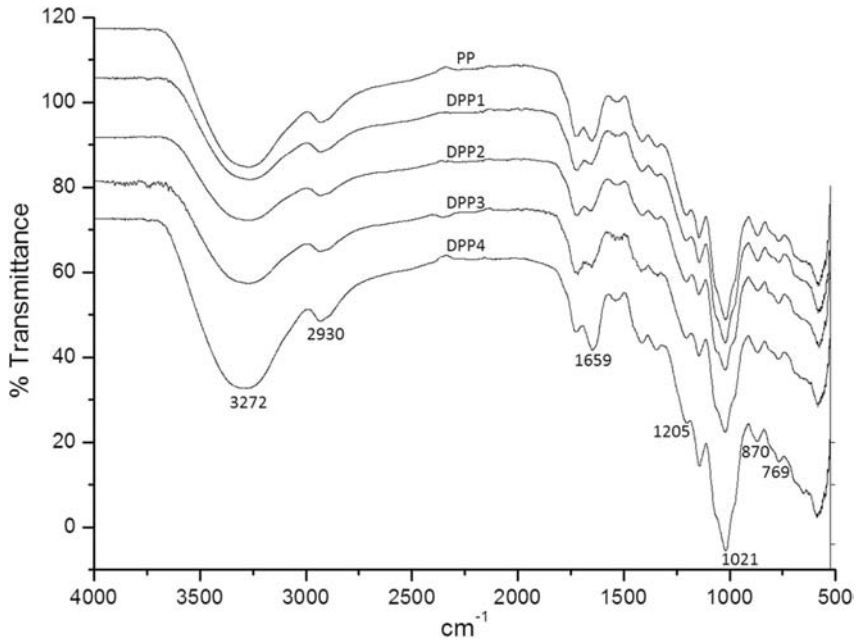


FIGURE 1 | FT-IR spectra of four degraded (DPP1,2,3,4) and natural polysaccharides (PP) from *P. yezoensis*.

the chemical nature of the polymer (Hou et al., 2012; Li et al., 2013).

Effect of Polysaccharides on Wheat Seedlings Under Salt Stress

Plant Growth and Biomass Accumulation

Increased soil salinity can cause abiotic stress and significantly inhibit crop germination, growth, and yield. As shown in **Table 2**, under NaCl stress, the root- and shoot lengths and the fresh- and dry weights of wheat seedlings were all significantly depressed ($P < 0.05$) relative to the untreated control. In contrast, all of these parameters increased relative to the negative control in plants treated with PPs of different MW.

Compared with the NaCl stress treatment, the exogenous application of DPPs and PP increased wheat seedling shoot lengths by 4.9, 5.2, 4.5, 5.0, and 6.1%, respectively ($P < 0.05$). Nevertheless, there were no statistically significant differences in wheat seedling shoot lengths among the PP treatments. Similarly, relative to the negative control, the application of DPPs and PP increased wheat seedling root lengths by 9.5, 5.0, 5.1, 6.1, and 5.6%, respectively ($P < 0.05$). PPs also increased the fresh- and dry weights of wheat seedlings under NaCl stress. DPPs and PP increased the wheat seedling fresh weights by 40.6, 20.2, 18.9, 19.4, and 19.5% and their dry weights by 52.5, 25.9, 26.1, 29.1, and 13.7%, respectively, relative to the negative control. The fresh- and dry seedling weights in the DPP1 group were significantly higher than those of the other groups. On the basis of these results, it could be suggested that the application of any of the PPs could improve the wheat seedling growth parameters tested in this study. Of the five PPs applied, DPP1 (3.2 kDa) was the most effective at improving the growth parameters of plants under salt stress.

Lipid Peroxidation

As a product of lipid peroxidation, MDA is an indicator of oxidative free radical damage to cell membranes. The results

TABLE 2 | Effects of four degraded (DPP1,2,3,4) and natural polysaccharides (PP) from *P. yezoensis* on growth parameters of wheat seedlings.

	Shoot length (cm)	Root length (cm)	Wet weight (g)	Dry weight (g)
Control	21.6 ± 1.8^{a}	23.5 ± 3.2^{a}	0.622 ± 0.189^{b}	0.086 ± 0.017^{b}
NaCl stress	20.2 ± 2.2^{b}	21.2 ± 2.0^{b}	0.538 ± 0.147^{c}	0.068 ± 0.015^{d}
DPP1 + NaCl stress	21.2 ± 1.0^{a}	23.2 ± 2.4^{a}	0.756 ± 0.113^{a}	0.104 ± 0.015^{a}
DPP2 + NaCl stress	21.3 ± 1.5^{a}	22.2 ± 2.0^{ab}	0.647 ± 0.100^{b}	0.086 ± 0.014^{b}
DPP3 + NaCl stress	21.1 ± 1.7^{a}	22.3 ± 1.8^{ab}	0.640 ± 0.095^{b}	0.086 ± 0.013^{b}
DPP4 + NaCl stress	21.2 ± 1.7^{a}	22.5 ± 1.6^{ab}	0.642 ± 0.103^{b}	0.088 ± 0.012^{b}
PP + NaCl stress	21.4 ± 1.8^{a}	22.4 ± 2.6^{ab}	0.643 ± 0.104^{b}	0.077 ± 0.014^{c}

Values are mean ± SD of 30 replicates. Different letters indicate significant differences at $P < 0.05$.

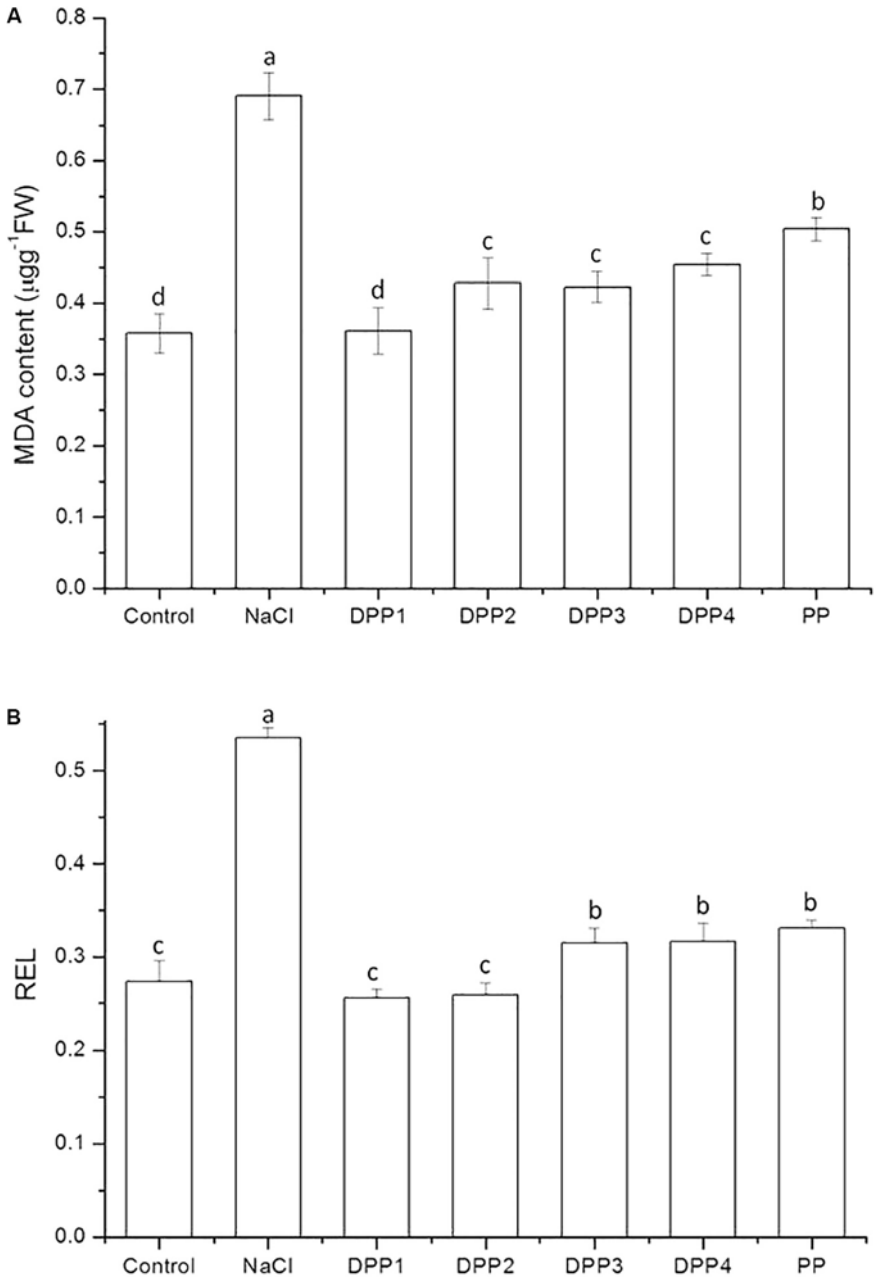


FIGURE 2 | Effect of four degraded (DPP1,2,3,4) and natural polysaccharides (PP) from *P. yezoensis* on MDA content **(A)** and REL **(B)** in leaves of Jimai-22. Values are the mean ± SD of three replicates. Different letters indicate significant differences at $P < 0.05$.

showed that the MDA content in the leaves of the wheat seedlings (**Figure 2A**) increased significantly by 93.0% in response to NaCl stress ($P < 0.05$). PPs of different MW reduced the MDA content to 47.6, 38.0, 38.7, 34.3, and 27.0% ($P < 0.05$) compared to NaCl-stressed plants. Similarly, the relative electric leakage (REL) significantly increased by 95.3% due to NaCl stress ($P < 0.05$, **Figure 2B**). PPs of different MW reduced the REL to 52.2, 51.6, 41.2, 40.8, and 38.1% ($P < 0.05$) relative to NaCl-stressed plants. Exogenous PPs treatment may mitigate NaCl-induced oxidative damage. PPs could scavenge free radicals and prevent lipid peroxidation caused by the active oxygen produced under salt stress (Bose et al., 2014). Moreover, DPP1 reduced the MDA content in the seedlings relative to NaCl-stressed plants significantly more than the other DPPs and PP ($P < 0.05$). Nevertheless, there were no statistically significant differences in terms of MDA content reduction among DPP2, DPP3, and DPP4.

Chlorophyll Content and Photosynthetic Characters

Chlorophyll content is widely used as an important indicator of abiotic stress tolerance in plants. The chlorophyll concentration

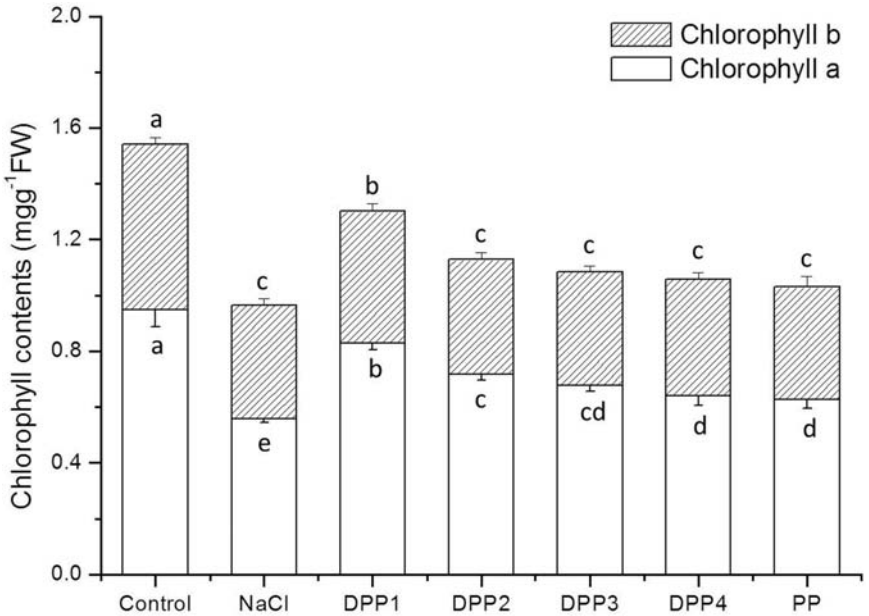


FIGURE 3 | Effect of four degraded (DPP1,2,3,4) and natural polysaccharides (PP) from *P. yezoensis* on chlorophyll content in wheat seedlings. Values are the mean ± SD of three replicates. Different letters indicate significant differences at $P < 0.05$.

in plants exposed to stressors like salinity tends to be significantly lower than that of control plants (Sudhir and Murthy, 2004). In this study, the Chl-a and Chl-b contents significantly decreased under NaCl stress by 41.1% ($P < 0.05$) and 31.6% ($P < 0.05$), respectively, in comparison with the control (**Figure 3**). The Chl-a contents of plants treated with PPs of different MW were 48.3, 28.7, 21.4, 14.7, and 12.1% greater than that of the negative control ($P < 0.05$). In contrast, DPP1 significantly increased Chl-b content by 16.5% relative to the salt-stressed plants whereas the Chl-b contents in the plants treated with DPP2, DPP3, DPP4, and PP did not significantly differ from those of the NaCl-stressed group.

Improvement in the growth of plants which are subjected to various stressors and are receiving PPs treatment is determined by the increases in chlorophyll content and photosynthetic efficiency (Mehta et al., 2010). In the present study, the salt-stressed plants exhibited significant decreases in Chl-a, Chl-b, and total chlorophyll contents compared with the control. On the other hand, the PPs with different MW significantly increased chlorophyll levels in plants under NaCl stress. The results suggested that PPs protected chlorophyll from degradation in salt-stressed wheat seedling leaves.

It is generally recognized that declined photosynthesis by salt stress is mainly due to stomatal closure, feedback inhibition because of reduced sink activity, and swelling or direct effect of salt stress on stomatal conductance (Zahra et al., 2014). The results showed that gs significantly reduced under salt stress ($P < 0.05$) (**Table 3**), which was caused by an excessive accumulation of Na^+ ion in the guard cells. And it leaded to a decrease in available Ci as the results showed. Closure of stomata and reduction of the availability of Ci resulted in a Pn reduction. In this work, salt-stressed wheat seedlings had a 30.8% reduction in Pn compared with control ($P < 0.05$), which is in accord with previous findings (Zou et al., 2015). When treated with DPPs and PP with different MWs, the value of Pn increased by 128.6, 93.9, 92.7, 68.4, and 54.9%, separately

TABLE 3 | Effects of four degraded (DPP1,2,3,4) and natural polysaccharides (PP) from *P. yezoensis* on growth parameters of wheat seedlings.

	Pn (μmol $m^{-2}s^{-1}$)	E (mol $m^{-2}s^{-1}$)	Gs (mol $m^{-2}s^{-1}$)	Ci (μmol $m^{-2}s^{-1}$)
Control	14.96 ± 1.44^{d}	11.21 ± 1.2^{a}	1.19 ± 0.13^{a}	361.90 ± 4.53^{a}
NaCl stress	10.36 ± 1.64^{e}	8.04 ± 0.64^{d}	0.60 ± 0.09^{c}	333.30 ± 6.77^{b}
DPP1 + NaCl stress	23.68 ± 3.30^{a}	10.46 ± 1.70^{ab}	0.86 ± 0.15^{b}	316.80 ± 13.27^{c}
DPP2 + NaCl stress	20.09 ± 3.47^{b}	9.70 ± 1.68^{bc}	0.83 ± 0.15^{b}	316.10 ± 20.55^{c}
DPP3 + NaCl stress	19.96 ± 2.23^{b}	9.71 ± 1.6^{bc}	0.79 ± 0.14^{b}	317.90 ± 10.73^{c}
DPP4 + NaCl stress	17.45 ± 2.24^{c}	8.97 ± 1.97^{cd}	0.79 ± 0.09^{b}	318.80 ± 18.72^{c}
PP + NaCl stress	16.05 ± 2.48^{cd}	8.89 ± 1.36^{cd}	0.75 ± 0.11^{b}	318.40 ± 14.47^{c}

Values are mean ± SD of 10 replicates. Different letters indicate significant differences at $P < 0.05$.

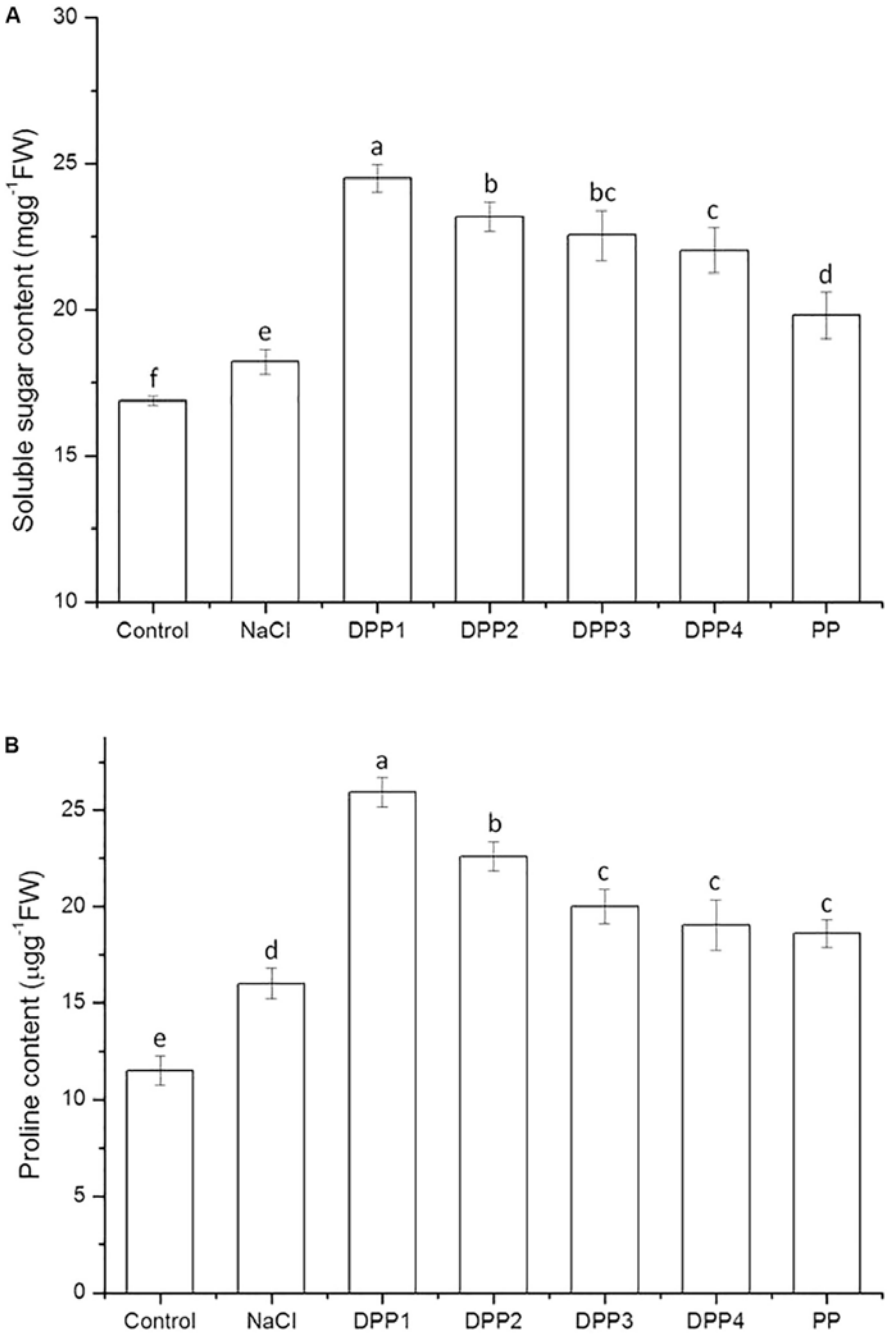


FIGURE 4 | Effect of four degraded (DPP1,2,3,4) and natural polysaccharides (PP) from *P. yezoensis* on soluble sugar **(A)** and proline content **(B)** in wheat seedlings. Values are the mean ± SD of three replicates. Different letters indicate significant differences at $P < 0.05$.

($P < 0.05$). The responding trends of gs and E were similar with Pn. Nevertheless, Ci had a contrary tendency. Under NaCl stress, Ci was much lower than the control group ($P < 0.05$), while Ci of PP groups with different MWs decreased as well compared to the salt stress group. This result indicated that PP relieved the stomata closure due to salt stress and promoted the utilization of CO_2. The alleviating of stomatal closure may also benefit from the regulation of ions in stomata guard cells. Moreover, DPP1 promoted the photosynthesis is much efficiently more than other groups when in comparison with NaCl-stressed plants.

Soluble Sugar Content

The effect of PPs of different MW on the soluble sugar content of wheat seedlings is shown in **Figure 4A**. The results indicated that the soluble sugar content significantly increased in plants under salt stress relative to the control ($P < 0.05$). In wheat seedlings

treated with exogenous PPs, the soluble sugar content increased by 34.4, 27.2, 23.7, 20.9, and 8.7%, respectively ($P < 0.05$) compared with the salt-stressed plants. The results showed that DPP1 more strongly induced soluble sugar production than the other DPPs and PP ($P < 0.05$).

Under salt stress, proteins and other macromolecules rapidly decompose. In the process, plant cell membranes are damaged and imbalances in osmotic pressure occur. Sugars act as osmoregulators and reduce membrane permeability. They lower the water potential in cells and stabilize membranes (Cao et al., 2014). The results showed that the content of soluble sugar increased in plants under NaCl stress (**Figure 4A**). The fact that the soluble sugar content increased in wheat seedlings treated with different PPs suggested that soluble sugar can help maintain osmotic balance and stabilize cell membranes in plants.

Proline Content

As shown in **Figure 4B**, the proline content in wheat seedling leaves increased by 39.3% ($P < 0.05$) relative to the control in response to the 10-days NaCl treatment. There was a significant increase in the proline content of wheat seedlings treated with PPs. After 10 days salt stress, PPs increased the proline content by 61.9, 41.1, 24.9, 19.0, and 16.2%, respectively ($P < 0.05$) compared with the NaCl stress group. The results showed that DPP1 more strongly promoted proline production and accumulation in the plants than the other groups ($P < 0.05$).

Under abiotic stress, low MW organic compounds, such as proline, betaine, and free amino acids were synthesized in the cytoplasm, to prevent cytoplasm dehydration caused by salt stress (Zeng et al., 2013). Proline not only occupies a compatible osmolyte and osmoprotectant under abiotic stresses, it also regulates osmotic potential, stabilizes cellular structure, and reduces damage to the photosynthetic apparatus. Zhang Z. et al. (2017) indicated that proline metabolic pathways exhibit significant differences during the salt stress response. Proline induces the expression of salt stress-responsive proteins, consequently increases the adaptation of plant to salt stress. The protective effect of proline may be explained by its ability to induce antioxidant enzymes to scavenge reactive oxygen species (ROS). There was a report that exogenous proline alleviated the oxidative damage by reducing H_2O_2 and lipid peroxidation levels and by increasing antioxidant activity (Ben Ahmed et al., 2010). The results of the present study showed that salt stress induced proline biosynthesis in salt-stressed plants to resist the osmotic imbalance caused by salt stress. This may be a stress reaction for plant to salt stress. PPs further increased the proline content in salt-stressed wheat seedlings in order to counteract the salt-induced osmotic stress. Most of all, the proline content in DPP1 group was obviously higher than that of other groups, indicated that the wheat seedlings of DPP1 group had stronger osmotic regulation ability.

Antioxidant Enzymes Activities

The results showed that soluble protein content decreased after 10 days salt stress. By that time, it was significantly lower than that of the control ($P < 0.05$). In wheat seedlings treated with exogenous PPs, the soluble protein content increased by 49.0, 40.2, 39.2, 40.4, and 30.1%, respectively ($P < 0.05$) relative to the negative control (**Figure 5**). There was, however, no significant difference in soluble protein content among the four DPP groups. Similarly, the application of PPs increased the POD activities by 45.3, 23.5, 15.6, 18.4, and 16.4% ($P < 0.05$) relative to the salt-stress treatment. Moreover, the SOD and POD activities in the DPP1 treatment were significantly higher than those in the other groups ($P < 0.05$).

In the present study, salt stress increased the SOD, POD, and CAT activities (**Figures 5B–D**) compared to the control. Compared with the control, the SOD activity rose to 69.1% after 10 days salt stress treatment ($P < 0.05$). Treatment with the various PPs further increased SOD activity. After 10 days NaCl stress, PPs increased the SOD activities by 62.9, 31.1, 25.2, 25.8, and 15.6%, respectively ($P < 0.05$) compared with the NaCl stress group. Similarly, PPs increased the POD activities by 45.3, 23.5, 15.6, 18.4, and 16.4%, respectively ($P < 0.05$) relative to the negative control. Moreover, the activities of SOD and POD in the DPP1 treatment were significantly higher than those in the other groups ($P < 0.05$). A similar trend was observed and recorded for the CAT activity but the levels of this enzyme did not significantly differ among the DPP1, DPP2, and DPP3 treatments.

Salinity-induced crop yield loss is a consequence of imbalances in mineral nutrients concentrations and osmotic effects which trigger the overproduction of ROS (Mittler et al., 2004). Under normal physiological conditions, ROS are constantly generated from aerobic metabolism in chloroplasts, mitochondria, and peroxisomes. Their overproduction, however, causes oxidative damage to lipids, proteins, and nucleic acids, and alters membrane permeability (Mittler et al., 2004). Adaptations which regulate ROS generation in plants may effectively defend them against oxidative damage and increase their stress tolerance.

The accumulation and activity of antioxidant enzymes help inhibit membrane protein and lipid peroxidation. There have been many studies to prove that SOD, POD, and CAT are strongly associated with salt resistance in plants (Ma et al., 2012; Rasool et al., 2012; Qiu et al., 2014). SOD detoxifies $O_2^{\bullet}$ by forming H_2O_2. The latter is also toxic and must be eliminated by the concerted actions of CAT and POD. In the present study, salt stress induced SOD, POD, and CAT activities to levels significantly greater than those of the control. Plants receiving PPs under NaCl stress also showed relative increases in their SOD, POD, and CAT activities. The results indicated that PPs effectively induce ROS scavenging in wheat seedlings by modulating their antioxidant enzymes activities. Therefore, PPs may enhance defense responses in plants under salt stress. In general, DPP1 (3.2 kDa) was significantly more effective at inducing antioxidant activity and ROS scavenging than the other PP and DPPs.

Na^+ and K^+ Accumulation in Different Tissues of Wheat Seedlings

The results showed that Na^+ content increased highly in different tissues of wheat seedlings after 10 days salt stress. In the root, sheath, and leaf, the increase in Na^+ content of salt stressed plants was 3.1, 2.2, 3.8-times, respectively, higher than that

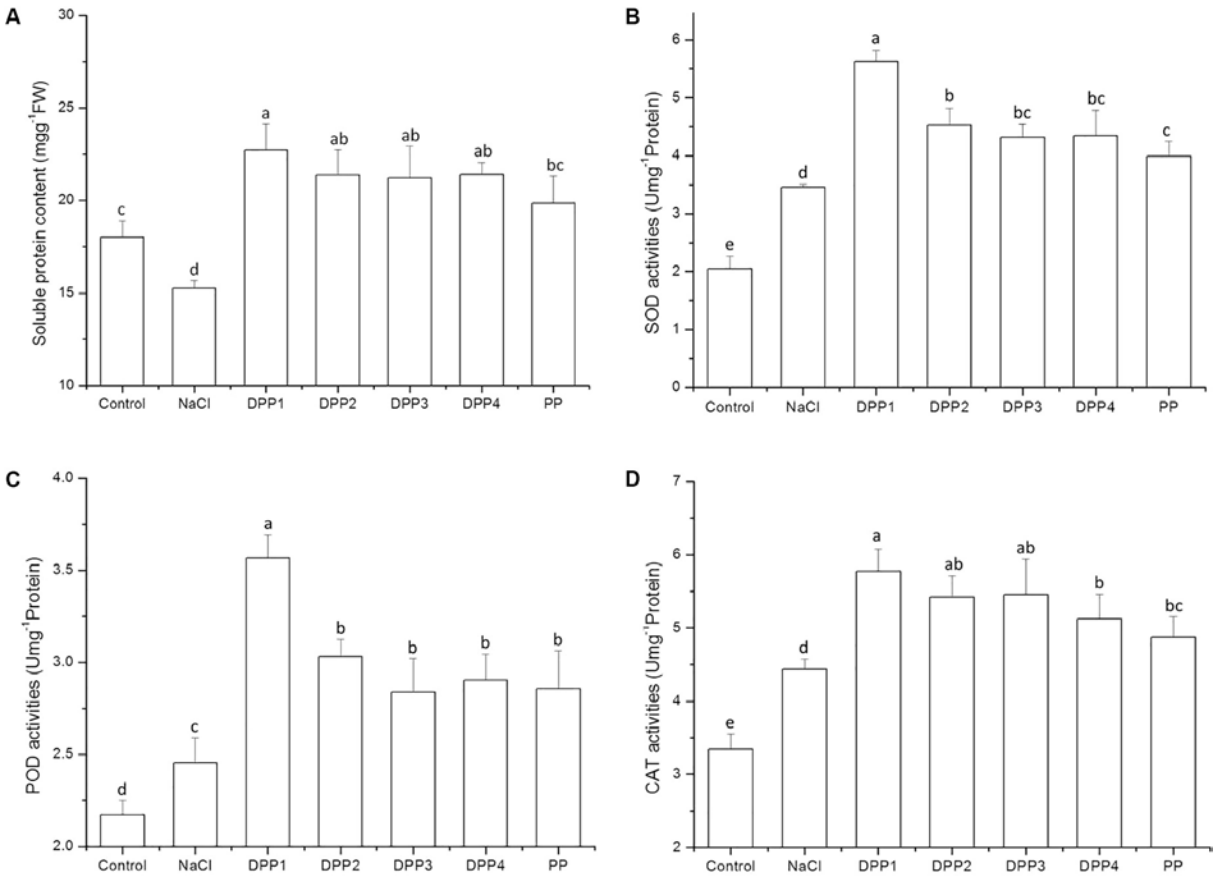


FIGURE 5 | Effect of four degraded (DPP1,2,3,4) and natural polysaccharides (PP) from *P. yezoensis* on soluble protein contents **(A)**, SOD **(B)**, POD **(C)**, and CAT **(D)** activities in wheat seedlings. Values are the mean ± SD of three replicates. Different letters indicate significant differences at $P < 0.05$.

of the control ($P < 0.05$, **Figure 6**). But there is a reverse trend in K^+ content (**Figure 7**). The K^+ content in the root, sheath, and leaf of salt stressed plants decreased by 40.5, 55.7, and 64.3%, respectively ($P < 0.05$). The Na^+ contents in plant tissues of different polysaccharide treatment groups were lower than that of salt stress group, but still higher than that of control group. The results showed that Na^+ accumulated in different tissues of plants under salt stress, especially in roots. In wheat seedlings treated with exogenous PPs, the K^+ content increased 3.4-, 2.9-, 2.8-, 2.7-, and 2.7-fold relative to the negative control (**Figure 7**). Similarly, the application of PPs increased the K^+ content in sheath and leaf compared to the salt-stress treatment. Consequently, a higher K^+/Na^+ ratio was observed in the root, sheath, and leaf of PP treated plants (**Figure 8**).

The ability to restrict the transport and accumulation of Na^+ in the leaves is the most important adaptation of plant to salt stress (Mekawy et al., 2015). In this study, a large amount of Na^+ was accumulated in roots of all the salt stressed plants. However, treated with exogenous PPs, leaf Na^+ content was significantly lower compared with the salt stressed group. Thus, wheat seedlings treated with DPPs excluded Na^+ selectively from leaves, the source tissue for photosynthesis (Lekshmy et al., 2015). Under salt stress, plants suffer from K^+ deficiencies stemming from the competitive inhibition of its uptake by Na^+, and this often leads to high Na^+/K^+ ratios that disrupt cellular homeostasis (Mekawy et al., 2015). In this work, the K^+ immense accumulation in PPs treated plants was accompanied by a higher K^+/Na^+ ratio, which contributed to the salt tolerance. Moreover, the K^+/Na^+ ratio in DPP1 treated plants was much higher than that of other groups.

Expression of Genes Encoding Na^+/K^+ Transporter

To determine the mechanisms underlying differential Na^+ and K^+ accumulation in salt stressed and PPs treated plants, genes expression of Na^+ and K^+ transporter were analyzed. The analysis showed that compared with the control, salt stress induced higher transcript levels of the *TaHKT2;1* gene (**Figure 9**). While treated with PPs, the expression of *TaHKT2;1* was down regulated in the root, sheath, and leaf significantly (**Figure 9**). By contrast, the analysis of *TaNHX2* revealed that under salt stress, the *TaNHX2* expression was obviously down regulated in the root of the salt stressed plants, but it there was no significant difference in sheath and leaf (**Figure 10**). After the application of different PPs, the *TaNHX2* expression was still

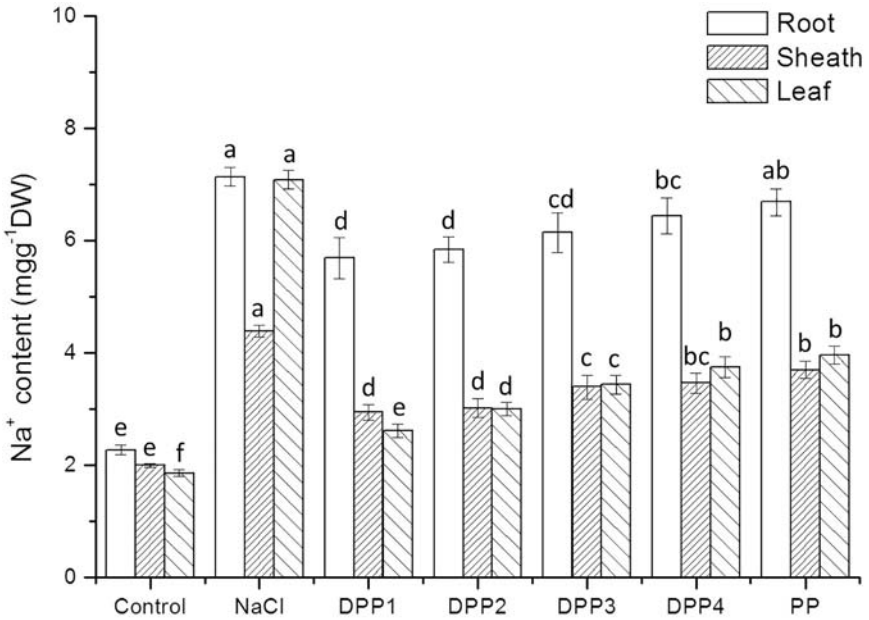


FIGURE 6 | Effect of four degraded (DPP1,2,3,4) and natural polysaccharides (PP) from *P. yezoensis* on Na^+ contents of root, sheath, and leaf in wheat seedlings. Values are the mean ± SD of three replicates. Different letters indicate significant differences at $P < 0.05$.

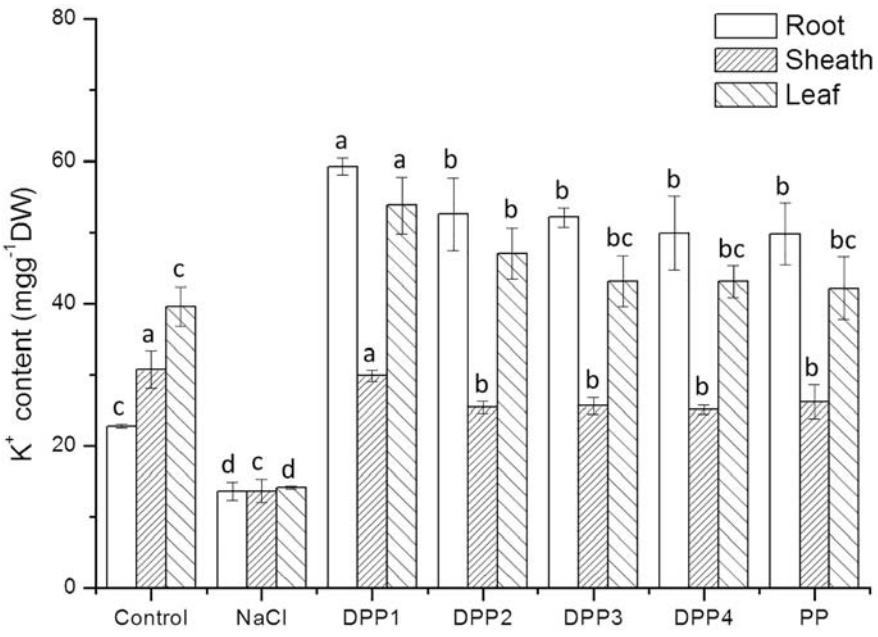


FIGURE 7 | Effect of four degraded (DPP1,2,3,4) and natural polysaccharides (PP) from *P. yezoensis* on K^+ contents of root, sheath, and leaf in wheat seedlings. Values are the mean ± SD of three replicates. Different letters indicate significant differences at $P < 0.05$.

down regulated in the root, while the gene was up regulated in the sheath and leaf. In the leaf, it was observed to be 2.3-, 2.1-, 1.8-, 1.7-, and 1.5-fold in the PPs treated plants, respectively, compared to the salt stress plants. There was a similar trend for the *TaNHX2* expression in sheath. The quantitative analysis of the *TaSOS1* showed that compared with the control group, salt stress induced higher transcript levels of the *TaSOS1* gene (**Figure 11**). While treated with PPs, the expression of *TaSOS1* was up regulated significantly in the root, sheath, and leaf (**Figure 11**). Moreover, the transcript levels of *TaSOS1* treated with DPP1 were significantly higher than those of the other groups ($P < 0.05$).

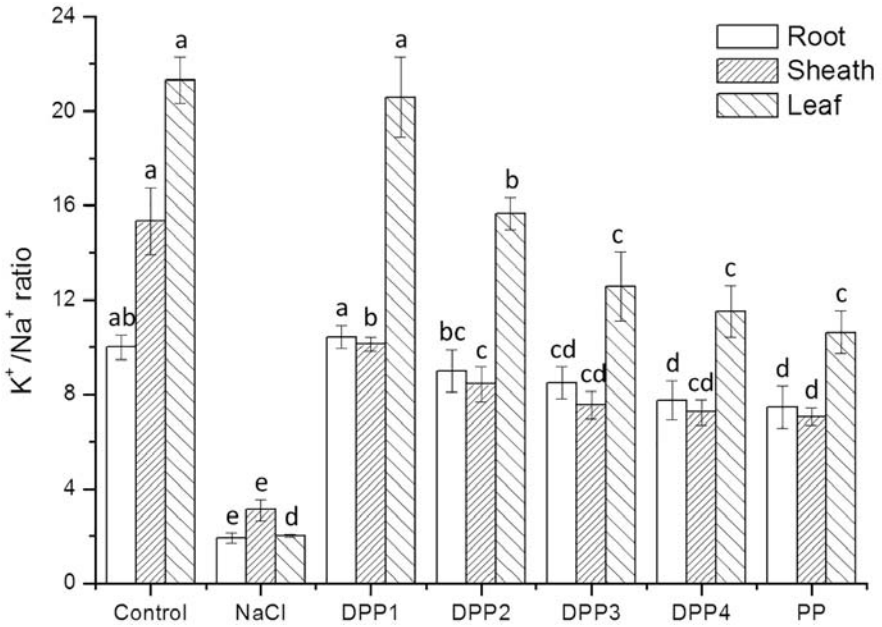


FIGURE 8 | Effect of four degraded (DPP1,2,3,4) and natural polysaccharides (PP) from *P. yezoensis* on K^+/Na^+ ratio of root, sheath, and leaf in wheat seedlings. Values are the mean ± SD of three replicates. Different letters indicate significant differences at $P < 0.05$.

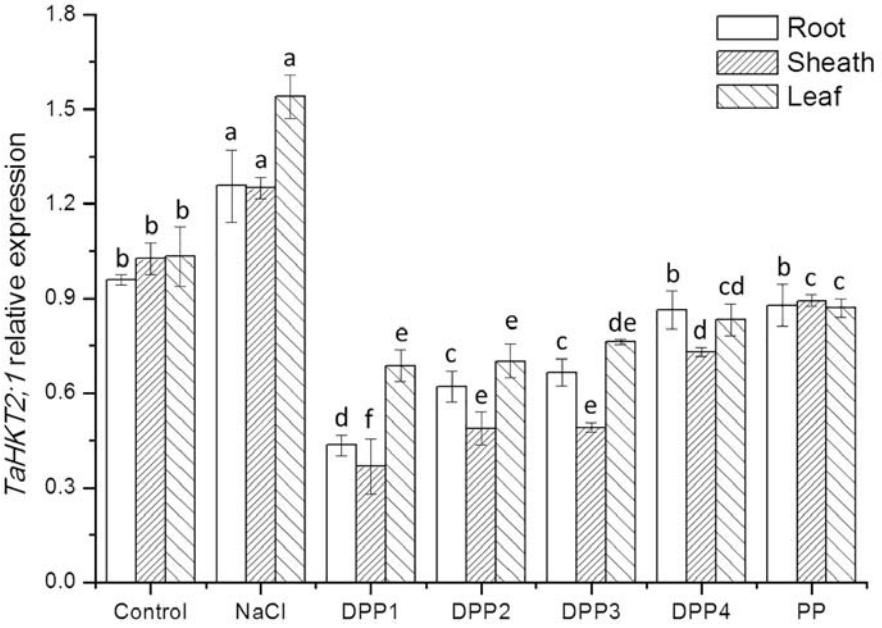


FIGURE 9 | Effect of four degraded (DPP1,2,3,4) and natural polysaccharides (PP) from *P. yezoensis* on *TaHKT2;1* expression of root, sheath, and leaf in wheat seedlings. Values are the mean ± SD of three replicates. Different letters indicate significant differences at $P < 0.05$.

High-affinity potassium transporters (HKTs) have been reported to function as Na^+/K^+ symporters as well as selective Na^+ uniporters (Horie et al., 2009). In the bread wheat, *TaHKT2;1* has been identified and presumed to function in Na^+ uptake from the soil (Ariyarathna et al., 2014). The expression analysis of *TaHKT2;1* in the contrasting wheat genotypes has been studied previously (Kumar et al., 2017). The expression of *TaHKT2;1* was observed to upregulate in the shoots of the salt-sensitive genotype, while it was downregulated in the salt-tolerant genotype. It shows that the inhibition of gene expression was related to its salt resistance. In this study, salt stress induced higher transcript

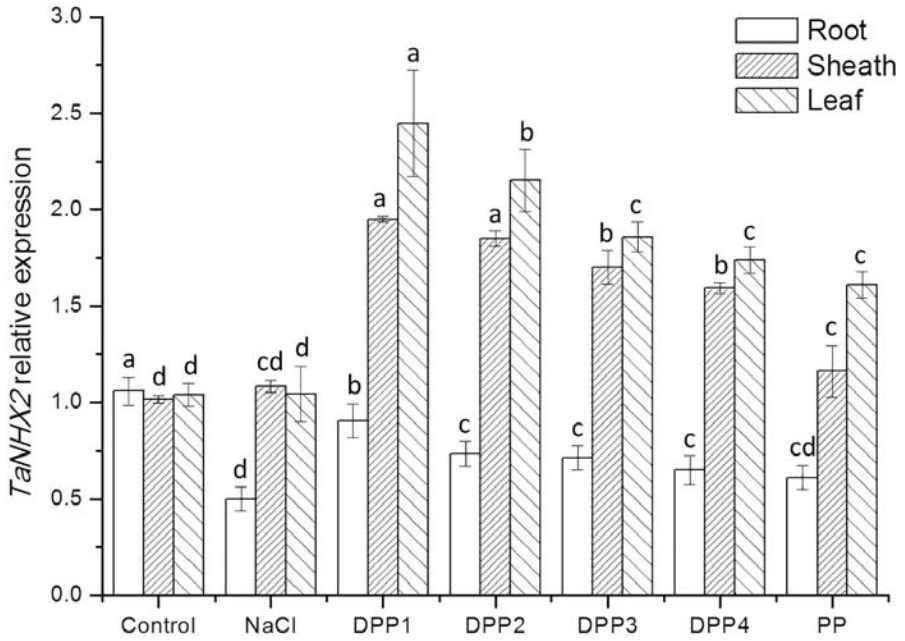


FIGURE 10 | Effect of four degraded (DPP1,2,3,4) and natural polysaccharides (PP) from *P. yezoensis* on *TaNHX2* expression of root, sheath, and leaf in wheat seedlings. Values are the mean ± SD of three replicates. Different letters indicate significant differences at $P < 0.05$.

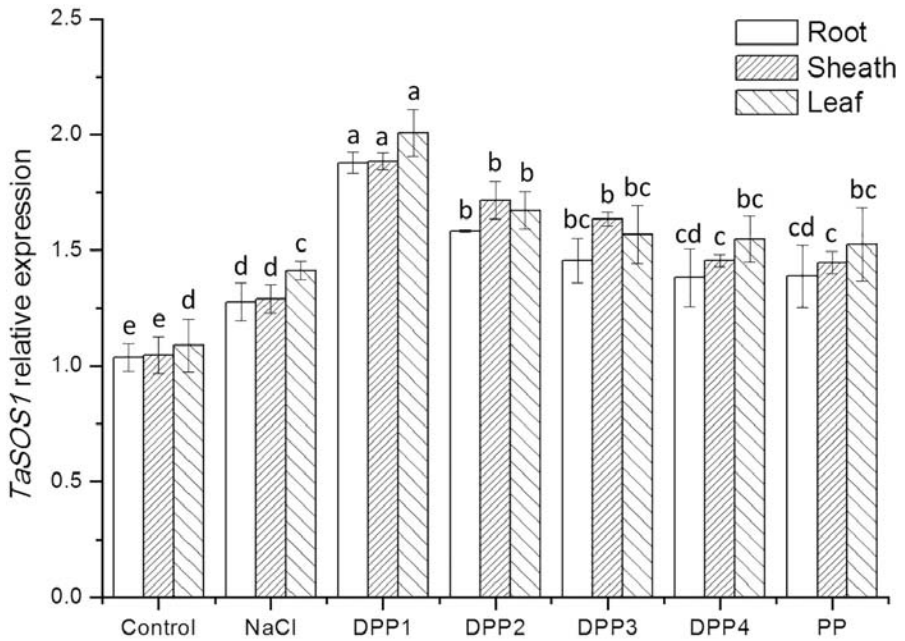


FIGURE 11 | Effect of four degraded (DPP1,2,3,4) and natural polysaccharides (PP) from *P. yezoensis* on *TaSOS1* expression of root, sheath, and leaf in wheat seedlings. Values are the mean ± SD of three replicates. Different letters indicate significant differences at $P < 0.05$.

levels of the *TaHKT2;1* gene (**Figure 9**). While treated with PPs, the expression of *TaHKT2;1* was down regulated in the root significantly. The results indicated that the role of *TaHKT2;1* in restricting uptake Na^+ from the soil to the roots and consequently a lower Na^+ level and Na^+/K^+ ratio in the leaves. Recently, Kumar et al. (2017) claimed that under salt stress the increase in cytosine methylation down regulated the expression of *TaHKT 2;1* and *TaHKT2;3* in the salt-tolerant wheat, thereby improved its salt-tolerance ability.

In addition to limiting the entry of Na^+ into cells, the mechanisms for salt tolerance also includes transporting Na^+ out of cells and compartmentalizing Na^+ into vacuoles (Yamaguchi et al., 2013). The Salt Overly Sensitive 1 (SOS1), a plasma membrane Na^+/K^+ antiporter mediates cytosolic Na^+ efflux at the root and regulates Na^+ transportation from root to shoot thereby maintaining appropriated K^+/Na^+ ratio in leaves, where the photosynthesis take place (Qiu et al., 2002). The Na^+ compartmentation into the vacuoles depends on the activity of endosomal Na^+/H^+ antiporters such as NHX (Gaxiola et al., 2001). It was reported that expression levels of *SOS1* and *NHX1* were significantly higher in salt tolerant wheat genotypes under 200 mM NaCl stress, and consequently correlated with improved sodium exclusion and lower Na^+/K^+ ratio (Lekshmy et al., 2015). In this work, after application of PPs, the overexpression of *TaSOS1* and *TaNHX2* was markly upregulated in the wheat seedlings which were much more resistant to high concentrations of NaCl. In general, the transcript levels of *TaSOS1* and *TaNHX2* genes treated with DPP1 were significantly higher than those of the other groups ($P < 0.05$). Our results indicated that DPP1 can effectively coordinate the efflux and regionalization of Na^+, alleviate the salt stress damage.

Effects of Polysaccharides From *Pyropia yezoensis* With Different MWs on the Plants' Defense Response Under Salt Stress

Polysaccharides from *P. yezoensis* are high-MW polysaccharides with high viscosity. This physical property may limit its practical application in agriculture, pharmaceutical, and other industries. Many studies demonstrated that polysaccharide MW distributions significantly influence their biological functions. Polysaccharides with relatively lower MWs have been reported to have higher antioxidant effects than those with higher MWs (Qi et al., 2006; Zha et al., 2016). Li et al. (2013) prepared six representative sulphated polysaccharides (446.5, 247.0, 76.1, 19.0, 5.0, and 3.1 kDa) using the microwave-assisted acid hydrolysis method and investigated their antioxidant activities. Samples with high MW inhibited superoxide radicals more effectively than did lower-MW polysaccharides. In contrast, samples with low MW more effectively inhibited hydroxyl radicals than those with higher MW. Nevertheless, Hou et al. (2012) reported that the relationship between the MW of fucoidans (1.0, 3.8, 8.3, 13.2, 35.5, 64.3, and 144.5 kDa) and their antioxidant activities is non-linear. The 3.8, 1.0, and >8.3 kDa samples had relatively superior hydroxyl radical scavenging activity, reducing power, and superoxide anion scavenging activity, respectively.

Sulfate content significantly influences the superoxide anion scavenging ability of fucoidans (Rocha de Souza et al., 2007). In the present study, all the samples had similar sulfate levels, so this factor could be ignored. The results suggest that lower-MW samples (DPP1, 3.2 kDa) are the most effective at protecting wheat seedlings against salt stress. The close correlation between the activity and the MW of polysaccharides reveals that the former is dependent on a specific chemical structure. Polysaccharides with different MW may form different configurations that interact with elicitor receptors on plant cell membranes. Polysaccharides that are too small or large are often inactive. The appropriate degree of polymerization will effectively induce a regulatory response. The capacity of functional oligosaccharides produced by alginase was studied on the growth promoting of *Brassica campestris* L. under salt stress. The plant growth experiment suggested that low MW oligosaccharides were more effective in stimulating root elongation and relieving salt stress (Tang et al., 2011). Seven chitooligomers (COSs) with determined degrees of polymerization (chitotetraose to chitooctaose, DP 8–10, DP 10–12) were applied to explore the relationship between the degrees of polymerization of COSs and its effect on the tolerance of wheat seedlings to salt stress. The results suggested that chitohexaose, chitoheptaose, and chitooctaose exhibited stronger activity compared with other COS samples, which suggested that its activity had a closely relationship with its degrees of polymerization (Zhang X. et al., 2017). Ulteriorly, Wei et al. (2009) investigated the effects of chitooligomers (COS) COS5 and COS6 *in vivo* and *in vitro* on the expression of the gene coding for the CR3 cell surface receptor. The results showed that both COS5 and COS6 promote the expression of CR3 mRNA, and this effect was stronger in response to COS6 than COS5. Both of these activated phagocytes and enhanced antibody transmission by binding to the CR3 receptor. COS6 has a higher MW and more exposed amino glucose groups than COS5. Consequently, relative to COS5, COS6 has more active binding sites available to combine with the CR3 receptor surfaces of macrophages and lymphocytes. What is more, COS6 can change the configuration of the CR3 receptor and increase its substrate affinity. The function of these plasma membrane proteins is related to the perception of the elicitor signal. Nevertheless, the mechanisms underlying elicitor recognition, including polysaccharides and signal transduction, require further investigation.

CONCLUSION

This study developed an efficient method for the degradation of *P. yezoensis* polysaccharide using microwave-assisted acid hydrolysis. Microwave exposures significantly accelerated the reaction rate. Polysaccharides with different MW could be obtained by strictly controlling the reaction conditions. Representative polysaccharides with different MW (3.2, 10.5, 29.0, and 48.8 kDa) were prepared and the relationship between their MW and their effects on plant salt tolerance was investigated. The results showed that degradation of *P. yezoensis* polysaccharide could protect plants from salt stress damage by regulating antioxidant enzyme activities and the content of permeable substances. The expression level examination analysis of several Na^+/K^+ transporter genes indicated that DPPs could restrict the transport and accumulation of Na^+ in the wheat

seedlings, maintain a higher K^+/Na^+ ratio, thereby protect plants from the damage of salt stress. Furthermore, the effect of polysaccharides on the tolerance of wheat seedlings to salt stress was closely correlated with polysaccharide MW.

AUTHOR CONTRIBUTIONS

PZ: Conceived the study and wrote the manuscript. LM and HZ: Preparation of polysaccharide. CJ and YY: Chemical analysis of polysaccharide. XL and YL: Study the effect of polysaccharides on wheat seedlings under salt stress. CZ and YL: Reviewed and edited the manuscript.

FUNDING

This work was supported by the Agricultural Science and Technology Innovation Program (ASTIP-TRIC07), Science Foundation for Young Scholars of Tobacco Research Institute of Chinese Academy of Agricultural Sciences (2016A01), and funded by Open Foundation of the State Key Laboratory of Bioactive Seaweed Substances (SKL-BASS1715).

ACKNOWLEDGMENTS

We would like to thank Editage (https://www.editage.cn) for English language editing.

SUPPLEMENTARY MATERIAL

The Supplementary Material for this article can be found online at: https://www.frontiersin.org/articles/10.3389/fpls.2018.00427/full#supplementary-material

FIGURE S1 | The Mw of four degraded (DPP1,2,3,4) and natural polysaccharides (PP) from *P. yezoensis*.

TABLE S1 | Reaction conditions of degraded polysaccharides from *P. yezoensis*.

TABLE S2 | Primers used for quantitative real-time RT-PCR.

REFERENCES

Ariyarathna, H. A., Ul-Haq, T., Colmer, T. D., and Francki, M. G. (2014). Characterization of the multigene family TaHKT 2;1 in bread wheat and the role of gene members in plant Na(+) and K(+) status. *BMC Plant Biol.* 14:159. doi: 10.1186/1471-2229-14-159

Ben Ahmed, C., Ben Rouina, B., Sensoy, S., Boukhriss, M., and Ben Abdullah, F. (2010). Exogenous Proline Effects on Photosynthetic Performance and Antioxidant Defense System of Young Olive Tree. *J. Agric. Food Chem.* 58, 4216–4222. doi: 10.1021/jf9041479

Bose, J., Rodrigo-Moreno, A., and Shabala, S. (2014). ROS homeostasis in halophytes in the context of salinity stress tolerance. *J. Exp. Bot.* 65, 1241–1257. doi: 10.1093/jxb/ert430

Cao, Y.-Y., Yang, M.-T., Li, X., Zhou, Z.-Q., Wang, X.-J., and Bai, J.-G. (2014). Exogenous sucrose increases chilling tolerance in cucumber seedlings by modulating antioxidant enzyme activity and regulating proline and soluble sugar contents. *Sci. Hortic.* 179, 67–77. doi: 10.1016/j.scienta.2014.09.016

Gaxiola, R. A., Li, J., Undurraga, S., Dang, L. M., Allen, G. J., Alper, S. L., et al. (2001). Drought- and salt-tolerant plants result from overexpression of the AVP1 H+-pump. *Proc. Natl. Acad. Sci. U.S.A.* 98, 11444–11449. doi: 10.1073/pnas.191389398

Horie, T., Hauser, F., and Schroeder, J. I. (2009). HKT transporter-mediated salinity resistance mechanisms in *Arabidopsis* and monocot crop plants. *Trends Plant Sci.* 14, 660–668. doi: 10.1016/j.tplants.2009.08.009

Hou, Y., Wang, J., Jin, W., Zhang, H., and Zhang, Q. (2012). Degradation of *Laminaria japonica* fucoidan by hydrogen peroxide and antioxidant activities of the degradation products of different molecular weights. *Carbohydr. Polym.* 87, 153–159. doi: 10.1016/j.carbpol.2011.07.031

Huang, C., Wang, D., Sun, L., and Wei, L. (2015). Effects of exogenous salicylic acid on the physiological characteristics of *Dendrobium officinale* under chilling stress. *Plant Growth Regul.* 79, 199–208. doi: 10.1007/s10725-015-0125-z

Kumar, S., Beena, A. S., Awana, M., and Singh, A. (2017). Salt-induced tissue-specific cytosine methylation downregulates expression of HKT genes in contrasting wheat (*Triticum aestivum* L.) genotypes. *DNA Cell Biol.* 36, 283–294. doi: 10.1089/dna.2016.3505

Lekshmy, S., Sairam, R. K., Chinnusamy, V., and Jha, S. K. (2015). Differential transcript abundance of salt overly sensitive (SOS) pathway genes is a determinant of salinity stress tolerance of wheat. *Acta Physiol. Plant.* 37:169. doi: 10.1007/s11738-015-1910-z

Li, B., Liu, S., Xing, R., Li, K., Li, R., Qin, Y., et al. (2013). Degradation of sulfated polysaccharides from *Enteromorpha prolifera* and their antioxidant activities. *Carbohydr. Polym.* 92, 1991–1996. doi: 10.1016/j.carbpol.2012.11.088

Li, H., Chang, J., Chen, H., Wang, Z., Gu, X., Wei, C., et al. (2017). Exogenous melatonin confers salt stress tolerance to watermelon by improving photosynthesis and redox homeostasis. *Front. Plant Sci.* 8:295. doi: 10.3389/fpls.2017.00295

Ma, L., Li, Y., Yu, C., Wang, Y., Li, X., Li, N., et al. (2012). Alleviation of exogenous oligochitosan on wheat seedlings growth under salt stress. *Protoplasma* 249, 393–399. doi: 10.1007/s00709-011-0290-5

Ma, X., Zheng, J., Zhang, X., Hu, Q., and Qian, R. (2017). Salicylic acid alleviates the adverse effects of salt stress on *Dianthus superbus* (Caryophyllaceae) by activating photosynthesis, protecting morphological structure, and enhancing the antioxidant system. *Front. Plant Sci.* 8:600. doi: 10.3389/fpls.2017.00600

Mehta, P., Jajoo, A., Mathur, S., and Bharti, S. (2010). Chlorophyll a fluorescence study revealing effects of high salt stress on Photosystem II in wheat leaves. *Plant Physiol. Biochem.* 48, 16–20. doi: 10.1016/j.plaphy.2009.10.006

Mekawy, A. M., Assaha, D. V., Yahagi, H., Tada, Y., Ueda, A., and Saneoka, H. (2015). Growth, physiological adaptation, and gene expression analysis of two Egyptian rice cultivars under salt stress. *Plant Physiol. Biochem.* 87, 17–25. doi: 10.1016/j.plaphy.2014.12.007

Mishra, P., Bhoomika, K., and Dubey, R. S. (2013). Differential responses of antioxidative defense system to prolonged salinity stress in salt-tolerant and salt-sensitive Indica rice (*Oryza sativa* L.) seedlings. *Protoplasma* 250, 3–19. doi: 10.1007/s00709-011-0365-3

Mittler, R., Vanderauwera, S., Gollery, M., and Van Breusegem, F. (2004). Reactive oxygen gene network of plants. *Trends Plant Sci.* 9, 490–498. doi: 10.1016/j.tplants.2004.08.009

Mohibbullah, M., Bhuiyan, M. M., Hannan, M. A., Getachew, P., Hong, Y. K., Choi, J. S., et al. (2015). The edible red alga *Porphyra yezoensis* promotes neuronal survival and cytoarchitecture in primary hippocampal neurons. *Cell. Mol. Neurobiol.* 36, 669–682. doi: 10.1007/s10571-015-0247-x

Qi, H., Zhao, T., Zhang, Q., Li, Z., Zhao, Z., and Xing, R. (2006). Antioxidant activity of different molecular weight sulfated polysaccharides from *Ulva pertusa* Kjellm (Chlorophyta). *J. Appl. Phycol.* 17, 527–534. doi: 10.1007/s10811-005-9003-9

Qian, L., Zhou, Y., and Ma, J.-X. (2014). Hypolipidemic effect of the polysaccharides from *Porphyra yezoensis*. *Int. J. Biol. Macromol.* 68, 48–49. doi: 10.1016/j.ijbiomac.2014.04.004

Qiu, Q. S., Guo, Y., Dietrich, M. A., Schumaker, K. S., and Zhu, J. K. (2002). Regulation of SOS1, a plasma membrane Na+/H+ exchanger in *Arabidopsis*

thaliana, by SOS2 and SOS3. *Proc. Natl. Acad. Sci. U.S.A.* 99, 8436–8441. doi: 10.1073/pnas.122224699

Qiu, Z., Guo, J., Zhu, A., Zhang, L., and Zhang, M. (2014). Exogenous jasmonic acid can enhance tolerance of wheat seedlings to salt stress. *Ecotoxicol. Environ. Saf.* 104, 202–208. doi: 10.1016/j.ecoenv.2014.03.014

Rasool, S., Ahmad, A., Siddiqi, T. O., and Ahmad, P. (2012). Changes in growth, lipid peroxidation and some key antioxidant enzymes in chickpea genotypes under salt stress. *Acta Physiol. Plant.* 35, 1039–1050. doi: 10.1007/s11738-012-1142-4

Rivero, R. M., Mestre, T. C., Mittler, R., Rubio, F., Garcia-Sanchez, F., and Martinez, V. (2014). The combined effect of salinity and heat reveals a specific physiological, biochemical and molecular response in tomato plants. *Plant Cell Environ.* 37, 1059–1073. doi: 10.1111/pce.12199

Rocha de Souza, M. C., Marques, C. T., Guerra Dore, C. M., Ferreira Da Silva, F. R., Oliveira Rocha, H. A., et al. (2007). Antioxidant activities of sulfated polysaccharides from brown and red seaweeds. *J. Appl. Phycol.* 19, 153–160. doi: 10.1007/s10811-006-9121-z

Sangha, J. S., Ravichandran, S., Prithiviraj, K., Critchley, A. T., and Prithiviraj, B. (2010). Sulfated macroalgal polysaccharides λ-carrageenan and ι-carrageenan differentially alter *Arabidopsis thaliana* resistance to *Sclerotinia sclerotiorum*. *Physiol. Mol. Plant Pathol.* 75, 38–45. doi: 10.1016/j.pmpp.2010.08.003

Stadnik, M. J., and de Freitas, M. B. (2014). Algal polysaccharides as source of plant resistance inducers. *Trop. Plant Pathol.* 39, 111–118. doi: 10.1590/S1982-56762014000200001

Sudhir, P. M., and Murthy, S. D. S. (2004). Effects of salt stress on basic processes of photosynthesis. *Photosynthetica* 42, 481–486. doi: 10.1007/S11099-005-0001-6

Tang, J., Zhou, Q., Chu, H., and Nagata, S. (2011). Characterization of alginase and elicitor-active oligosaccharides from *Gracilibacillus* A7 in alleviating salt stress for *Brassica campestris* L. *J. Agric. Food Chem.* 59, 7896–7901. doi: 10.1021/jf201793s

Van Oosten M. J., Silletti, S., Guida, G., Cirillo, V., Di Stasio E., Carillo, P., et al. (2017). A benzimidazole proton pump inhibitor increases growth and tolerance to salt stress in tomato. *Front. Plant Sci.* 8:1220. doi: 10.3389/fpls.2017.01220

Vera, J., Castro, J., Contreras, R. A., González, A., and Moenne, A. (2012). Oligo-carrageenans induce a long-term and broad-range protection against pathogens in tobacco plants (var. Xanthi). *Physiol. Mol. Plant Pathol.* 79, 31–39. doi: 10.1016/j.pmpp.2012.03.005

Wei, X., Wang, Y., Xiao, J., and Xia, W. (2009). Separation of chitooligosaccharides and the potent effects on gene expression of cell surface receptor CR3. *Int. J. Biol. Macromol.* 45, 432–436. doi: 10.1016/j.ijbiomac.2009.07.003

Yamaguchi, T., Hamamoto, S., and Uozumi, N. (2013). Sodium transport system in plant cells. *Front. Plant Sci.* 4:410. doi: 10.3389/fpls.2013.00410

Yu, X., Zhou, C., Yang, H., Huang, X., Ma, H., Qin, X., et al. (2015). Effect of ultrasonic treatment on the degradation and inhibition cancer cell lines of polysaccharides from *Porphyra yezoensis*. *Carbohydr. Polym.* 117, 650–656. doi: 10.1016/j.carbpol.2014.09.086

Zahra, J., Nazim, H., Cai, S., Han, Y., Wu, D., Zhang, B., et al. (2014). The influence of salinity on cell ultrastructures and photosynthetic apparatus of barley genotypes differing in salt stress tolerance. *Acta Physiol. Plant.* 36, 1261–1269. doi: 10.1007/s11738-014-1506-z

Zeng, J., Chen, A., Li, D., Yi, B., and Wu, W. (2013). Effects of salt stress on the growth, physiological responses, and glycoside contents of *Stevia rebaudiana* Bertoni. *J. Agric. Food Chem.* 61, 5720–5726. doi: 10.1021/jf401237x

Zha, S., Zhao, Q., Zhao, B., Ouyang, J., Mo, J., Chen, J., et al. (2016). Molecular weight controllable degradation of *Laminaria japonica* polysaccharides and its antioxidant properties. *J. Ocean Univ. China* 15, 637–642. doi: 10.1007/s11802-016-2943-7

Zhang, M., Cao, Y., Wang, Z., Wang, Z. Q., Shi, J., Liang, X., et al. (2018). A retrotransposon in an HKT1 family sodium transporter causes variation of leaf Na(+) exclusion and salt tolerance in maize. *New Phytol.* 217, 1161–1176. doi: 10.1111/nph.14882

Zhang, Q., Li, N., Zhou, G., Lu, X., Xu, Z., and Li, Z. (2003). In vivo antioxidant activity of polysaccharide fraction from *Porphyra haitanesis* (Rhodephyta) in aging mice. *Pharmacol. Res.* 48, 151–155. doi: 10.1016/s1043-6618(03)00103-8

Zhang, S., Gan, Y., and Xu, B. (2016). Application of plant-growth-promoting fungi trichoderma longibrachiatum t6 enhances tolerance of wheat to salt stress through improvement of antioxidative defense system and gene expression. *Front. Plant Sci.* 7:1405. doi: 10.3389/fpls.2016.01405

Zhang, X., Li, K., Liu, S., Zou, P., Xing, R., Yu, H., et al. (2017). Relationship between the degree of polymerization of chitooligomers and their activity affecting the growth of wheat seedlings under salt stress. *J. Agric. Food Chem.* 65, 501–509. doi: 10.1021/acs.jafc.6b03665

Zhang, Z., Mao, C., Shi, Z., and Kou, X. (2017). The amino acid metabolic and carbohydrate metabolic pathway play important roles during salt-stress response in tomato. *Front. Plant Sci.* 8:1231. doi: 10.3389/fpls.2017.01231

Zhao, T., Zhang, Q., Qi, H., Zhang, H., Niu, X., Xu, Z., et al. (2006). Degradation of porphyran from *Porphyra haitanensis* and the antioxidant activities of the degraded porphyrans with different molecular weight. *Int. J. Biol. Macromol.* 38, 45–50. doi: 10.1016/j.ijbiomac.2005.12.018

Zhou, C., Yu, X., Zhang, Y., He, R., and Ma, H. (2012). Ultrasonic degradation, purification and analysis of structure and antioxidant activity of polysaccharide from *Porphyra yezoensis* Udea. *Carbohydr. Polym.* 87, 2046–2051. doi: 10.1016/j.carbpol.2011.10.026

Zou, P., Li, K., Liu, S., He, X., Zhang, X., Xing, R., et al. (2016). Effect of sulfated chitooligosaccharides on wheat seedlings (*Triticum aestivum* L.) under salt stress. *J. Agric. Food Chem.* 64, 2815–2821. doi: 10.1021/acs.jafc.5b05624

Zou, P., Li, K., Liu, S., Xing, R., Qin, Y., Yu, H., et al. (2015). Effect of chitooligosaccharides with different degrees of acetylation on wheat seedlings under salt stress. *Carbohydr. Polym.* 126, 62–69. doi: 10.1016/j.carbpol.2015.03.028

Conflict of Interest Statement: The authors declare that the research was conducted in the absence of any commercial or financial relationships that could be construed as a potential conflict of interest.

frontiers in Plant Science

ORIGINAL RESEARCH
published: 15 May 2018
doi: 10.3389/fpls.2018.00635

5-Aminolevulinic Acid (ALA) Alleviated Salinity Stress in Cucumber Seedlings by Enhancing Chlorophyll Synthesis Pathway

*Yue Wu[1†], Xin Jin[1†], Weibiao Liao[1], Linli Hu[1], Mohammed M. Dawuda[1,2], Xingjie Zhao[3], Zhongqi Tang[1], Tingyu Gong[1] and Jihua Yu[1]**

[1] College of Horticulture, Gansu Agricultural University, Lanzhou, China, [2] Department of Horticulture, Faculty of Agriculture, University for Development Studies, Tamale, Ghana, [3] School of Environmental and Municipal Engineering, Lanzhou Jiaotong University, Lanzhou, China

5-Aminolevulinic acid (ALA) is a common precursor of tetrapyrroles as well as a crucial growth regulator in higher plants. ALA has been proven to be effective in improving photosynthesis and alleviating the adverse effects of various abiotic stresses in higher plants. However, little is known about the mechanism of ALA in ameliorating the photosynthesis of plant under abiotic stress. In this paper, we studied the effects of exogenous ALA on salinity-induced damages of photosynthesis in cucumber (*Cucumis sativus* L.) seedlings. We found that the morphology (plant height, leave area), light utilization capacity of PS II [qL, Y(II)] and gas exchange capacity (Pn, gs, Ci, and Tr) were significantly retarded under NaCl stress, but these parameters were all recovered by the foliar application of 25 mg L^{-1} ALA. Besides, salinity caused heme accumulation and up-regulation of gene expression of ferrochelatase (*HEMH*) with suppression of other genes involved in chlorophyll synthesis pathway. Exogenously application of ALA under salinity down-regulated the heme content and *HEMH* expression, but increased the gene expression levels of glutamyl-tRNA reductase (*HEMA1*), Mg-chelatase (*CHLH*), and protochlorophyllide oxidoreductase (*POR*). Moreover, the contents of intermediates involved in chlorophyll branch were increased by ALA, including protoporphyrin IX (Proto IX), Mg-protoporphyrin IX (Mg-Proto IX, protochlorophyllide (Pchlide), and chlorophyll (Chl *a* and Chl *b*) under salt stress. Ultrastructural observation of mesophyll cell showed that the damages of photosynthetic apparatus under salinity were fixed by ALA. Collectively, the chlorophyll biosynthesis pathway was enhanced by exogenous ALA to improve the tolerance of cucumber under salinity.

Keywords: 5-aminolevulinic acid, salinity, photosynthesis, tetrapyrrol biosynthesis, cucumber seedlings

OPEN ACCESS

Edited by:
Youssef Rouphael, University of Naples Federico II, Italy

Reviewed by:
Sheng Shu, Nanjing Agricultural University, China
Rosa Porcel, Universitat Politècnica de València, Spain

****Correspondence:***
Jihua Yu
yujihuagg@163.com

† These authors have contributed equally to this work as co-first authors.

Specialty section:
This article was submitted to Crop and Product Physiology, a section of the journal Frontiers in Plant Science

Received: *12 December 2017*
Accepted: *24 April 2018*
Published: *15 May 2018*

Citation:
Wu Y, Jin X, Liao W, Hu L, Dawuda MM, Zhao X, Tang Z, Gong T and Yu J (2018) 5-Aminolevulinic Acid (ALA) Alleviated Salinity Stress in Cucumber Seedlings by Enhancing Chlorophyll Synthesis Pathway. Front. Plant Sci. 9:635. doi: 10.3389/fpls.2018.00635

INTRODUCTION

5-aminolevulinic acid (ALA) has been considered as a growth regulator or potential plant hormone in higher plants. Its promotive role in enhancing plant biomass, photosynthesis and fruit quality under normal growth condition has been confirmed in rice (*Oryza sativa* L.) (Nguyen et al., 2016), strawberry (*Fragaria ananassa* Duch.) (Sun et al., 2017), and peach (*Prunus persica* L.)

(Ye et al., 2017). In addition, ALA is known to be effective against the harmful effects caused by various abiotic stresses in plants. For example, foliar application of ALA alleviated the peroxidation of membrane and inhibition of net photosynthetic rate caused by salinity stress in creeping bentgrass (*Agrostis stolonifera* L.) (Yang et al., 2014). The application of ALA to roots significantly reduced the harmful effects of waterlogging stress by enhancing the activities of lactate dehydrogenase (LDH) and alcohol dehydrogenase (ADH) in *Ficus carica* L. (An et al., 2016). Moreover, exogenous ALA improved the resistance of peach (*Prunnus persica* L.) (Ye et al., 2016), tomato (*Lycopersicon esculentum* Mill.) (Zhang Z.-P. et al., 2015), rice (*Oryza sativa* L.) (Nunkaew et al., 2014), swiss chard (*Beta vulgaris* L.) (Liu et al., 2014), sicklepod (*Cassia obtusifolia* L.) (Zhang et al., 2013), and cucumber (*Cucumis sativus* L.) (Zhen et al., 2012) to salt stress. Furthermore, as a key precursor in the biosynthesis pathway of chlorophyll, ALA was found to have promotive role in photosynthesis under various stresses. Exogenously supplied ALA increased the content of chlorophyll which was suppressed by UV-B stress in lettuce (*Lactuca sativa* L.) (Aksakal et al., 2017). In another study, foliar application of ALA up-regulated the chlorophyll fluorescence indexes, including qP, φPSII, and Fv/Fm, in oilseed rape (*Brassica napus* L.) under drought stress (Liu et al., 2013). Besides, gas exchange indexes, such as net photosynthetic rate (Pn), stomatal conductance (gs), intercellular CO_2 concentration (Ci) and transpiration rate (Tr), which were adversely affected by abiotic stress, were, however, promoted by ALA application in cauliflower (*Brassica oleracea botrytis* L.) under chromium stress (Ahmad et al., 2017). The relative gene expressions, like fructose-1,6-bisphosphatase (*FBP*), triose-3-phosphate isomerase (*TPI*) and ribulose-1,5-bisphosphate carboxylase/oxygenase small subunit (*RBCS*) of enzymes in Calvin cycle of photosynthesis were up-regulated by ALA and the carbohydrate contents were enhanced in oilseed rape (*Brassica napus* L.) under drought stress (Liu et al., 2016a).

Recently, exogenous application of ALA has been shown to have a positive effect on chlorophyll synthesis in de-etiolated cotyledon of oilseed rape under water-deficit stress (Liu et al., 2016b). Moreover, as another metabolic branch downstream of ALA, endogenous heme content was increased significantly by exogenous ALA in maize (*Zea mays* L.) under non-stressful conditions (Yonezawa et al., 2015). However, the regulative mechanisms of exogenous ALA to tetrapyrrol biosynthesis pathway and photosynthesis under salt stress have not been evaluated yet. Keeping in view of the crucial role ALA playing in tetrapyrrol synthesis and its alleviative effects to stress-damages in plant, the present study was designed to test a hypothesis that exogenous ALA could enhance plant stress tolerance by heightening the chlorophyll synthesis pathway. In this paper, the intermediate contents and relative gene expression levels of crucial enzymes among branches downstream of ALA metabolic pathway (including Fe-branch and Mg-branch) in cucumber under salinity stress were determined. Then, the photosynthesis capacity, intrinsic water use efficiency and the ultrastructure in mesophyll cell of cucumber leaves were determined to verify the stimulative effects of ALA. Thus, the main objective of the study was to explore the mechanism of ALA in improving plants tolerance to salt stress using cucumber as a test crop.

MATERIALS AND METHODS

Plant Material and Growth Conditions

Cucumber seeds (*Cucumis sativus* L. cv. Xinchun No. 4) were surface sterilized with liquor potassii permanganatis (0.03%) for 10 min, and rinsed with distilled water. The seeds were soaked in distilled water for 6 h and then exposed to germination conditions. The moistened seeds were placed on double-layer filter paper and kept at 28 ± 1°C under dark condition. At 5 days after germination, seedlings with uniform size, fully spread cotyledons, and well-formed roots were transferred to 1-L opaque plastic containers containing half-strength Yamasaki's cucumber nutrient solution ($Ca(NO_3)_2$ 1.75 mmol L^{-1}, KNO_3 3 mmol L^{-1}, $NH_4H_2PO_4$ 0.5 mmol L^{-1}, $MgSO_4{\cdot}7H_2O$ 1 mmol L^{-1}). One repetition of each treatment consisted of two 1-L opaque plastic containers and each container contained four seedlings. Each treatment was repeated three times. The seedlings were grown in artificial climate chamber throughout the experiment. The light intensity in the climate chamber was 350–450 μmol m^{-2} s^{-1}, temperature was 18–28°C and the relative humidity was 50–60%. The nutrient solution was changed at 2-day intervals.

Experiments and Data Collection

In experiment 1, the 30-day-old seedlings which were raised in half-strength Yamasaki's cucumber nutrient solution were used to determine the NaCl concentration that could cause moderate salt stress in the cucumber seedlings. The seedlings were subjected to five levels of NaCl concentrations including 0, 25, 50, 75, and 100 mmol L^{-1} for 10 days in the nutrient solution. The 50 mmol L^{-1} NaCl was found to have caused moderate salt stress in cucumber seedlings based on the morphological characteristics studied in experiment 1. In experiment 2, the most effective ALA (Sigma Aldrich, United States) concentration in alleviating moderate NaCl stress in cucumber seedlings was determined. There were five treatments including (1) normal nutrient solution only; (2) 50 mmol L^{-1} NaCl in nutrient solution + 0 mg L^{-1} ALA; (3) 50 mmol L^{-1} NaCl in nutrient solution + 10 mg L^{-1} ALA; (4) 50 mmol L^{-1} NaCl in nutrient solution + 25 mg L^{-1} ALA, and (5) 50 mmol L^{-1} NaCl in nutrient solution + 50 mg L^{-1} ALA. The ALA was applied with hand-held nebulize by thoroughly spraying both the upper and lower surfaces of leaves. ALA application was done twice, as soon as the seedlings were exposed to the salt treatment and at 24 h afterwards. Meanwhile, the treatments without ALA sprayed distilled water to the same extent. The 25 mg L^{-1} ALA was found to have the most appropriate alleviative effect towards moderate salt stress based on the morphological characteristics studied in the experiment 2. In experiment 3, after concentration screening of NaCl and ALA, the treatments were (1) CK: normal nutrient solution only; (2) NaCl: 50 mmol L^{-1} NaCl in nutrient solution + 0 mg L^{-1} ALA; (3) NaCl + ALA: 50 mmol L^{-1} NaCl in nutrient solution + 25 mg L^{-1} ALA, and (4) ALA: normal nutrient solution + 25 mg L^{-1} ALA. The ALA application was

done as in experiment 2. All indexes were measured at 10 days after treatments application.

Morphological Indexes

To selected appropriate concentration of chemicals (NaCl and ALA) in this study, leaf area and plant height of seedlings were measured at 10 days after treatment application. The leaf areas of fully opened true leaves of the seedlings were determined by a leaf area analyzer (YMJ-C, Tuopu Instruments Inc., China). Plant height was determined by tracing a string along the length of the stem and the length obtained was measured with a meter rule.

Chlorophyll Fluorescence Parameters

Chlorophyll fluorescence induction parameters were measured using an Imaging-PAM Chlorophyll Fluorometer (Walz, Effeltrich, Germany). The seedlings from each treatment were kept in darkness for 30 min to fully open the reaction centers of photosystems II before measurement. Through the application of a saturation pulse, which was 2700 μmol m^{-2} s^{-1}, Fo (minimum fluorescence of the dark-adapted leaves) and Fm (maximum fluorescence yield of the dark-adapted leaves) were obtained from dark-adapted leaves. Then, the actinic light (81 μmol m^{-2} s^{-1}), which was opened every 20 s and lasted for 0.8 s, the light-adaptation time was 5 min. Under the application of actinic light, indexes like Fo' (minimum fluorescence of the light-adapted leaves), Fs (steady chlorophyll fluorescence of light-adapted leaves) and Fm' (maximum fluorescence yield of the light-adapted leaves) could be collected from the fully light adapted leaves.

The actual photosynthetic efficiency [Y(II)] was calculated as described by Genty et al. (1989). The quantum yield of regulated energy dissipation in PS II [Y(NPQ)] and the quantum yield of non-regulated energy dissipation in PS II [Y(NO)] were calculated according to Kramer et al.'s (2004) method . The coefficient of actinic light quenching (qL) was calculated according to the method of Klughammer and Schreiber (Klughammer and Schreiber, 2008). The specific computational formulas were as follows:

$$Y(II) = (Fm' - Fs)/Fm'$$
$$Y(NO) = 1/(NPQ + 1 + qL(Fm/Fo - 1))$$
$$Y(NPQ) = 1 - Y(II) - 1/(NPQ + 1 + qL(Fm/Fo - 1))$$
$$Y(II) + Y(NO) + Y(NPQ) = 1$$
$$NPQ = (Fm - Fm')/Fm'$$
$$qL = (Fm' - Fs)/(Fm' - Fo') \times Fo'/Fs$$

Gas Exchange Parameters

Gas exchange indexes including the net photosynthetic rate (Pn), intercellular CO_2 concentration (Ci), stomata conductance (gs) and transpiration rate (Tr) of the fourth true leaf of sampled seedlings were determined with portable photosynthetic system (CIRAS-2, PP System, United Kingdom). The photosynthetic photon flux density (400 μmol m^{-2} s^{-1}), ambient CO_2 concentration (380 μmol mol^{-1}), leaf temperature (25°C), and relative humidity (70%) were maintained throughout the measurements. In addition, the intrinsic water use efficiency (iWUE) of each treatment was derived based on Pn and gs according to Pimentel et al.'s (1999) method, which was calculated by dividing Pn by gs .

Intermediates Contents on ALA Metabolic Pathway

ALA Content

The measurement of ALA was performed according to the methods of Morton (1975). A fresh leaf sample (5 g) was homogenized with 6 mL acetate buffer (pH 4.6) in ice bath; then, the homogenate was centrifuged at 5000 *g* for 15 min at 4°C. After that the supernatant (5 mL) was mixed with four drops of acetylacetic ester, and incubated at 100°C, 10 min for condensation reaction in a water bath. After cooling to room temperature, fresh Ehrlich's reagent solution (containing 42 mL glacial acetic acid, 8 mL 70% perchloric acid, 1 g dimethylaminobenzaldehyde) in the same volume was mixed and allowed for 15 min. The absorbance was measured at 554 nm and concentration was calculated using a standard curve of ALA reference standards. Specifically, the concentrations of ALA standard curve were 0, 5, 15, 20, and 25 μg mL^{-1}.

Uro III Content

Uroporphyrinogen III (Uro III) was determined according to Bogorad with some modifications (Bogorad, 1962). Fresh leaf sample (1 g) was homogenized with 5 mL Tris–HCl buffer (pH 7.2) in ice bath, and then the homogenate was centrifuged at 5000 *g* for 15 min at 4°C. The glacial acetic acid was used to adjust the supernatant to pH 4.0, then, centrifuged at 5000 *g* for 15 min at 4°C. The sediment was mixed with 5 mL distilled water and centrifuged at 5000 *g* for 15 min at 4°C. Then, precooled ammonia spirit (4 mL) was added into the sediment to extract Uro III, and centrifuged at 5000 *g* for 15 min at 4°C. The supernatant was evaporated to dryness at 55°C. Then, sulfuric acid-methanol (4 mL, 5%) was added into it to esterify for 48 h. Then, 20 mL distilled water was added to the mixture and extracted by mixing with 4 mL chloroform. The mixture was evaporated to dryness at 55°C, and then, chloroform (4 mL) was added and the absorbance was measured at 405 nm and the content of Uro III was calculated by the following formula. In the formula, $\varepsilon = 5.48 \times 10^5$ L mol^{-1} cm^{-1} is the molar extinction coefficient of Uro III under 405 nm. $d = 1$ cm is the optical path length of determine solution. $V = 0.004$ L is the dissolved volume of Uro III. FW = 1 g is the weight of fresh sample. The 10^9 is to convert the unit from mol g FW^{-1} to nmol g FW^{-1}.

$$\text{Uro III (nmol g FW}^{-1}) = [A405/(\varepsilon \times d)] \times V/FW \times 10^9$$

Proto IX, Mg-Proto IX and Pchlide Contents

The content of protoporphyrin IX (Proto IX), Mg-protoporphyrin IX (Mg-Proto IX), and protochlorophyllide (Pchlide) were determined according to the method of Hodgins and Van Huystee (1986) with some modifications . Fresh leaf sample (0.3 g) was homogenized with 5 mL 80% alkaline acetone, and then 80% alkaline acetone was added to the volume of 25 mL. The homogenate was incubated in dark condition until the tissue was bleached. After that, the homogenate was centrifuged at 1500 *g* for 10 min. The absorbance was measured at 575 nm,

590 nm, and 628 nm using the supernatant; then, the results were calculated by the corresponding formulas (Liu et al., 2015). In the formula, V is the dissolved volume of determined solution; FW is the weight of fresh sample.

$$\text{ProtoIX}(\mu\text{mol g FW}^{-1}) = (0.18016 \times A575 - 0.04036 \times A628 - 0.04515 \times \text{A590}) \times \text{V/FW}$$

$$\text{Mg} - \text{ProtoIX}(\mu\text{mol g FW}^{-1}) = (0.06077 \times \text{A590} - 0.01937 \times \text{A575} - 0.003423 \times \text{A628}) \times \text{V/FW}$$

$$\text{Pchlide}(\mu\text{mol g FW}^{-1}) = (0.03563 \times \text{A628} + 0.007225 \times \text{A590} - 0.02955 \times \text{A575}) \times \text{V/FW}$$

Heme Content

The measurement of heme was performed according to the methods of Marsh et al. (1963)with some modifications . The fresh leaf sample (2 g) was ground in liquid nitrogen, and then mixed with 5 mL of extract I (containing 0.5 mL 0.1 mol L^{-1} ammonia and 4.5 mL pure acetone).The mixture was centrifuged at 8000 *g* for 10 min. This process was repeated until the chlorophyll was completely removed. After that, extract II (5 mL; containing 80% acetone, 16% dimethyl sulfoxide and 4% concentrated sulfuric acid) was added into the sediment, and centrifuged at 8000 *g* for 10 min. The supernatant was mixed with 0.7 mL ethanol and the absorbance was determined at 386 nm. The heme concentration was calculated using a standard curve of heme reference standards. And the concentrations of heme standard curve were 0, 1, 3, 5, 7, and 10 μg mL^{-1}.

Chlorophyll Content

The chlorophyll content of leaves was extracted with an 80% buffered aqueous acetone according to Porra et al.'s (1989) method . The supernatant was determined at 646 nm and 663 nm, and the content of chlorophyll (Chl *a* and Chl *b*) was calculated using the following formulas according to Lichtenthaler and Wellburn (1983).

$$\text{Chl } a \text{ (mg g FW}^{-1}) = (12.21 \times \text{A663} - 2.81 \times \text{A646}) \times \text{V/FW}$$
$$\text{Chl } b \text{ (mg g FW}^{-1}) = (20.13 \times \text{A646} - 5.03 \times \text{A663}) \times \text{V/FW}$$

Total RNA Extraction and Gene Expression Analysis

Total RNA was extracted using TaKaRa MiniBEST Plant RNA Extraction Kit (TaKaRa Biomedicals, Japan) according to the manufacturer's protocol. Synthesis of cDNA was executed with RevertAid First Strand cDNA Synthesis Kit (Thermo Scientific, United States). RNA solution (contain 2 μg RNA) and 1 μL oligo(dT)18 were added into PCR tube, and incubated under 65°C for 5 min in a thermal cycler (Bio-Rad, United States), and then rapidly cooled in ice. The PCR tube was then incubated under 42°C for 60 min in the thermal cycler with 4 μL 5× buffer, 2 μL 10 mmol L^{-1} dNTPs, 1 μL RNA inhibitor, and 1 μL reverse transcriptase. After that, the reverse transcriptase was inactivated under 80°C. The real time quantitative RT-PCR was implemented to analyze the expression of enzyme genes among ALA metabolic pathway in cucumber seedlings through a SYBR Premix Ex Taq II (Tli RNaseH Plus; TaKaRa Biomedicals, Japan). The cucumber α-*tubulin* gene was used as an internal control. Gene bank accession numbers of the sequences used to design the primers are shown in **Table 1**. The q-PCR test was executed in a fluorescence ration PCR instrument (LightCycler® 96 System, Roche, United Kingdom). The reaction system contained 10 μL 2 × Tli RNaseH Plus, 0.8 μL forward primer, 0.8 μL reverse primer, 2 μL cDNA, 6.4 μL RNase Free dH_2O. Samples for RT-qPCR were obtained from three seedlings for each treatment (n = 3) in each qPCR test, and every sample on the sample plate contained three wells of target gene and three wells of negative control (by adding components of the reaction system except for templet cDNA, and the templet was replaced by RNase Free dH_2O). The PCR conditions were: initial denaturation at 95°C for 30 s, then cycle steps (40 t) at 95°C for 5 s, 60°C for 30 s and the melt curve conditions were 95°C for 5 s, 60°C for 60 s and 95°C. The last step of cooling was 30 s at 50°C. Each qPCR manipulation was replicated three times. Quantification analysis was performed by the comparative CT method (Livak and Schmittgen, 2001). The CT value of α-tubulin was subtracted from that of the target gene to obtain a ΔCT value. The average CT value of the CK sample in this experiment was subtracted from the ΔCT value to obtain a ΔΔCT value. Then the relative expression level to the control for each sample was expressed as $2^{-\Delta\Delta CT}$.

Chloroplast Ultrastructure Observation

Small pieces (about 1 mm^2) of fresh leaf samples were fixed in 3% glutaraldehyde in 0.1 M phosphate buffer (pH 7.4) for 24 h

TABLE 1 | Primer sequences and Genebank accession number of the *HEMA1*, *HEMH*, *CHLH*, *POR*, *CAO* and α-*tubulin* gene sequences.

Gene symbol	Accession number	Forward primer (5′-3′)	Reverse primer (5′-3′)
HEMA1	D50407	5′-TTTGTCTCAGCATCGTGGAG-3′	5′-ATGTTGTGTGGCATCGTTGT-3′
HEMH	AB037113	5′-TGGAGTGTTGTTGCTGAACC-3′	5′-TTGGAGGAAACGGAACAATC-3′
CHLH	JW942287	5′-TTCGGTTGTGTCGCTTACTG-3′	5′-ACCAAAGGCAAAGCAACAAT-3′
POR	D50085	5′-AATGATCGACGGTGGTGAGT-3′	5′-CAAATGTTATGCCGGTTTCC-3′
CAO	AB512416	5′-AGCAGATTCCCTTCATGCAC-3′	5′-AAATGTTTGCTCCGTTGACC-3′
α-*tubulin*	AJ715498	5′-ACGCTGTTGGTGGTGGTAC-3′	5′-GAGAGGGGTAAACAGTGAATC-3′

at 4°C. The leaf samples were then fixed in 1% H_2OsO_4 for 5 h. After that, dehydration was carried out using graded ethanol series (70%, 80%, 90%, and 100%); then, acetone-infiltrated and embedded in Epon812 epoxy resin. Ultrathin sections were cut on a microtome (Leica EM UC6 ultra-microtome, Japan), and then stained with uranyl acetate and lead citrate for 15 min. Ultrathin sections of cucumber leaf was examined and photographed with a transmission electron microscope (TEM, Joel JEM-1230, Japan).

Statistical Analysis

All the experiments were performed with three replicates and results were expressed as mean ± SE. Analysis of variance was performed using SPSS 22.0 (SPSS Institute Inc., United States) and treatments means were compared using the Tukey's test at a 0.05 level of probability. All figures were prepared with OriginPro 2017 (OriginLab Institute Inc., United States).

RESULTS

Leaf Area and Plant Height

Salinity stress, which was administered by various concentrations of NaCl in 1/2 Yamasaki cucumber nutrient solution reduced the leaf area of seedlings (**Figure 1**). Except the 25 mmol L^{-1} NaCl treatment, leaf area, and plant height were decreased significantly with the higher concentrations of NaCl, where the highest concentration of NaCl (100 mmol L^{-1}) even caused the death of the seedlings. Compared with the control, the 25-mmol L^{-1} NaCl caused 13.00% reduction in leaf area but increased plant height by 3.36%. The 50-mmol L^{-1} NaCl resulted in 41.67% reduction in leaf area and 17.68% in plant height. The 75-mmol L^{-1} NaCl decreased leaf area by 60.52% and plant height by 45.95%. The 100-mmol L^{-1} NaCl caused 82.21% reduction in leaf area and 64.13% in plant height. Based on the effects of the various NaCl concentrations on leaf area and plant height, we considered the 25 mmol L^{-1} as mild stress, 50 mmol L^{-1} as moderate stress, 75 mmol L^{-1} as severe stress and 100 mmol L^{-1} as lethal dosage. The 50-mmol L^{-1} NaCl treatment which provided moderate salt stress was employed in the subsequent experiment.

As shown in **Figure 2**, the effects of various concentrations of ALA sprayed on the seedlings showed dose-dependent effect under moderate salt stress. With the increase of ALA concentration, the leaf area and plant height of cucumber seedlings showed a tendency of increasing first and then decreasing. Moreover, both the two indexes reached the highest values when treated with 25 mg L^{-1} ALA under NaCl stress. Otherwise, the leaf area in 25 mg L^{-1} ALA treatment had no significant difference with that in CK treatment. However, cucumber leaves treated with high level of ALA (50 mg L^{-1}) showed growth inhibition and chlorotic symptoms. Consequently, 25 mg L^{-1} ALA was the optimal concentration against moderate NaCl stress and was used for further experiments.

Chlorophyll Fluorescence Parameters

As shown in **Figure 3**, Y(II) and qL markedly declined in leaves treated with NaCl alone and Y(NO) increased significantly compared with CK. When the leaves were sprayed with ALA under salt condition, it significantly promoted Y(II) and qL of the seedlings but Y(NO) was suppressed to the level of CK. Furthermore, the Y(NPQ) of leaves in CK treatment was lower than those of the other treatments.

Gas Exchange Parameters

Figure 4 showed the results on Pn, gs, Ci, Tr and iWUE of cucumber leaves under salt stress with or without ALA application. All the indexes were decreased by 50 mmol L^{-1} NaCl when compared to CK. Compared with seedlings treated with NaCl alone, application of ALA under salt condition showed positive effect on the photosynthetic gas exchange parameters, among them, Pn was increased by 255.84%, gs 130.50%, Ci 8.97%, and Tr 220.35%. Meanwhile, exogenous application of ALA stimulated the leave iWUE of seedlings which was depressed under salt stress. The value of iWUE increased by 63.28% compared to NaCl treatment. For the treatment with ALA alone, Pn and Ci decreased but gs and Tr showed no significant difference compared to CK.

Intermediates Contents on ALA Metabolic Pathway

The contents of several important intermediates among the ALA downstream metabolic pathway were determined to explore the mechanism of exogenous ALA on photosynthesis of cucumber seedlings under salt stress. As shown in **Figure 5A**, the contents of ALA and Uro III increased markedly under stressful condition, while, foliar application of ALA recovered ALA and Uro III contents to levels similar to that in the CK. Under salt stress, the Proto IX content reduced by 40.37% in contrast to the control group, but was remarkably increased when ALA was applied to seedlings. In seedlings treated with ALA alone, the content of Proto IX reached the highest value among all treatments (**Figure 5B**). On the contrary, the heme content significantly increased by 147.01% compared to CK under salt stress. When ALA was applied to seedlings subjected to the stress condition, the heme content decreased. Moreover, heme content showed no significant difference compared to CK when ALA was applied under normal growth condition (**Figure 5B**). The contents of Mg-Proto IXand Pchlide were both decreased by salinity but exogenous ALA application increased their levels evidently in cucumber leaves under salt stress. In addition, treatment with ALA only showed relative high concentrations of Mg-Proto IX and Pchlide (**Figure 5C**). Salt stress inhibited the total content of chlorophyll markedly, which was due to the reduction of both chlorophyll *a* and chlorophyll *b*. Exogenous application of ALA reversed the inhibitory effects of salinity on chlorophyll content. Besides, as for the treatment with ALA only, the contents of Chl *a* and Chl *b* were decreased compared to CK (**Figure 5D**).

Relative Expressions of Genes on ALA Metabolic Pathway

The relative expression of genes related to tetrapyrrol biosynthesis pathway is presented in **Figure 6**. Under salinity, the level of *HEMA1* was down-regulated significantly compared

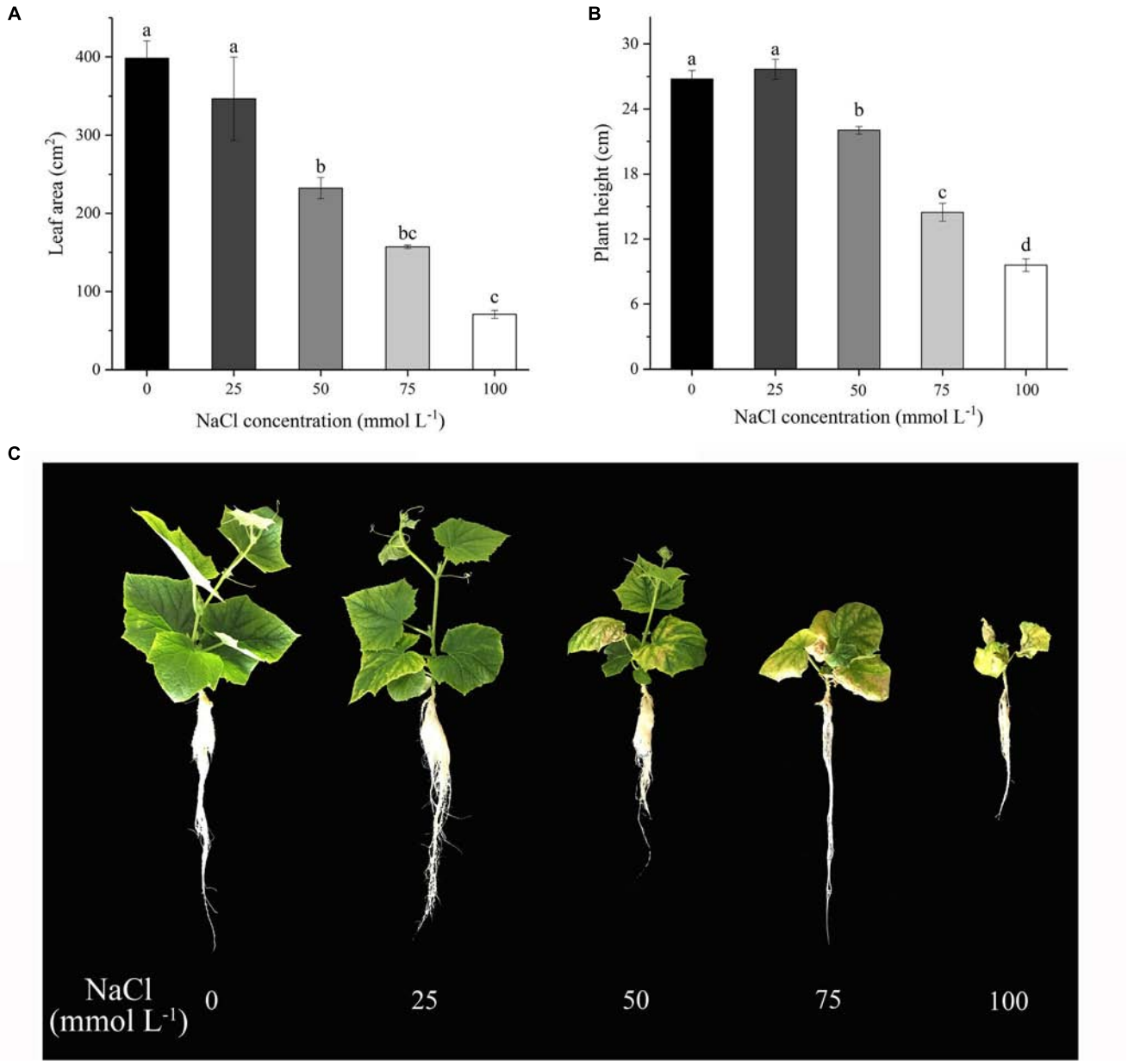


FIGURE 1 | Effects of different NaCl concentrations on leaf morphology and plant height of cucumber seedlings. **(A)** Leaf area changes of cucumber seedlings under different concentration of NaCl. **(B)** Plant height of cucumber seedlings under different concentration of NaCl. **(C)** Morphological changes of cucumber seedlings under various concentration of NaCl, including 0, 25, 50, 75, 100 mmol L^{-1}. The data presented were the means of three plants ± SE of means (n = 3, with 6 plants per replication). Treatment means with different letters indicate significant differences according to Tukey's test ($p < 0.05$).

to untreated plants, but up-regulated by applying ALA under stress condition. In addition, treatment with ALA alone resulted in a threefold expression of *HEMA1* compared to control (**Figure 6A**). *HEMH* expression was up-regulated significantly by salt stress, and then showed slight decrease by spraying ALA. But *HEMH* gene level in seedlings treated with ALA solely had no significant difference compared to that CK (**Figure 6B**). Although suppressed by salinity, the expression of *CHLH* was stimulated by ALA application, which was 2.17-fold compared to control (**Figure 6C**). As shown in **Figure 6D**, the gene level of *POR* remained stable under moderate salt stress, while ALA treatments up-regulated its expression to 4.79 and 7.41 fold with or without stress condition, respectively, compared to the control. The expression of *CAO* was inhibited by salt but recovered by exogenous ALA. Moreover, *CAO* gene in treatment with ALA alone revealed relative low level compared with CK (**Figure 6E**).

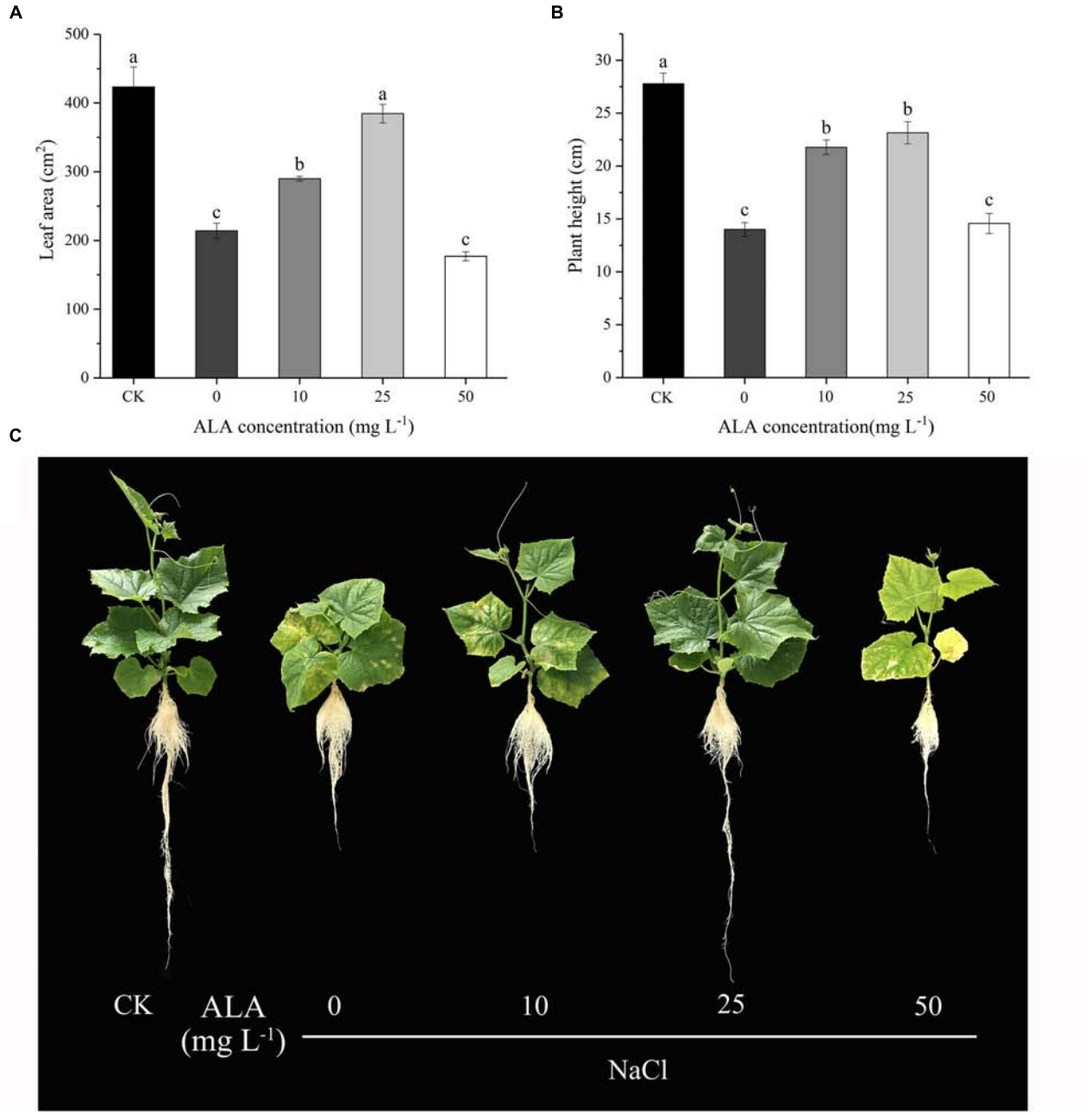


FIGURE 2 | Effects of different ALA concentrations on leaf morphology and plant height of cucumber seedlings under salt stress. **(A)** Leaf area changes of cucumber seedlings treated with different concentration of ALA. **(B)** Plant height of cucumber seedlings treated with different concentration of ALA. **(C)** Morphological changes of cucumber seedlings under various ALA concentrations. The data presented were the means of three plants ± SE of means (n = 3, with 6 plants per replication). Treatment means with different letters indicate significant differences according to Tukey's test ($p < 0.05$).

Ultrastructure Morphometric Changes

Changes of whole mesophyll cells and chloroplasts are shown in **Figure 7**. Seedlings grown in normal condition exhibited regular cell shape and typical chloroplast; meanwhile, there were smoothly arrayed grana lamellae, number of well packed starch grains, and a small quantity of osmiophilic granules (**Figures 7A–D**). However, cell morphological disturbance and plasmolysis occurred when seedlings were treated with 50 mmol L^{-1} NaCl, but the number of mitochondria markedly increased (**Figures 7E,F**). The grana lamellae of thylakoid were loosed and the shapes of chloroplasts severely swollen. Furthermore, there were plenty of osmiophilic granules as well as less starch grains in chloroplast compared with control (**Figures 7G,H**). For the ALA treated seedlings under salt

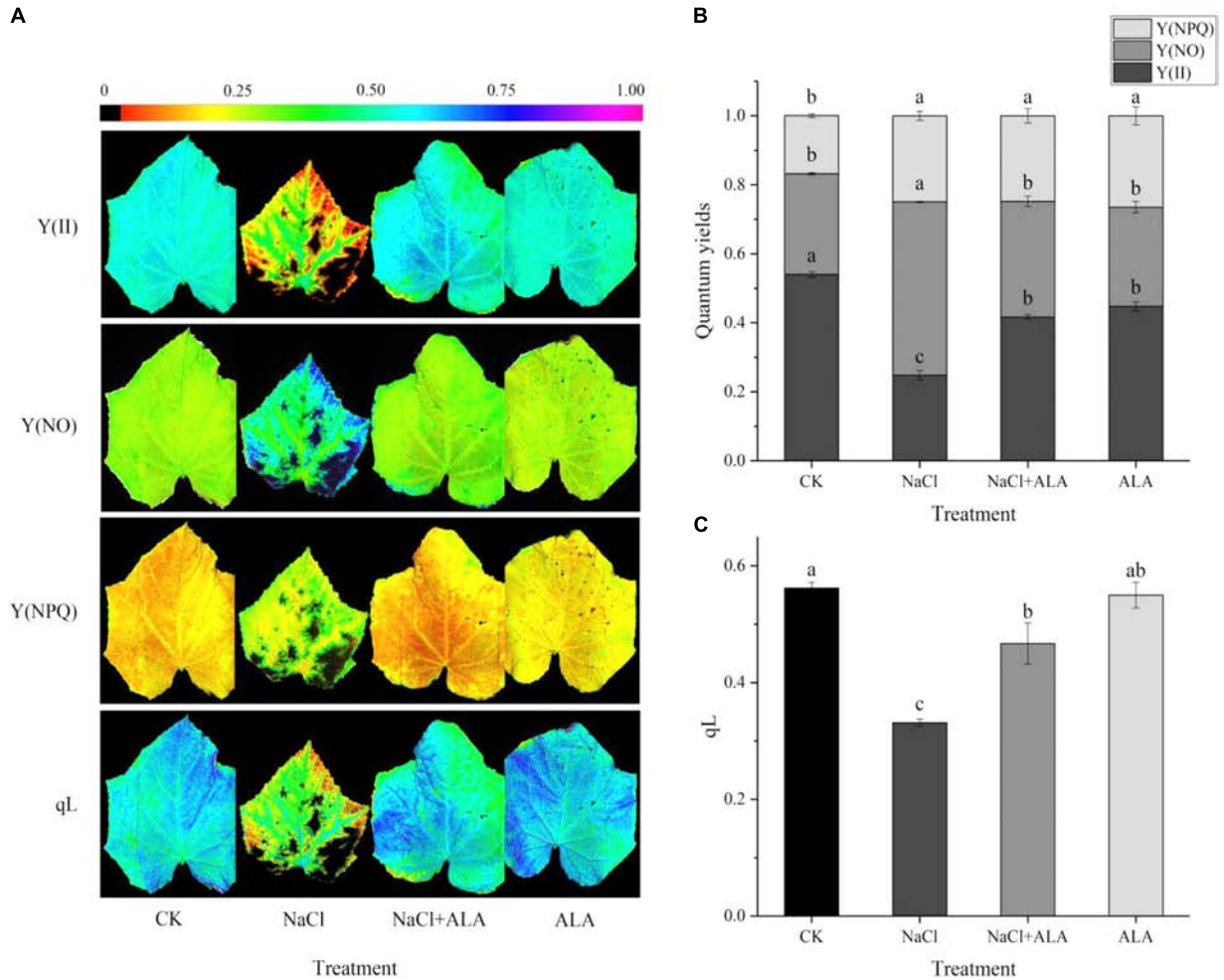


FIGURE 3 | Effects of ALA on chlorophyll fluorescence parameters under salt condition. **(A)** Chlorophyll fluorescence images, the fluorescence images of the Y(II), Y(NO), and Y(NPQ) are given in corresponding colors that represent the absolute values of the ratio ranged from 0 (black) to 1.0 (purple). **(B)** Quantum yield of PSII, including Y(II) (actual photosynthetic efficiency of PS II), Y(NO) (the quantum yield of non-regulated energy dissipation) and Y(NPQ) (the effective quantum yield of PS II). **(C)** Changes of qL (photochemical quenching coefficient). The data presented were the means of three plants ± SE of means (n = 3). Treatment means with different letters indicate significant differences according to Tukey's test ($p < 0.05$).

stress condition, although there was a little improvement of cell morphology, the shapes of chloroplast become typically fusiform (**Figures 7I,J**). Moreover, the chloroplasts contained more orderly grana lamellae, more starch grains and fewer osmiophilic granules than those of seedlings under NaCl stress (**Figures 7K,L**). Under normal growth condition, ALA-treated seedlings were very similar to that of the CK plants. However, exogenous ALA application significantly increased the number of starch grains in chloroplast of the treated seedlings (**Figures 7M–P**).

DISCUSSIONS

Salt stress is one of the common abiotic stresses in agricultural production, and salinization in soil or culture substrates is a growing problem for agriculture worldwide (Deinlein et al., 2014). The harmful effects caused by rhizospheric salt stress involve various physiological and biochemical mechanisms related to plant growth and development. Accordingly, improving the growth and tolerance of plants under salt conditions is gaining prominence in research field.

Alleviation Effects of Exogenous ALA on Growth Under NaCl Stress

In recent years, it has been well established that ALA, a natural plant growth regulator, could effectively improve plants tolerance to many environmental stresses (Akram and Ashraf, 2013). In the present study, we found that leaf growth of cucumber seedlings was suppressed by different NaCl concentrations of salt, and the inhibitory effect aggravated with the increase in salt level. However, application of ALA at a relative low concentration reversed the adverse effects caused by moderate NaCl stress. It has been reported that exogenous ALA increased plant dry

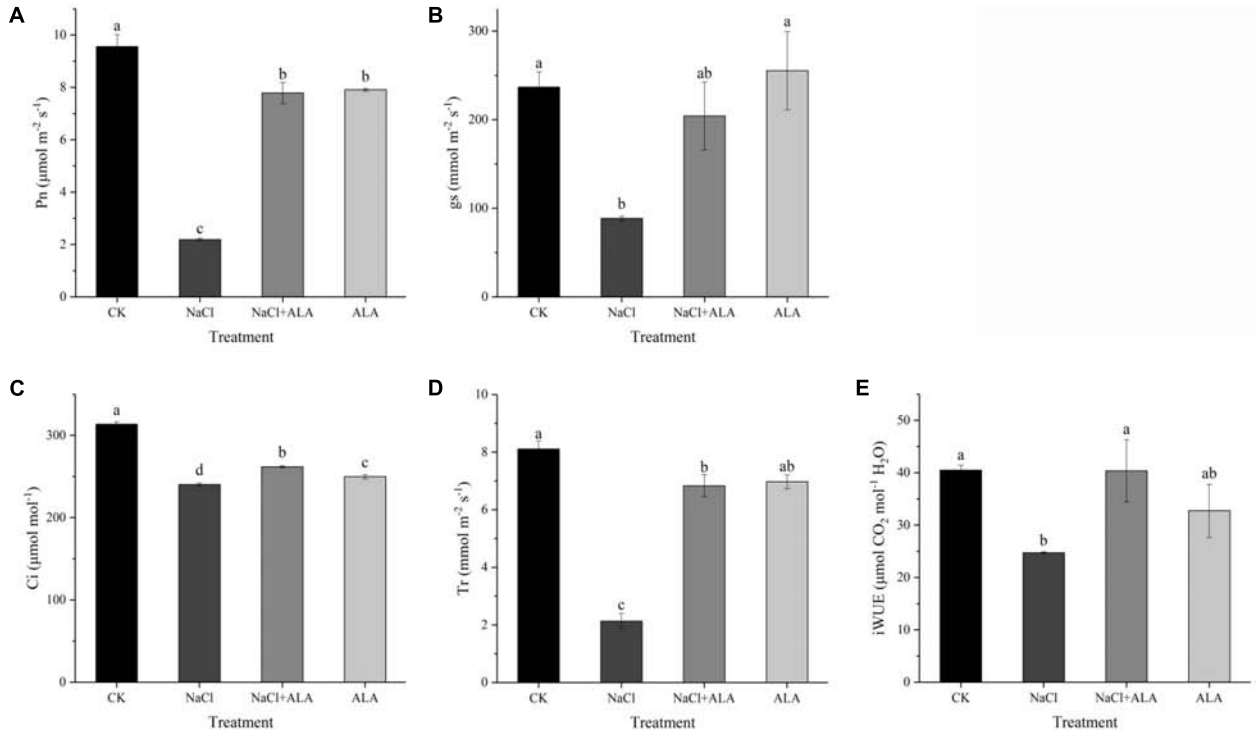


FIGURE 4 | Effects of ALA on photosynthetic gas exchange parameters under salt stress. **(A)** Net photosynthetic rate (Pn). **(B)** Stomatal conductance (gs). **(C)** Intercellular CO_2 concentration (Ci). **(D)** Transpiration rate (Tr). **(E)** Intrinsic water use efficiency (iWUE). The data presented were the means of 3 plants ± SE of means ($n = 3$). Treatment means with different letters indicate significant differences according to Tukey's test ($p < 0.05$).

weight, relative growth rate (RGR) and relative water content (RWC) under stressful conditions (Liu et al., 2014; An et al., 2016). In addition, ALA at 2 mg L^{-1} increased the hypocotyl length of oilseed rape under cadmium stress, but when ALA was added at 10 mg L^{-1}, hypocotyl length decreased significantly (Ali et al., 2013a). These findings have shown that low levels of ALA stimulated the growth of seedlings, while high levels inhibited growth under stress condition.

Regulation of Chlorophyll and Heme Biosynthesis Pathway by Exogenous ALA Under NaCl Stress

In order to explore the mechanism of the regulative effects of ALA on photosynthesis under stress, the pathway downstream of ALA, which is associated with photosynthetic pigment, was studied in the present research. The rate-limiting enzyme among this pathway which is encoded by *HEMA1*, Glu-tRNA reductase enzyme (GluTR), can be feedback regulated by downstream products. The relative expression of *HEMA1* was suppressed under salt condition, which was accompanied by increasing content of endogenous heme, indicating that the accumulation of heme restrained *HEMA1* gene expression by a feedback regulation. It has been demonstrated by other studies that reduction of *HEMA1* could weaken the activity of GluTR and inhibit heme and chlorophyll biosynthesis, while a notable quantity of heme could decrease the activity of GluTR (Kumar and Söll, 2000; Zhang M. et al., 2015). These results suggest that feedback inhibition manner of heme towards GluTR was mainly at the gene expression level. In addition, what made the large accumulation of heme under NaCl stress was the up-regulated *HEMH* gene which encoded ferrochelatase (FECH). The accumulation of heme was coupled with reduction of Proto IX, Mg-Proto IX and Pchlide, and we also noticed that endogenous ALA and Uro III accumulated at moderate salt concentration. It is possible to speculate that the metabolic pathway of ALA under moderate salt stress can redirect its focus from the pathway of chlorophyll branch to the heme branch; since the stress resistance effects of heme and its oxydates have been proven in higher plants. Catalyzed by heme oxygenase (HO), heme can be transformed into CO, free iron (Fe^{2+}), and biliverdin (BV), then, BV will turn to bilirubin (BR) (Kwon et al., 2011). Among them, BR could inhibit protein oxidation *in vitro* in the presence of a variety of oxidants including superoxide and hydroxyl radicals; and CO played a critical role as signaling molecule and participated in regulating against various abiotic stress in plants (Wegiel et al., 2014; Wang and Liao, 2016). The study of transgenic rice (*Oryza sativa* L.), which overexpressed *Bradyrhizobium japonicum* FECH gene, resulted in increasing activity of FECH, raising content of heme and enhancing tolerance of oxidative stress (Kim et al., 2014). Apparently, this metabolic focus switch might be an adaptive mechanism in cucumber under NaCl stress (see regulation manner of metabolic pathway in

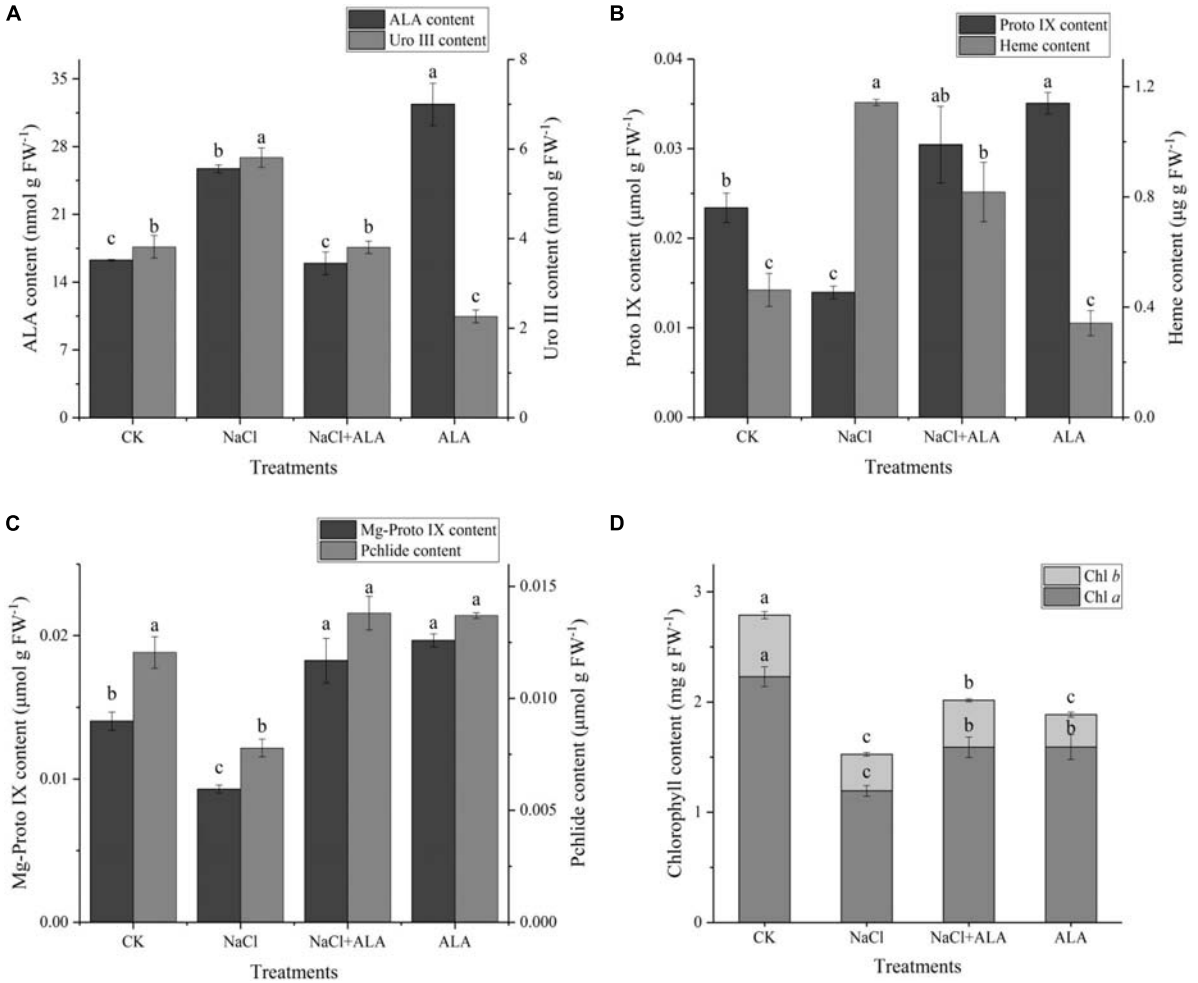


FIGURE 5 | Contents of intermediates on ALA metabolic pathway. **(A)** Contents of ALA (5-aminolevulinic acid) and Uro III (uroporphyrinogen III). **(B)** Contents of Proto IX (protoporphyrin IX) and heme. **(C)** Contents of Mg-Proto IX (Mg-protoporphyrin IX) and Pchlide (protochlorophyllide). **(D)** Content of chlorophyll, including Chl *a* and Chl *b*. The data presented were the means of 3 plants ± SE of means ($n = 3$). Treatment means with different letters indicate significant differences according to Tukey's test ($p < 0.05$).

Figure 8). In another study, water-deficit stress up-regulated the ferrochelatase gene and weakened the expression of Mg-chelatase gene in oilseed cotyledons (Liu et al., 2016b). Nevertheless, ALA applied exogenously reversed those phenomena caused by stress. For example, the expression of genes (including *HEMA1*, *CHLH* and *POR*) involved in chlorophyll biosynthesis was up-regulated under stressful condition. Especially for the gene *CHLH*, which encodes the key H-subunit of Mg-chelatase, is the crucial regulator in Fe-branch (Chen, 2014). MCH consists of three subunits, ChlH, ChlI, and ChlD in higher plants, among which it is ChlH that is primarily responsible for catalytic action of MCH (Richter and Grimm, 2013). The content of intermediate products in chlorophyll pathway, such as Proto IX, Mg-Proto IX, and Pchlide, were all increased by ALA application. Finally, chlorophyll *a* (Chl *a*) content was significantly increased by the strengthening of whole Mg-branch. Moreover, the content of chlorophyll *b* (Chl *b*) was also raised by up-regulating *CAO* gene which could be inhibited by salt. Chl *a* and Chl *b* are known as the major light-harvesting pigments in photosystems of all oxygenic photosynthetic organisms. Meanwhile, CAO is a unique enzyme responsible for Chl *b* synthesis (Sakuraba et al., 2010; Tsuchiya et al., 2012). Therefore, photosynthetic capacity of photosystems II (PS II) was enhanced by increasing light-harvesting pigments in our study. Exogenous ALA increased the content of chlorophyll in rice (*Oryza sativa* L.) (Nguyen et al., 2016). Subsequently, we also noticed that at stressful condition, heme content and *HEMH* expression were not reduced to the level of CK. Moreover, the Fe-branch of seedlings treated with ALA under normal condition remained stable similar to the level of control while the Mg-branch was stimulated markedly. This interesting phenomenon might suggest that stress environment is a prerequisite for heme pathway strengthening; and the effects of exogenous ALA in enhancing chlorophyll biosynthesis coordinated with the slight retard of heme biosynthesis (see regulation manner of metabolic pathway in **Figure 8**). Therefore, exogenous ALA can reverse

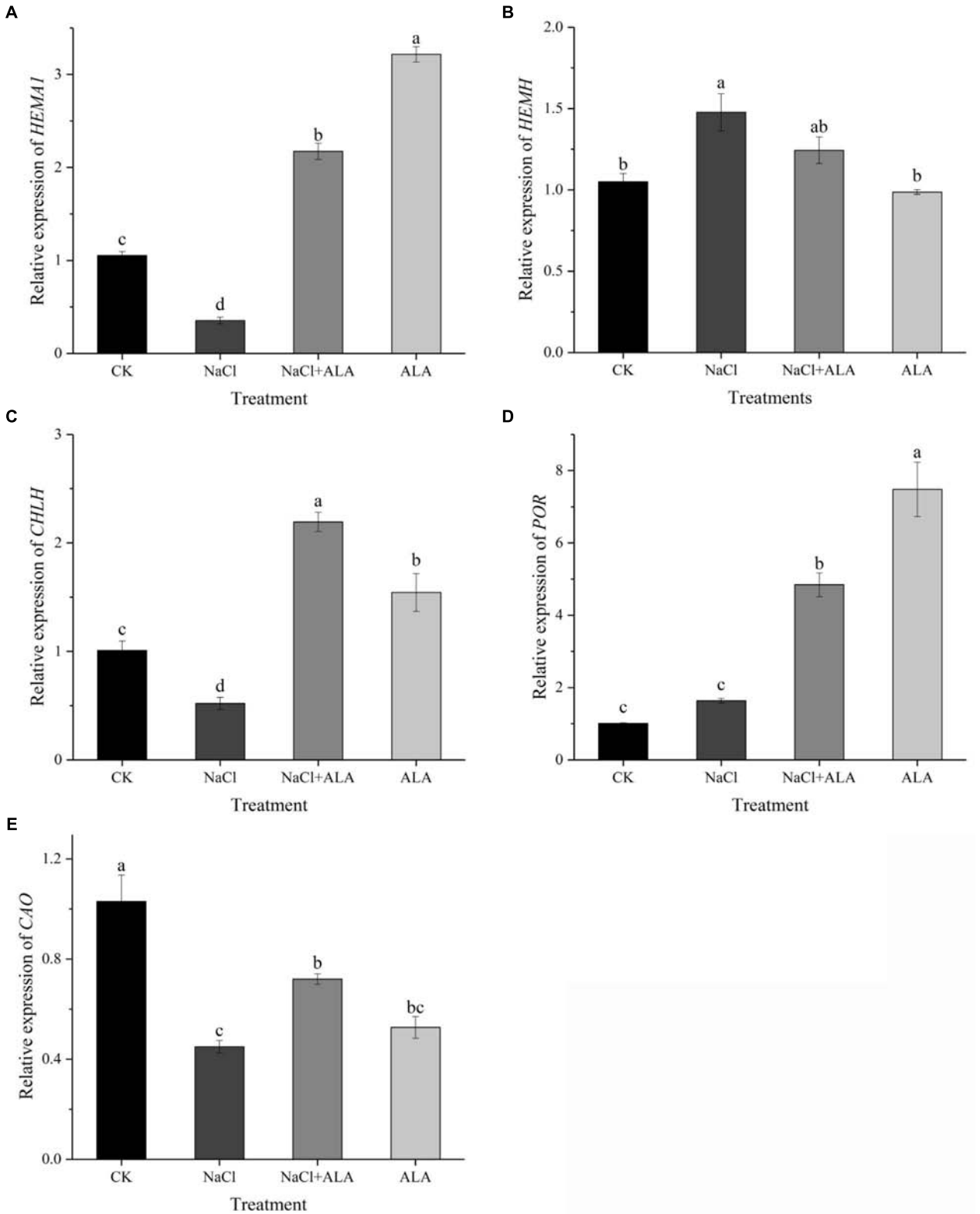


FIGURE 6 | Relative expressions of genes involved in ALA metabolic pathway. **(A)** Relative expression of *HEMA1*, encoding glutamyl-tRNA reductase (Glu-TR). **(B)** Relative expression of *HEMH*, encoding ferrochelatase (FECH). **(C)** Relative expression of *CHLH*, encoding Mg-chelatase (MCH). **(D)** Relative expression of *POR*, encoding protochlorophyllide oxidoreductase (POR). **(E)** Relative expression of *CAO*, encoding chlorophyllide *a* oxygenase (CAO). The data presented were the means of 3 plants ± SE of means ($n = 3$). Treatment means with different letters indicate significant differences according to Tukey's test ($p < 0.05$).

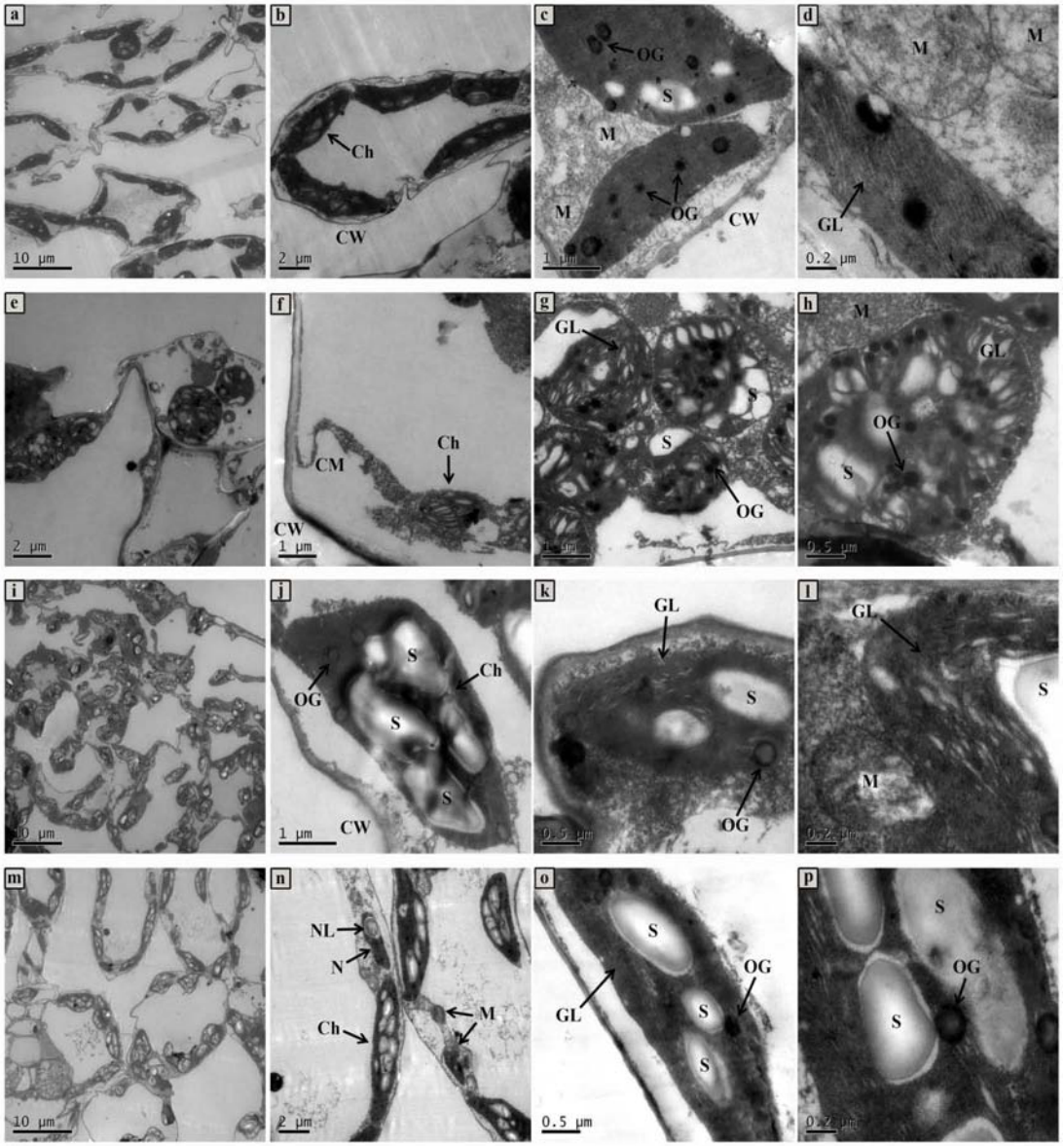


FIGURE 7 | The ultrastructural observation of mesophyll cell and chloroplast of cucumber seedlings. **(a–d)** Seedlings grown in normal condition. **(e–h)** 50 mmol L^{-1} NaCl treated seedlings. **(i–l)** Seedlings treated with 50 mmol L^{-1} NaCl and 25 mg L^{-1} ALA simultaneously. **(m–p)** Seedlings sprayed 25 mg L^{-1} ALA only. Abbreviations: CW, cell wall; CM, cell membrane; N, nucleus; NL, nucleolus; Ch, chloroplast; OG, osmiophilic globules; GL, grana lamella; S, starch; M, mitochondria.

the suppression of chlorophyll biosynthesis pathway which is caused by stressful conditions in plants. Meanwhile, the heme biosynthesis pathway can be enhanced under stress as an adaptive mechanism of the plant, and can also be retarded under enhanced chlorophyll synthesis.

Promotion Effects of Exogenous ALA on Photosynthetic Capacity Under NaCl Stress

The effectiveness of photosystems II (PS II) can be reflected by chlorophyll fluorescence indexes. The present study has shown that saline rhizosphere and foliar application of ALA affected various chlorophyll fluorescence indexes of cucumber seedlings, including Y(II) (actual photosynthetic efficiency); Y(NO) (quantum yield of non-regulated energy dissipation in PSII) and Y(NPQ) (quantum yield of regulated energy dissipation in PS II) quantum yield of regulated energy dissipation in PS II) (Klughammer and Schreiber, 2008). Moreover, these three indexes represent the energy distribution and the activity of the photosynthetic reaction center in PS II. In this study, Y(II) and qL were significantly declined by NaCl stress while Y(NO) and Y(NPQ) were increased, suggesting that salt stress reduced the quantity of light quantum absorbed by the reaction center,

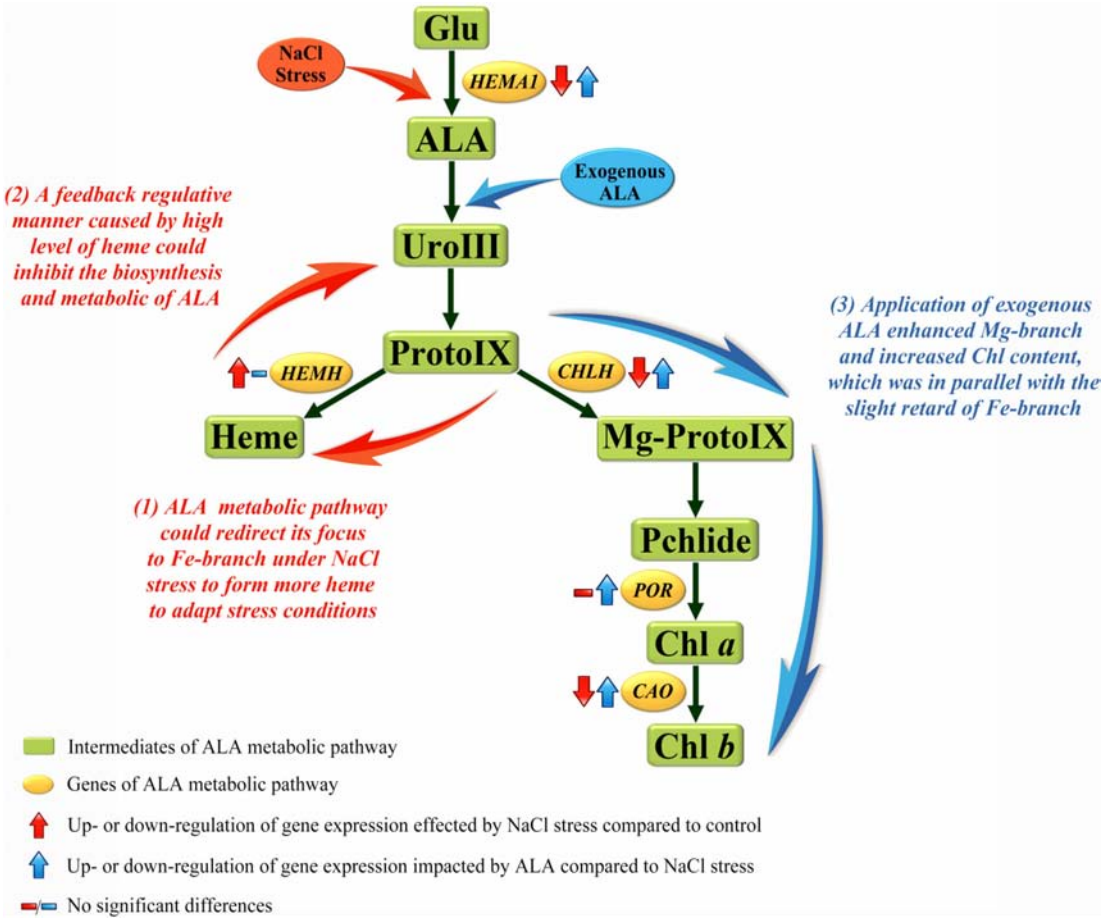


FIGURE 8 | Regulation manners among ALA metabolic pathway of cucumber under NaCl stress. The metabolic pathway downstream of ALA in higher plants can mainly fall into Fe-branch and Mg-branch, for heme biosynthesis and chlorophyll biosynthesis, respectively. Results showed that (1) Fe-branch was enhanced under salt stress to form more heme, this could be conjectured as an adaptive or against response in plants; (2) The accumulation of heme under stress condition feed-back inhibited the biosynthesis and metabolic of endogenous ALA; (3) Application of exogenous ALA under stress enhanced the Mg-branch to produce more chlorophyll and kept the Fe-branch enhanced at the same time.

and shut down PS II. The excess light energy couldn't dissipate through regulatory mechanism of seedling, implicated by the high Y(NO), the damage had been caused to photosynthetical system under salt condition. These results were similar to the report on oil rape (*Brassica napus* L.), where herbicide stress aggravated the non-regulated heat dissipation and weakened photosynthetic efficiency (Jin et al., 2011). However, exogenous ALA effectively diminished the proportion of Y(NO) and enhanced photochemical energy conversion although Y(NPQ) remained stable. As mentioned above, under NaCl stress, application of ALA could enhance the relevant gene and intermediates of Mg-branch, which ultimately led to the increase in Chl *a* and Chl *b* contents. Therefore, excess light energy was reduced through the improved absorption and transmission of light energy since Chl *a* and Chl *b* were the main constituents in light-harvesting complex II (LHC II) (Akihito and Graham, 2010). Besides, photosynthetic efficiency in PS II of treatment with ALA alone was retarded, which was coupled with the reduction of chlorophyll content. This suggested that application of ALA to plants under normal growth environment would not enhance photosynthesis. In contrast, light-sensitive intermediate products (such as Proto IX and Pchlide) accumulated in thylakoid, and easily caused photo-damage (Lee et al., 2003). The reason behind the enhancement of light harvesting efficiency under salt stress is due to heightening of Mg-branch through applied-ALA which mainly increased the chlorophyll content.

Carbon assimilation is a vital procedure of photosynthesis in higher plants. Previous studies have shown that gas exchange indexes under salinity stress could be enhanced by ALA application (Liu et al., 2014). In our experiment, we found that ALA significantly increased the net photosynthetic rate and gas exchange capacity in cucumber seedlings under NaCl stress conditions. Salinity could inhibit the stomatal aperture and led to the reduction of gs. It has been demonstrated that under water-stress the aquaporins (such as *OePIP1.1* and *OePIP2.1*) are closely involved in the regulation of gs (Perez-Martin et al., 2014). Stomatal conductivity and net photosynthesis rate have

been reported to be sensitive to saline environment (Nelson et al., 2007). Cadmium stress decreased the net photosynthetic rate by decreasing gs in oil seed rape but the ALA-treated plants exhibited distinct improvement of the gas exchange indexes and photosynthesis activity (Ali et al., 2013b). In another experiment, lead toxicity negatively affected Ci and Tr of *Brassica napus*, which were repaired by exogenous ALA (Tian et al., 2014). Relative high gs could increase CO_2 uptake of mesophyll cell, and the assimilatory efficiency of Calvin cycle could be enhanced. Coupled with the photoreaction we discussed above, exogenous ALA enhanced photosynthetic efficiency in PS II under stress, which could bring more energy to dark reaction. Moreover, ALA reversed the reduction of gs and Ci caused by stress condition, which could bring more carbon source to form assimilation product. Therefore, net photosynthetic rate could be ameliorated by ALA under salt stress. The index of iWUE is often used to evaluate gas exchange adaptation. Researchers have found that it was closely related to environmental CO_2 concentration for plants in nature (Leavitt et al., 2003; van der Sleen et al., 2014). In the facility environment with equal CO_2 level, it could be regulated by stress conditions. For example, under mild water-deficit stress, the iWUE of *Phaseolus vulgaris* increased obviously, indicated that plants might have adaptation mechanism to against stress condition (Santos et al., 2009). However, under NaCl stress, in the present study, the value of iWUE in cucumber leaves decreased significantly. Similarly, in a pot-experiment that simulated salt stress in groundwater, with the increasing penetration depths of ground salinity water (0.3–0.8 m), the iWUE of quinoa (*Chenopodium quinoa* Willd.) continued to decrease (Talebnejad and Sepaskhah, 2016). Since cucumber seedlings were suffered in moderate salt stress for ten days, it indicated that the iWUE of plant is sensitive to stress degree. Nevertheless, exogenous ALA increased the iWUE of seedlings under stress condition. This indicated that ALA could enhance stomatal conductance and water use for photosynthesis then alleviated the harmful effects of salt stress. This promotive role of ALA might relative to the enhanced function of aquaporins (AQPs). Since the AQP genes could be up-regulated by exogenous ALA under salinity condition, and overexpression *NtAQP1* in tomato resulted in improved iWUE, because some aquaporin protein functions as both water and CO_2 channel (Sade et al., 2010; Yan et al., 2014).

Enhancement Effects of Exogenous ALA on Ultrastructure of Mesophyll Cell Under NaCl Stress

The alleviation effect of ALA on chloroplast under stressful condition has been well proved by researches (Naeem et al., 2012; Ali et al., 2013a; Tian et al., 2014). In this study, leaves of cucumber seedlings which were grown in normal condition had typical shape of mesophyll cell and chloroplast, whereas those under salt stress showed swollen chloroplast and abnormal cellular morphology. Under lead toxicity, the chloroplasts became swollen and ruptured; cell wall and cell membrane were diffused, but in the TEM micrographs of leaves treated with ALA, the adverse effects of lead were fixed (Tian et al., 2014). In addition, the accumulation of starch grains in chloroplasts indicated the activation of carbon assimilation of photosynthesis in plants (Wang et al., 2015). The number and size of osmiophilic globules can also be used as an indicator of thylakoid disintegration (Ding et al., 2015). Osmiophilic granules are regarded as the collection of lipid produced by thylakoid degradation. In the present study, osmiophilic granules accumulated obviously in chloroplast, suggesting that salt stress caused cell senescence to the mesophyll cells of cucumber. The exogenous ALA repaired the lamellar structure in chloroplast and gradually decreased the number of osmiophilic granules. This observation was consistent with an earlier report of a study conducted on *Brassica napus* L (Ali et al., 2013b). Besides, the treatment with ALA under normal condition, the starch grains were obviously accumulated more than ALA applied under stress. It could be suggested that the starch grains probably decomposed to form energy and osmotic adjustment substances which worked against abiotic stress. Therefore, application of ALA repaired the disturbance of thylakoid and the accelerated cell senescence of mesophyll cell caused by stressful condition, which provided a necessary site for the normal operation of photosynthesis in plant.

CONCLUSION

The results of our experiments have demonstrated that the tetrapyrrol biosynthesis pathway downstream of ALA could redirect its focus to heme branch to adapt salt condition. In addition, the chlorophyll biosynthesis pathway was enhanced by exogenous ALA which was accompanied with the retarding of heme synthesis pathway under salinity stress. Exogenous ALA application enhanced the tolerance of the seedlings to salt stress through improvement in chlorophyll synthesis, light harvesting capacity, photosynthesis capacity, and also retarded thylakoid degradation. Therefore, application of ALA on cucumber seedlings could ameliorate the harmful effects caused by NaCl stress.

AUTHOR CONTRIBUTIONS

YW and JY conceived and designed the research. YW, XJ, and XZ conducted the experiments. YW, ZT, and TG analyzed the data and prepared the figures and illustrations. YW wrote the manuscript. WL, LH, and MD read the manuscript and made valuable inputs. All authors read and approved the submission of the manuscript.

FUNDING

This work was supported by the National Natural Science Foundation of China (No. 31660584), China Agriculture Research System (CARS-25-C-07), and Natural Science Foundation of Gansu References Province, China (1610RJZA098).

REFERENCES

Ahmad, R., Ali, S., Hannan, F., Rizwan, M., Iqbal, M., Hassan, Z., et al. (2017). Promotive role of 5-aminolevulinic acid on chromium-induced morphological, photosynthetic, and oxidative changes in cauliflower (*Brassica oleracea* botrytis L.). *Environ. Sci. Pollut. Res.* 24, 8814–8824. doi: 10.1007/s11356-017-8603-7

Akihito, I., and Graham, R. F. (2010). Quantum superpositions in photosynthetic light harvesting: delocalization and entanglement. *N. J. Phys.* 12, 055004. doi: 10.1088/1367-2630/12/5/055004

Akram, N. A., and Ashraf, M. (2013). Regulation in plant stress tolerance by a potential plant growth regulator, 5-aminolevulinic acid. *J. Plant Growth Regul.* 32, 663–679. doi: 10.1007/s00344-013-9325-9

Aksakal, O., Algur, O. F., Icoglu Aksakal, F., and Aysin, F. (2017). Exogenous 5-aminolevulinic acid alleviates the detrimental effects of UV-B stress on lettuce (*Lactuca sativa* L) seedlings. *Acta Physiol. Plant.* 39:55. doi: 10.1007/s11738-017-2347-3

Ali, B., Huang, C. R., Qi, Z. Y., Ali, S., Daud, M. K., Geng, X. X., et al. (2013a). 5-aminolevulinic acid ameliorates cadmium-induced morphological, biochemical, and ultrastructural changes in seedlings of oilseed rape. *Environ. Sci. Pollut. Res.* 20, 7256–7267. doi: 10.1007/s11356-013-1735-5

Ali, B., Wang, B., Ali, S., Ghani, M. A., Hayat, M. T., Yang, C., et al. (2013b). 5-aminolevulinic acid ameliorates the growth, photosynthetic gas exchange capacity, and ultrastructural changes under cadmium stress in *Brassica napus* L. *J. Plant Growth Regul.* 32, 604–614. doi: 10.1007/s00344-013-9328-6

An, Y., Qi, L., and Wang, L. (2016). ALA pretreatment improves waterlogging tolerance of fig plants. *PLoS One* 11:e0147202. doi: 10.1371/journal.pone.0147202

Bogorad, L. (1962). Porphyrin synthesis. *Methods Enzymol.* 5, 885–895. doi: 10.1016/S0076-6879(62)05334-3

Chen, M. (2014). Chlorophyll modifications and their spectral extension in oxygenic photosynthesis. *Annu. Rev. Biochem.* 83, 317–340. doi: 10.1146/annurev-biochem-072711-162943

Deinlein, U., Stephan, A. B., Horie, T., Luo, W., Xu, G., and Schroeder, J. I. (2014). Plant salt-tolerance mechanisms. *Trends Plant Sci.* 19, 371–379. doi: 10.1016/j.tplants.2014.02.001

Ding, G., Li, C., Han, X., Chi, C., Zhang, D., and Liu, B. (2015). Effects of lead on ultrastructure of isoetes sinensis palmer (Isoetaceae), a critically endangered species in China. *PLoS One* 10:e0139231. doi: 10.1371/journal.pone.0139231

Genty, B., Briantais, J.-M., and Baker, N. R. (1989). The relationship between the quantum yield of photosynthetic electron transport and quenching of chlorophyll fluorescence. *Biochim. Biophys. Acta* 990, 87–92. doi: 10.1016/S0304-4165(89)80016-9

Hodgins, R. R., and Van Huystee, R. B. (1986). Rapid simultaneous estimation of protoporphyrin and Mg-porphyrins in higher plants. *J. Plant Physiol.* 125, 311–323. doi: 10.1016/S0176-1617(86)80153-5

Jin, Z. L., Tian, T., Naeem, M. S., Jilani, G., Zhang, F., and Zhou, W. J. (2011). Chlorophyll fluorescence responses to application of new herbicide ZJ0273 in winter oilseed rape species. *Int. J. Agric. Biol.* 13, 43–50.

Kim, J.-G., Back, K., Lee, H. Y., Lee, H.-J., Phung, T.-H., Grimm, B., et al. (2014). Increased expression of Fe-chelatase leads to increased metabolic flux into heme and confers protection against photodynamically induced oxidative stress. *Plant Mol. Biol.* 86, 271–287. doi: 10.1007/s11103-014-0228-3

Klughammer, C., and Schreiber, U. (2008). Complementary PS II quantum yields calculated from simple fluorescence parameters measured by PAM fluorometry and the saturation pulse method. *Pam Appl. Notes* 1, 27–35.

Kramer, D. M., Johnson, G., Kiirats, O., and Edwards, G. E. (2004). New fluorescence parameters for the determination of QA redox state and excitation energy fluxes. *Photosynth. Res.* 79:209. doi: 10.1023/B:PRES.0000015391.99477.0d

Kumar, A. M., and Söll, D. (2000). Antisense HEMA1 RNA expression inhibits heme and chlorophyll biosynthesis in Arabidopsis. *Plant Physiol.* 122, 49–56. doi: 10.1104/pp.122.1.49

Kwon, S. W., Sohn, E. J., Kim, D. W., Jeong, H. J., Kim, M. J., Ahn, E. H., et al. (2011). Anti-inflammatory effect of transduced PEP-1-heme oxygenase-1 in Raw 264.7 cells and a mouse edema model. *Biochem. Biophys. Res. Commun.* 411, 354–359. doi: 10.1016/j.bbrc.2011.06.147

Leavitt, S. W., Idso, S. B., Kimball, B. A., Burns, J. M., Sinha, A., and Stott, L. (2003). The effect of long-term atmospheric CO2 enrichment on the intrinsic water-use efficiency of sour orange trees. *Chemosphere* 50, 217–222. doi: 10.1016/S0045-6535(02)00378-8

Lee, K. P., Kim, C., Lee, D. W., and Apel, K. (2003). TIGRINA d, required for regulating the biosynthesis of tetrapyrroles in barley, is an ortholog of the FLU gene of *Arabidopsis thaliana*. *FEBS Lett.* 553, 119–124. doi: 10.1016/S0014-5793(03)00983-9

Lichtenthaler, H. K., and Wellburn, A. R. (1983). Determination of total carotenoids and chlorophylls A and B of leaf in different solvents. *Biochem. Soc. Trans.* 11, 591–592. doi: 10.1042/bst0110591

Liu, D., Hu, L. Y., Ali, B., Yang, A. G., Wan, G. L., Xu, L., et al. (2016a). Influence of 5-aminolevulinic acid on photosynthetically related parameters and gene expression in *Brassica napus* L. under drought stress. *Soil Sci. Plant Nutr.* 62, 254–262. doi: 10.1080/00380768.2016.1198216

Liu, D., Kong, D. D., Fu, X. K., Ali, B., Xu, L., and Zhou, W. J. (2016b). Influence of exogenous 5-aminolevulinic acid on chlorophyll synthesis and related gene expression in oilseed rape de-etiolated cotyledons under water-deficit stress. *Photosynthetica* 54, 468–474. doi: 10.1007/s11099-016-0197-7

Liu, D., Wu, L., Naeem, M. S., Liu, H., Deng, X., Xu, L., et al. (2013). 5-Aminolevulinic acid enhances photosynthetic gas exchange, chlorophyll fluorescence and antioxidant system in oilseed rape under drought stress. *Acta Physiol. Plant.* 35, 2747–2759. doi: 10.1007/s11738-013-1307-9

Liu, J., Wang, J., Yao, X., Zhang, Y., Li, J., Wang, X., et al. (2015). Characterization and fine mapping of thermo-sensitive chlorophyll deficit mutant1 in rice (*Oryza sativa* L.). *Breed. Sci.* 65, 161–169. doi: 10.1270/jsbbs.65.161

Liu, L., Nguyen, N. T., Ueda, A., and Saneoka, H. (2014). Effects of 5-aminolevulinic acid on Swiss chard (*Beta vulgaris* L. subsp. cicla) seedling growth under saline conditions. *Plant Growth Regul.* 74, 219–228. doi: 10.1007/s10725-014-9913-0

Livak, K. J., and Schmittgen, T. D. (2001). Analysis of relative gene expression data using real-time quantitative PCR and the $2^{-\Delta\Delta^{CT}}$ method. *Methods* 25, 402–408. doi: 10.1006/meth.2001.1262

Marsh, H. Jr., Evans, H., and Matrone, G. (1963). Investigations of the role of iron in chlorophyll metabolism. II. Effect of iron deficiency on chlorophyll synthesis. *Plant Physiol.* 38, 638–642. doi: 10.1104/pp.38.6.638

Morton, R. A. (1975). *Biochemical Spectroscopy*. Cambridge, MA: Academic Press.

Naeem, M. S., Warusawitharana, H., Liu, H., Liu, D., Ahmad, R., Waraich, E. A., et al. (2012). 5-Aminolevulinic acid alleviates the salinity-induced changes in *Brassica napus* as revealed by the ultrastructural study of chloroplast. *Plant Physiol. Biochem.* 57, 84–92. doi: 10.1016/j.plaphy.2012.05.018

Nelson, D. E., Repetti, P. P., Adams, T. R., Creelman, R. A., Wu, J., Warner, D. C., et al. (2007). Plant nuclear factor Y (NF-Y) B subunits confer drought tolerance and lead to improved corn yields on water-limited acres. *Proc. Natl. Acad. Sci. U.S.A.* 104, 16450–16455. doi: 10.1073/pnas.0707193104

Nguyen, H., Kim, H.-S., and Jung, S. (2016). Altered tetrapyrrole metabolism and transcriptome during growth-promoting actions in rice plants treated with 5-aminolevulinic acid. *Plant Growth Regul.* 78, 133–144. doi: 10.1007/s10725-015-0080-8

Nunkaew, T., Kantachote, D., Kanzaki, H., Nitoda, T., and Ritchie, R. J. (2014). Effects of 5-aminolevulinic acid (ALA)-containing supernatants from selected *Rhodopseudomonas palustris* strains on rice growth under NaCl stress, with mediating effects on chlorophyll, photosynthetic electron transport and antioxidative enzymes. *Electron. J. Biotechnol.* 17, 19–26. doi: 10.1016/j.ejbt.2013.12.004

Perez-Martin, A., Michelazzo, C., Torres-Ruiz, J. M., Flexas, J., Fernández, J. E., Sebastiani, L., et al. (2014). Regulation of photosynthesis and stomatal and mesophyll conductance under water stress and recovery in olive trees: correlation with gene expression of carbonic anhydrase and aquaporins. *J. Exp. Bot.* 65, 3143–3156. doi: 10.1093/jxb/eru160

Pimentel, C., Laffray, D., and Louguet, P. (1999). Intrinsic water use efficiency at the pollination stage as a parameter for drought tolerance selection in *Phaseolus vulgaris*. *Physiol. Plant.* 106, 184–189. doi: 10.1034/j.1399-3054.1999.106206.x

Porra, R. J., Thompson, W. A., and Kriedemann, P. E. (1989). Determination of accurate extinction coefficients and simultaneous equations for assaying chlorophylls a and b extracted with four different solvents: verification of the concentration of chlorophyll standards by atomic absorption spectroscopy. *Biochim. Biophys. Acta* 975, 384–394. doi: 10.1016/S0005-2728(89)80347-0

Richter, A. S., and Grimm, B. (2013). Thiol-based redox control of enzymes involved in the tetrapyrrole biosynthesis pathway in plants. *Front. Plant Sci.* 4:371. doi: 10.3389/fpls.2013.00371

Sade, N., Gebretsadik, M., Seligmann, R., Schwartz, A., Wallach, R., and Moshelion, M. (2010). The role of tobacco aquaporin1 in improving water use efficiency, hydraulic conductivity, and yield production under salt stress. *Plant Physiol.* 152, 245–254. doi: 10.1104/pp.109.145854

Sakuraba, Y., Yokono, M., Akimoto, S., Tanaka, R., and Tanaka, A. (2010). Deregulated chlorophyll b synthesis reduces the energy transfer rate between photosynthetic pigments and induces photodamage in *Arabidopsis thaliana*. *Plant Cell Physiol.* 51, 1055–1065. doi: 10.1093/pcp/pcq050

Santos, M. G., Ribeiro, R. V., Machado, E. C., and Pimentel, C. (2009). Photosynthetic parameters and leaf water potential of five common bean genotypes under mild water deficit. *Biol. Plant.* 53, 229–236. doi: 10.1007/s10535-009-0044-9

Sun, Y. P., Liu, J., Cao, R. X., Huang, Y. J., Hall, A. M., Guo, C. B., et al. (2017). Effects of 5-aminolevulinic acid treatment on photosynthesis of strawberry. *Photosynthetica* 55, 276–284. doi: 10.1007/s11099-016-0667-y

Talebnejad, R., and Sepaskhah, A. R. (2016). Physiological characteristics, gas exchange, and plant ion relations of quinoa to different saline groundwater depths and water salinity. *Arch. Agron. Soil Sci.* 62, 1347–1367. doi: 10.1080/03650340.2016.1144925

Tian, T., Ali, B., Qin, Y., Malik, Z., Gill, R. A., Ali, S., et al. (2014). Alleviation of lead toxicity by 5-aminolevulinic acid is related to elevated growth, photosynthesis, and suppressed ultrastructural damages in oilseed rape. *BioMed Res. Int.* 2014:11. doi: 10.1155/2014/530642

Tsuchiya, T., Akimoto, S., Mizoguchi, T., Watabe, K., Kindo, H., Tomo, T., et al. (2012). Artificially produced [7-formyl]-chlorophyll d functions as an antenna pigment in the photosystem II isolated from the chlorophyllide a oxygenase-expressing *Acaryochloris marina* bioenergetics. *Biochim. Biophys. Acta* 1817, 1285–1291. doi: 10.1016/j.bbabio.2012.02.021

van der Sleen, P., Groenendijk, P., Vlam, M., Anten, N. P. R., Boom, A., Bongers, F., et al. (2014). No growth stimulation of tropical trees by 150 years of CO2 fertilization but water-use efficiency increased. *Nat. Geosci.* 8, 24–28. doi: 10.1038/ngeo2313

Wang, M., and Liao, W. (2016). Carbon monoxide as a signaling molecule in plants. *Front. Plant Sci.* 7:572. doi: 10.3389/fpls.2016.00572

Wang, X. Y., Xu, X. M., and Cui, J. (2015). The importance of blue light for leaf area expansion, development of photosynthetic apparatus, and chloroplast ultrastructure of *Cucumis sativus* grown under weak light. *Photosynthetica* 53, 213–222. doi: 10.1007/s11099-015-0083-8

Wegiel, B., Nemeth, Z., Correa-Costa, M., Bulmer, A. C., and Otterbein, L. E. (2014). Heme oxygenase-1: a metabolic nike. *Antioxid. Redox Signal.* 20, 1709–1722. doi: 10.1089/ars.2013.5667

Yan, F., Qu, D., Zhao, Y., Hu, X., Zhao, Z., Zhang, Y., et al. (2014). Effects of exogenous 5-aminolevulinic acid on *PIP1* and *NIP* aquaporin gene expression in seedlings of cucumber cultivars subjected to salinity stress. *Genet. Mol. Res.* 13, 2563–2573. doi: 10.4238/2014.January.22.12

Yang, Z., Chang, Z., Sun, L., Yu, J., and Huang, B. (2014). Physiological and metabolic effects of 5-aminolevulinic acid for mitigating salinity stress in creeping bentgrass. *PLoS One* 9:e116283. doi: 10.1371/journal.pone.0116283

Ye, J., Yang, X., Chen, Q., Xu, F., and Wang, G. (2017). Promotive effects of 5-aminolevulinic acid on fruit quality and coloration of *Prunus persica* (L.) Batsch. *Sci. Hortic.* 217, 266–275. doi: 10.1016/j.scienta.2017.02.009

Ye, J. B., Chen, Q. W., Tao, T. T., Wang, G., and Xu, F. (2016). Promotive effects of 5-aminolevulinic acid on growth, photosynthetic gas exchange, chlorophyll, and antioxidative enzymes under salinity stress in *Prunus persica* (L.) Batseh seedling. *Emirates J. Food Agric.* 28:786. doi: 10.9755/ejfa.2016-06-647

Yonezawa, T., Sunohara, Y., and Matsumoto, H. (2015). Involvement of heme synthesis in the growth stimulation of maize seedlings by 5-aminolevulinic acid. *Weed Biol. Manag.* 15, 53–60. doi: 10.1111/wbm.12064

Zhang, C.-P., Li, Y.-C., Yuan, F.-G., Hu, S.-J., Liu, H.-Y., and He, P. (2013). Role of 5-aminolevulinic acid in the salinity stress response of the seeds and seedlings of the medicinal plant *Cassia obtusifolia* L. *Bot. Stud.* 54, 18. doi: 10.1186/1999-3110-54-18

Zhang, M., Zhang, F., Fang, Y., Chen, X., Chen, Y., Zhang, W., et al. (2015). The Non-canonical Tetratricopeptide-repeat (TPR) Domain of FLUORESCENT (FLU) mediates complex formation with Glutamyl-tRNA reductase. *J. Biol. Chem.* 290, 17559–17565. doi: 10.1074/jbc.M115.662981

Zhang, Z.-P., Miao, M.-M., and Wang, C.-L. (2015). Effects of ALA on photosynthesis, antioxidant enzyme activity, and gene expression, and regulation of proline accumulation in tomato seedlings under NaCl stress. *J. Plant Growth Regul.* 34, 637–650. doi: 10.1007/s00344-015-9499-4

Zhen, A., Bie, Z., Huang, Y., Liu, Z., and Fan, M. (2012). Effects of 5-aminolevulinic acid on the H2O2-content and antioxidative enzyme gene expression in NaCl-treated cucumber seedlings. *Biol. Plant.* 56, 566–570. doi: 10.1007/s10535-012-0118-y

Conflict of Interest Statement: The authors declare that the research was conducted in the absence of any commercial or financial relationships that could be construed as a potential conflict of interest.

frontiers in Microbiology

ORIGINAL RESEARCH
published: 15 May 2018
doi: 10.3389/fmicb.2018.00968

Genetic Characterization and Diversity of *Rhizobium* Isolated From Root Nodules of Mid-Altitude Climbing Bean (*Phaseolus vulgaris* L.) Varieties

Gilbert Koskey[1], Simon W. Mburu[1], Jacinta M. Kimiti[2], Omwoyo Ombori[3], John M. Maingi[1] and Ezekiel M. Njeru[1]*

[1] Department of Microbiology, Kenyatta University, Nairobi, Kenya, [2] Department of Forestry and Land Resources Management, South Eastern Kenya University, Kitui, Kenya, [3] Department of Plant Sciences, Kenyatta University, Nairobi, Kenya

The increasing interest in the use of rhizobia as biofertilizers in smallholder agricultural farming systems of the Sub-Saharan Africa has prompted the identification of a large number of tropical rhizobia strains and led to studies on their diversity. Inoculants containing diverse strains of rhizobia have been developed for use as biofertilizers to promote soil fertility and symbiotic nitrogen fixation in legumes. In spite of this success, there is paucity of data on rhizobia diversity and genetic variation associated with the newly released and improved mid-altitude climbing (MAC) bean lines (*Phaseolus vulgaris* L.). In this study, 41 rhizobia isolates were obtained from the root nodules of MAC 13 and MAC 64 climbing beans grown in upper and lower midland agro-ecological zones of Eastern Kenya. Eastern Kenya was chosen because of its high production potential of diverse common bean cultivars. The rhizobia isolates were characterized phenotypically on the basis of colony morphology, growth and biochemical features. Rhizobia diversity from the different regions of Eastern Kenya was determined based on the amplified ribosomal DNA restriction analysis (ARDRA) of PCR amplified 16S rRNA genes using *Msp* I, *EcoR* I, and *Hae* III restriction enzymes. Notably, native rhizobia isolates were morphologically diverse and grouped into nine different morphotypes. Correspondingly, the analysis of molecular variance based on restriction digestion of 16S rRNA genes showed that the largest proportion of significant ($p < 0.05$) genetic variation was distributed within the rhizobia population (97.5%) than among rhizobia populations (1.5%) in the four agro-ecological zones. The high degree of morphological and genotypic diversity of rhizobia within Eastern Kenya shows that the region harbors novel rhizobia strains worth exploiting to obtain strains efficient in biological nitrogen fixation with *P. vulgaris* L. Genetic sequence analysis of the isolates and testing for their symbiotic properties should be carried out to ascertain their identity and functionality in diverse environments.

Keywords: *Rhizobium*, climbing beans, ARDRA, genetic diversity, 16S rRNA genes

Abbreviations: AEZ, agroecological zones; ELM, Embu Lower Midland; EUM, Embu Upper Midland; MAC, Mid-altitude climbers; TLM, Tharaka Nithi Lower Midland; TUM, Tharaka Nithi Upper Midland.

OPEN ACCESS

Edited by:
Giuseppe Colla, Università degli Studi della Tuscia, Italy

Reviewed by:
Bhim Pratap Singh, Mizoram University, India
Mohammad Oves, King Abdulaziz University, Saudi Arabia

****Correspondence:***
John M. Maingi
maingijohn@gmail.com

Specialty section:
This article was submitted to Plant Microbe Interactions, a section of the journal Frontiers in Microbiology

Received: *17 January 2018*
Accepted: *25 April 2018*
Published: *15 May 2018*

Citation:
Koskey G, Mburu SW, Kimiti JM, Ombori O, Maingi JM and Njeru EM (2018) Genetic Characterization and Diversity of Rhizobium Isolated From Root Nodules of Mid-Altitude Climbing Bean (Phaseolus vulgaris L.) Varieties. Front. Microbiol. 9:968. doi: 10.3389/fmicb.2018.00968

INTRODUCTION

Rhizobia bacteria play a significant role in provision of agricultural ecosystem services due to their ability to form symbiotic association with a wide range of leguminous plants that results in biological nitrogen fixation (Orrell and Bennett, 2013). Some of the rhizobia strains are reported to enhance the production of phytohormones, mineral uptake and reduce toxic effects of metals, thereby, indirectly promoting plant growth and development (Karthik et al., 2017) in polluted agricultural soils. Modern agriculture has shifted to the use of sustainable farming practices that are eco-friendly, efficient, and affordable to the resource-limited smallholder farmers. For instance, the use of rhizobia biofertilizers in tropical areas of the Sub-Saharan Africa (SSA) has relatively increased compared to the previous decades due to the agronomic benefits associated with biofertilizers such as yield increase, cost saving, and improved soil health (Meng et al., 2015). However, lack of awareness, absence of supportive infrastructure and limited research targeting the diverse and elite rhizobia strains associated with the newly improved bean lines such as MAC constraints the utilization of biofertilizers in bean production (Ramaekers et al., 2013). In Kenya, limited information on the diversity of rhizobia species that nodulate with the newly released lines of MAC bean varieties and their genetic variation in different AEZ with contrasting environmental conditions is available, hence the need to carry out this study. The data on distribution and genetic variation among the native rhizobia isolates would aid in selecting novel rhizobia strains that could be developed and used as biofertilizers in bean production.

Rhizobia-legume symbiosis is a host-specific association and hence the need to determine the strains and the diversity of rhizobia associated with specific type of legume for better exploitation of the benefits associated with the rhizobia biofertilizers (Batista et al., 2015). Rhizobia distribution and diversity is also affected greatly by the geographical locality and determining their phylogeny could highlight their evolutionary origin. Taxonomically, the diverse heterogenous groups of rhizobia comprise of the alpha group which forms the majority of the rhizobia species and the beta group, which interacts with *Mimosa* genus (Saikia and Jain, 2007). Until recently, about 40 rhizobia species belonging to the seven genera of Alpha-proteobacteria have been identified and includes: *Rhizobium*, *Sinorhizobium*, *Azorhizobium*, *Bradyrhizobium*, *Allorhizobium*, *Mesorhizobium*, *Bradyrhizobium,* and *Methylobacterium* (Lemaire et al., 2015). However, there are other nitrogen fixing bacteria, which have been recently identified from beta and gamma Proteobacteria, that form symbiotic relationship with legumes. Rhizobia from each genera are known to nodulate and fix nitrogen with specific legumes. For instance, *Rhizobium* strains are largely associated with common beans (*Phaseolus vulgaris* L.) and chickpeas (*Cicer arietinum* L.), while *Bradyrhizobium* strains are often found to nodulate soybeans (*Glycine max* L. Merrill), cowpeas (*Vigna unguiculata* L.) and green grams (*Vigna radiata* L.). However, in some cases, cross inoculation by both genera has been documented in some legumes such as mungbeans (*V. radiata* L.) and this is considered advantageous and economical to farmers growing different types of legumes (Hameed et al., 2004).

Like other common beans (*P. vulgaris* L.), the MAC beans establish a mutual and a beneficial symbiotic partnership with *Rhizobium* leading to the formation of root nodules that catalyze nitrogen fixation from the atmospheric air (Baginsky et al., 2015). The species are commonly the fast growing *Rhizobium* and includes; *Rhizobium tropici*, *Rhizobium etli*, *Rhizobium phaseoli*, *Rhizobium leguminosarum*, *Rhizobium gallicum* and *Sinorhizobium meliloti* (Adhikari et al., 2013). The MAC beans were produced through vigorous breeding and were designed to have superior symbiotic nitrogen fixation potential and grain yield output compared to the commonly grown bush beans (Ramaekers et al., 2013). In the recent past, studies investigating the distribution of rhizobia nodulating different legumes in Kenya and other tropical areas of the SSA have revealed a significant diversity in terms of both phenotypic and genotypic traits (Zahran, 2001). This has led to the identification of several distinct groups and the description of novel strains (Berrada et al., 2012; Boakye et al., 2016; Onyango et al., 2015). From these previous researches, there exists a large heterogeneity among the rhizobia strains within the Sub-Saharan region and thus more studies should be channeled on rhizobia distribution, diversity determination and their nitrogen fixation performance.

The tropical soil of the SSA has a great diversity of rhizobia in spite of the pressure on the agricultural resources and harsh climatic conditions which adversely affect the soil ecosystem and its biodiversity (Grönemeyer et al., 2014). In this case, efficient methods of rhizobia classification should be used to fully characterize different rhizobia genotypes and exploit the diverse taxonomic groups that exist in the African tropical areas. Molecular techniques that are more accurate, faster and efficient have been developed to aid the traditional phenotypic and morpho-cultural techniques in distinguishing the different microbial genera, species and strains (Ismail et al., 2013). The polymerase chain reaction (PCR), electrophoresis and gene sequencing tools have revolutionized the characterization of microorganisms by distinguishing even close members of a species up to the strain level based on distinct genetic trait patterns (Baginsky et al., 2015). Moreover, PCR, metagenomics and third generation gene sequencing methods have higher reliability, faster and more sensitive as compared to other conventional methods (Baratto et al., 2012). In other studies, housekeeping genes such as *rec*A and *atp*D, nodulation and symbiotic genes such as *nod*A and *nif* H have been recently used to assess rhizobia diversity and phylogeny in different geographic locations due to their high resolution ability (Ampomah and Huss-Danell, 2016; Wang et al., 2016). This study utilizes PCR and amplified ribosomal DNA restriction analysis (ARDRA) tools to determine the genetic diversity of native rhizobia found in different AEZ of Eastern Kenya. ARDRA involves amplification and digestion of 16S rDNA region using specific restriction enzymes. This method has a higher taxonomic resolution, less expensive and does not

require additional equipment used by other fingerprinting techniques such as 16S rRNA gene sequencing (Park et al., 2014).

Apart from ARDRA tools, other PCR-based techniques such as random amplified polymorphic DNA (RAPD), restriction fragment length polymorphism (RFLP) and amplified fragment length polymorphism (AFLP) have been used to evaluate the diversity of rhizobia (Silva et al., 2012; Onyango et al., 2015; Boakye et al., 2016). In addition, the 16S rRNA and 16S-23S rRNA genes, which are highly conserved, have been sequenced to determine the taxonomic position of different rhizobia strains (Rahmani et al., 2011; Adhikari et al., 2013; Karthik et al., 2017). Several rhizobia specific and universal primers have been used in amplification and sequencing and have produced distinguishable rhizobia phylogenies. In recent times, 16S-23S rRNA ITS region is getting consideration among researchers as phylogenetic marker for species and sub-species delineation among rhizobia (Grönemeyer et al., 2014). The high sequence variation of the ITS region allows discrimination between closely related species. According to the PCR-RFLP analysis of 16S-23S ITS region of common bean nodulating rhizobia isolated by Rahmani et al. (2011) in Iran, it was reported that the rhizobia isolated in the study exhibited high genetic diversity and contained 43 ITS genotypes that were clustered into 10 groups at the similarity of 64%.

In this study, we hypothesized that there exists a large heterogeneity among the rhizobia isolates nodulating MAC beans grown in different AEZ with contrasting environmental conditions in Eastern Kenya. The aim of this study was to isolate, characterize morphologically and genetically and determine the diversity of the rhizobia populations associated with MAC 13 and MAC 64 climbing beans grown in different AEZ of Eastern Kenya.

MATERIALS AND METHODS

Field Trapping of Rhizobia

Field trapping of rhizobia was carried out during the first rainy season between March and August 2015 in four different sites of Eastern Kenya with contrasting agro-climatic conditions using MAC 13 and MAC 64 climbing beans as the host plants. Eastern Kenya was chosen because of its high production potential of diverse common bean cultivars (Mburu et al., 2016). The four farmers' fields used in rhizobia trapping were located in upper and lower midland AEZ of Embu (0.53°S, 37.45°E) and Tharaka Nithi (0.30°S, 38.06°E) Counties of Eastern Kenya. Three plots in each farm measuring 3 m × 3 m were prepared and climbing bean seeds were sown using the recommended spacing of 75 cm × 30 cm. Destructive sampling was done after 45 days since planting by randomly but carefully uprooting five plants within the mid-rows of each plot. A sterile clean spade was used to dig for approximately 15 cm sideways and up to a depth of about 20 cm. The clump of soil and the roots were uplifted carefully, placed in sterile aluminum foil where the nodules were detached from the roots and kept in screw-capped vials containing silica gel to prevent from desiccation. Extraction was done a day after nodules were harvested.

Isolation of Rhizobia From the Root Nodules

Ten healthy and undamaged root nodules of MAC 13 and MAC 64 climbing beans from each site were selected and used in the isolation of rhizobia following the standard protocols described by Somasegaran and Hoben (1994). The nodules were washed, surface sterilized in 1% sodium hypochlorite solution for 3 min, rinsed in seven changes of sterile distilled water and then crushed with a sterilized glass rod (Muthini et al., 2014). A loop-full of the resulting suspension was streaked on Yeast extract mannitol agar (YEMA) supplemented with Congo red (0.00125 mg/kg) and incubated in the dark at 28°C for 3–5 days (Vincent, 1970). Emerging single colonies, which were typical of rhizobia species were sub-cultured by repeated streaking on YEMA, and YEMA containing bromothymol blue (BTB) plates (0.00125 mg/kg). A total of 41 rhizobia isolates were obtained. All isolates were maintained on screw-capped McCartney YEMA slant bottles and preserved at 4°C and on vials containing 25% glycerol-Yeast extract mannitol broth (YEMB) where they were preserved at −20°C.

Morpho-Cultural and Biochemical Characterization of the Isolates

Using the standard microbiological techniques described by Somasegaran and Hoben (1994), all the isolates were characterized for selected morphological parameters such as colony size, shape, border, elevation, color, mucosity, transparency, and capacity to produce the exopolysaccharide gum (Liu, 2014; Muthini et al., 2014). Other tests that were carried out included Gram staining where young pure isolates (3–4 days old) cultured on YEMA were smeared on clean microscope slides. The wet smears were air-dried, heat fixed and then Gram stained as described by Beck et al. (1993). The prepared slides were observed under oil immersion on a compound light microscope. The production of acid or alkali was determined in YEMA medium containing bromothymol blue (YEMA-BTB). The plates were incubated at 28°C for 7 days in the dark. The isolates that changed the green color of YEMA-BTB to yellow were identified as acid producers and fast growers. Isolates that changed the YEMA-BTB medium to blue were considered as alkaline producers and slow growers. Lastly, rhizobia isolates were cultured in peptone glucose agar plates, incubated at 28°C for 4 days in the dark. The absence of bacteria growth was a clear indication of the presence of *Rhizobium*. Based on the differences in the observed morpho-cultural, Gram stain and growth features, the isolates were placed into different morphotype groups.

Genotypic Characterization

The molecular study involved 41 rhizobia isolates and was carried out at the Department of Microbiology, Kenyatta University. The analysis of genetic relatedness of the native isolates from

Eastern Kenya was carried out using amplified rDNA restriction analysis (ARDRA) of the 16s rRNA genes using three restriction enzymes *Hae* III, *EcoR* I and *Msp* I (Biolabs, England). Three reference strains; *Rhizobium tropici* CIAT 899, *Rhizobium etli* USDA 2667 strain and *Rhizobium leguminosarum* strain 446 were used. The three reference *Rhizobium* strains were obtained from the Microbiological Resource Centre (MIRCEN), University of Nairobi, Kenya.

Genomic DNA Extraction of Rhizobia

The extraction of total genomic DNA of rhizobia isolates was carried out following modification of a method outlined by Young et al. (1991). The young rhizobia cultures grown in YEMA plates for 3 days in the dark at 28°C were re-suspended in sterile eppendorf tubes containing 400 µl of normal saline, vortexed for 20 s and centrifuged at 13,000 rpm for 10 min. The supernatant was poured out carefully leaving the cell pellets. The cell pellets were washed five times with normal saline to remove the slimy exopolysaccharide (EPS). The cell pellets were then harvested and re-suspended in 400 µl of cetyltrimethylammonium bromide (CTAB) lysis buffer, vortexed and incubated in a water bath at 65°C for 2 h (Silva et al., 2012). The tubes were intermittently inverted every 20 min during the incubation period. The DNA was extracted by adding equal volumes of phenol: chloroform: isoamyl alcohol solution (25:24:1), vortexed and centrifuged at 13,000 rpm for 10 min at 4°C (Berrada et al., 2012). The supernatant was transferred carefully to a new sterile 1.5 ml eppendorf tube where an equal volume (400 µl) of absolute ethanol was added and incubated at 4°C for 10 min to allow precipitation. The precipitated DNA was centrifuged at 13,000 rpm for 8 min and the supernatant was discarded. The DNA pellet was air-dried for 40 min and dissolved in 40 µl of DNase free water. It was then stored at −20°C.

PCR Amplification of the 16S rRNA Genes

The PCR amplification was carried out in 30 µl reaction with 23.55 µl sterile PCR water, 3.0 µl buffer (Biolabs), 0.6 µl dNTP (10 mM), 0.3 µl of each Y1 and Y3 primers (10 µM), 0.6 µl 5% Tween 20, 0.15 µl Taq DNA polymerase (Biolabs) and 1.5 µl of DNA template. The sequences of the primers used for PCR amplification of the 16S rRNA gene were: Y1 forward primer (5′-TGGCTCAGAACGAACGCTGGCGGC-3′) that corresponds to positions 20–43 and Y3 reverse primer (5′-TA CCTTGTTACGACTTCACCCCAGTC-3′) corresponding to positions 1482–1507 for 16S rDNA sequence of *Escherichia coli* (Young et al., 1991). A negative control without DNA was used. The DNA was amplified in a Techgene Thermal Cycler (Techne) programmed to run as follows; initial DNA denaturation at 94°C for 2 min; 35 cycles (denaturation at 94°C for 45 s, annealing at 62°C for 45 s and extension at 72°C for 2 min). Final extension was carried out at 72°C for 5 min. Amplified PCR products were visualized by gel electrophoresis on a 1% agarose gel. The stain used was SYBR-Green (Biolabs). DNA ladder of 1 Kb (Biolabs) was used to estimate the band sizes. The gel was run in Tris-Borate Ethylenediaminetetraacetic acid (0.5X TBE) buffer at 80 V for 50 min and was visualized using a UV trans-illuminator and photographed using a digital camera (Nikon).

Amplified Ribosomal DNA Restriction Analysis of 16S rRNA Genes

Polymerase chain reaction products of the isolates and reference strains were digested using *Hae* III, *Eco*R I, and *Msp* I restriction enzymes (Biolabs) (Laguerre et al., 1996). A master-mix (10.0 µl) containing 3.8 µl PCR water, 1.0 µl reaction buffer, 0.2 µl restriction enzyme and 5.0 µl PCR amplicons were digested for 1 h at 37°C. The restriction digests (fragments) were then stained using SYBR-Green and separated on a 2% agarose gel. A 100 bp DNA ladder (Biolabs) was used to estimate the fragment sizes. The gel was run at 80 V for 50 min and was visualized using a UV trans-illuminator and photographed using a digital camera. The different band patterns were noted and the frequency of similar patterns was scored.

Data Analysis

The data on rhizobia genetic diversity based on the different band patterns formed after PCR restriction were coded in binary form and analyzed as described by Silva et al. (2012) using Gene Alex Software version 6.5. Euclidean distance similarity, Nei's unbiased genetic distance (Nei and Li, 1979), and single linkage (nearest neighbor) methods available in the PAST program (version 1.92) and Darwin software (version 6) were used to construct dendrograms for clustering the rhizobia isolates and to assess their diversity and similarity across the four AEZ of Eastern Kenya (Hammer et al., 2001). Shannon–Wiener diversity index (*H*) was also used to determine the rhizobia diversity in different AEZ of Eastern Kenya.

RESULTS

Morpho-Cultural Characteristics of Isolates

From the field trapping experiment using MAC 13 and MAC 64 climbing beans, 41 pure rhizobia isolates were obtained, which were placed in to nine distinct morphotype groups based on their morpho-cultural and biochemical features (**Table 1**). Morphotype III was the most abundant (29%) and morphotype II accounted for a partly 2% of the total number of isolates. All isolates were Gram negative rods and did not absorb Congo red dye when incubated in the dark on YEMA-CR medium (**Figure 1A**). Notably, all isolates turned BTB indicator from deep green to yellow when grown on YEMA-BTB indicating that the isolates were acid producers and fast growers (**Figure 1B**). The colony color varied with milky white, cream white, cream yellow and watery colonies being observed which were either opaque or translucent with either firm gummy or smooth mucoid texture (**Figure 1C**). Most of the isolates had a mucoid texture due to the production of

TABLE 1 | Morpho-cultural and biochemical characteristics of the rhizobia isolates trapped from the study farms in Eastern Kenya.

Isolate characteristic	Morphotypes								
	I	II	III	IV	V	VI	VII	VIII	IX
CR absorption	Na	Na	Na	Na	Na	Na	Na	Na	Na
Peptone G growth	No	No	No	No	No	No	No	No	No
BTB reaction	Y	Y	Y	Y	Y	Y	Y	Y	Y
Gram reaction	−ve	−ve	−ve	−ve	−ve	−ve	−ve	−ve	−ve
Cell shape	Rod	Rod	Rod	Rod	Rod	Rod	Rod	Rod	Rod
Elevation	Cvx	Cvx	Cvx	Cvx	Cvx	Cvx	Cvx	Raised	Cvx
Margin	Entire	Entire	Entire	Entire	Entire	Entire	Entire	Entire	Entire
Colony color	Cy	Cw	Mw	Mw	Mw	Mw	W	Mw	Mw
Transparency	Op	Trl	Trl	Op	Op	Trl	Trl	Trl	Op
Colony texture	Fg	Fg	Sm	Sm	Fg	Sm	Sm	Fg	Sm
Percentage%	4	2	29	7	9	20	18	4	7

Na, non-absorbing; Peptone G, Peptone Glucose growth; No, no growth; Y, yellow on BTB; −ve, negative; Cvx, convex elevation; Cy, Cream yellow; Cw, cream white; Mw, milky white; W, watery; Op, opaque; Trl, translucent; Fg, firm gummy; Sm, soft mucoid.

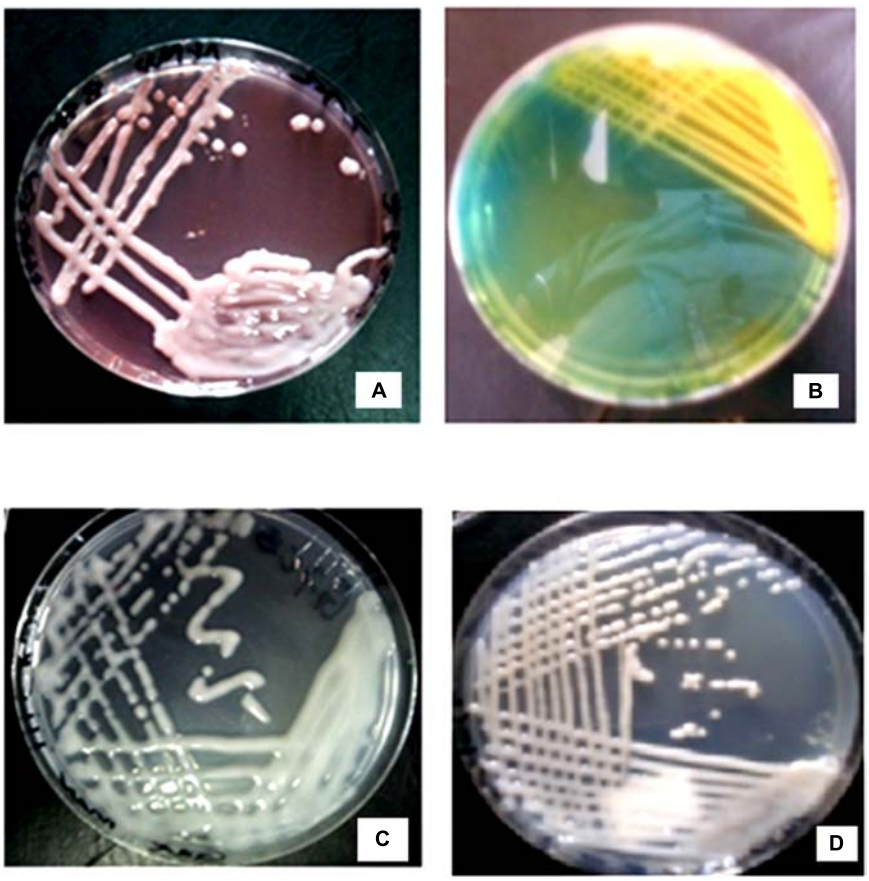


FIGURE 1 | Morphological characteristics of rhizobia isolates obtained from field trapping. **(A)** Native rhizobia isolate EUM7 on YEMA with Congo red dye. **(B)** Acidic reaction of native rhizobia isolate ELM3 on YEMA with BTB. **(C)** Growth of native rhizobia isolate ELM5 on YEMA media. **(D)** Native rhizobia isolate TUM2 on YEMA media.

excess EPSs. As expected, all isolates had an entire colony margin but the colony elevation varied consistently with convex and raised colonies being observed on YEMA media (**Figure 1D**). The morphological characteristics of the reference strains *Rhizobium tropici* CIAT 899 and *Rhizobium etli* USDA 2667 were closely similar to those of morphotype III while *Rhizobium leguminosarum* strain 446 closely resembled morphotype V (**Table 1**).

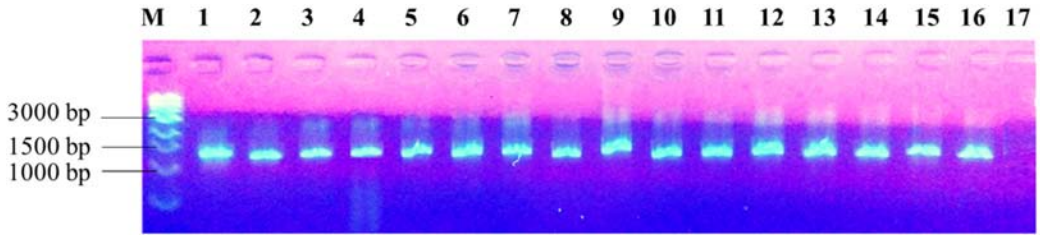


FIGURE 2 | PCR amplified 16S rDNA of the isolates in 1% agarose gel. Lane M, 1 kb DNA ladder (Biolabs); Lanes 1–13, genomic DNA of selected native rhizobia isolates (ELM1, TUM2, ELM3, ELM4, ELM5, EUM6, EUM7, ELM8, ELM9, TLM10, TUM11, TLM12, and EUM13); Lane 14, CIAT 899; Lane 15, USDA 2667; Lane 16, strain 446; Lane 17, Negative control.

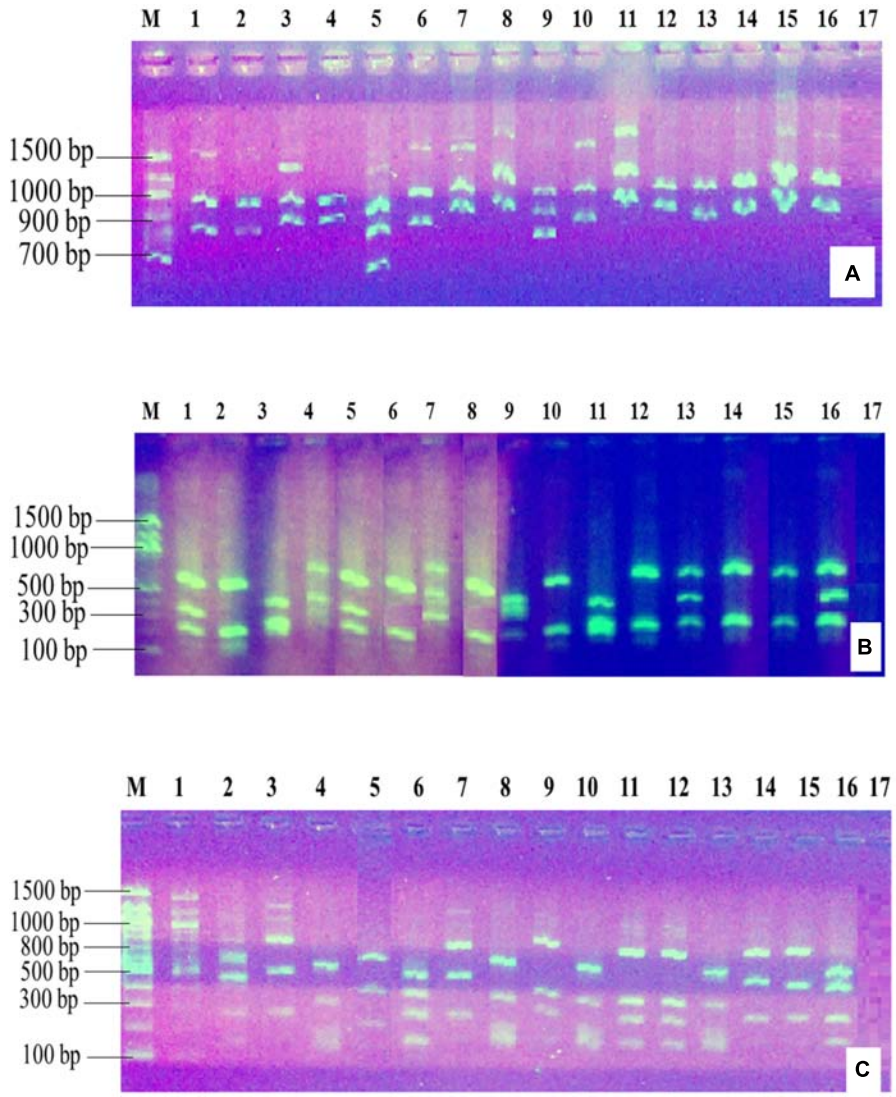


FIGURE 3 | Gel electrophoresis of the restriction digestion products of 16S rDNA of selected native rhizobia isolates in 2% agarose gel. Lane M, 100 bp DNA ladder (Biolabs); Lanes 1–13, genomic DNA of native rhizobia isolates (ELM1, TUM2, ELM3, ELM4, ELM5, EUM6, EUM7, ELM8, ELM9, TLM10, TUM11, TLM12, and EUM13); Lane 14, CIAT 899; Lane 15, USDA 2667; Lane 16, strain 446; Lane 17, Negative control. **(A)** Restriction digestion with *EcoR* I; **(B)** restriction digestion with *Hae* III; **(C)** restriction digestion with *Msp* I.

TABLE 2 | Mean number of different alleles (Na), number of effective alleles (Ne), Shannon's Information Index I (H), expected Heterozygosity (He) and percentage of Polymorphic Loci (% *P*) of native rhizobia populations from Eastern Kenya based on ARDRA analyses.

Population	Na	Ne	I(*H*)	He	% *P*
ELM	1.92 ± 0.08	1.55 ± 0.10	0.47 ± 0.07	0.32 ± 0.05	92.31
EUM	1.54 ± 0.18	1.39 ± 0.11	0.33 ± 0.08	0.22 ± 0.06	61.54
TLM	1.54 ± 0.18	1.37 ± 0.12	0.31 ± 0.08	0.21 ± 0.06	61.54
TUM	1.62 ± 0.18	1.41 ± 0.10	0.37 ± 0.08	0.25 ± 0.05	69.23

ELM, Embu Lower Midland; EUM, Embu Upper Midland; TLM, Tharaka Nithi Lower Midland; TUM, Tharaka Nithi Upper Midland.

Genetic Diversity of Native Rhizobia Isolates From Eastern Kenya

DNA Extraction, PCR Amplification and ARDRA Analysis of 16S rRNA Genes

The PCR amplification of the 16S rDNA region produced a single band of approximately 1500 bp (**Figure 2**). Restriction of the 16S rRNA amplicons of the climbing bean rhizobia isolates using enzymes *EcoR* I (**Figure 3A**), *Hae* III (**Figure 3B**) and *Msp* I (**Figure 3C**) produced multiple band patterns. Restriction enzyme *Msp* I produced the most diverse polymorphic patterns.

Based on ARDRA analysis of 16S rRNA amplicons, rhizobia population from ELM had the highest average number of different alleles (*Na* = 1.92 ± 0.08) and effective alleles (*Ne* = 1.55 ± 0.10) compared to other populations (**Table 2**). Rhizobia isolates from ELM had the highest percentage polymorphic loci (92.31% *P*) while isolates from EUM and TLM recorded the lowest at 61.54% *P*. The mean Shannon–Wiener diversity (*H*) estimate showed that the four rhizobia populations from Eastern Kenya were genetically diverse with rhizobia population from ELM having the highest genetic diversity estimate of *H* = 0.47 (**Table 2**). The rhizobia population from zone TLM recorded the lowest genetic diversity estimate of *H* = 0.31. The average expected heterozygosity (He) varied among the four populations and ranged from 0.21 for rhizobia population from TLM to 0.32 for ELM population (**Table 2**).

Analysis of molecular variance (AMOVA) for the four rhizobia populations in Eastern Kenya showed a significantly high genetic variation (*P* = 0.0406; 97%) within populations (within a test agroecological zone). The variation among the rhizobia populations from the four AEZ (2.5%) and two Counties (0.5%) were, however, not significant (**Table 3**).

The principal coordinate analysis (PCA) of 41 native isolates from the four zones of Eastern Kenya and three rhizobia reference strains showed considerable differentiation (**Figure 4**). Isolates from TUM zone were the most distributed and appeared in all the four quadrants, while isolates from ELM zone were the least distributed (**Figure 4**).

Based on the pairwise population matrix of Nei unbiased genetic distance and Euclidian similarity index, the neighbor joining dendrogram clustered rhizobia populations from Eastern Kenya into two main groups (**Figure 5**). Rhizobia populations from ELM and TUM clustered together with a bootstrap value of 85% while rhizobia populations from EUM and TLM clustered with a bootstrap value of 82% (**Figure 5**).

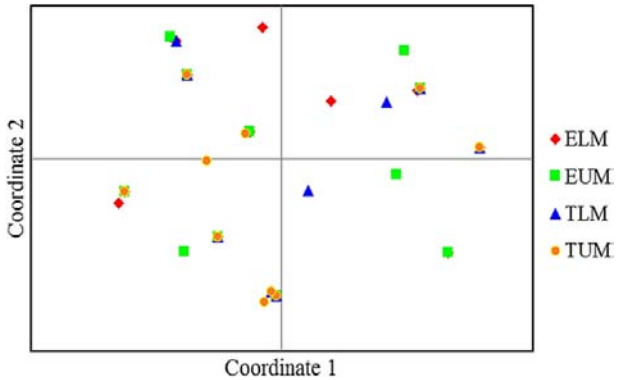


FIGURE 4 | Principle coordinate analyses (PCA) of 41 native rhizobia isolates from Eastern Kenya based on ARDRA restriction patterns. Percentage variation explained by the first 2 coordinates; 1, 33.82%; 2, 20.74%. ELM, Embu Lower Midland; EUM, Embu Upper Midland; TLM, Tharaka Nithi Lower Midland; TUM, Tharaka Nithi Upper Midland.

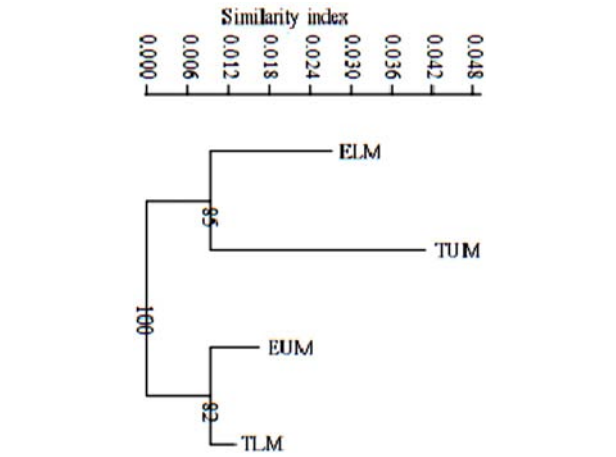


FIGURE 5 | A neighbor joining dendrogram based on Nei's unbiased genetic distance and Euclidian similarity index showing the genetic distance of four rhizobia populations from Eastern Kenya. Numbers shown at the nodes of the dendrogram indicate the percentage bootstrap support for 1000 iterations. ELM, Embu Lower Midland; EUM, Embu Upper Midland; TLM, Tharaka Nithi Lower Midland; TUM, Tharaka Nithi Upper Midland.

Based on the genetic distance after amplified rDNA restriction analyses and Euclidian similarity index, the phylogenetic tree clustered the native rhizobia isolates into three main clusters I, II, and III (**Figure 6**). Cluster I comprised of majority of the isolates while cluster III had only one isolate ELM5. The reference strain *Rhizobium tropici* CIAT 899 which was placed in cluster I, clustered together with native rhizobia isolates ELM33 and TLM32, indicating their close genetic relationship. Native isolates TUM26, TLM28, and EUM23 clustered together

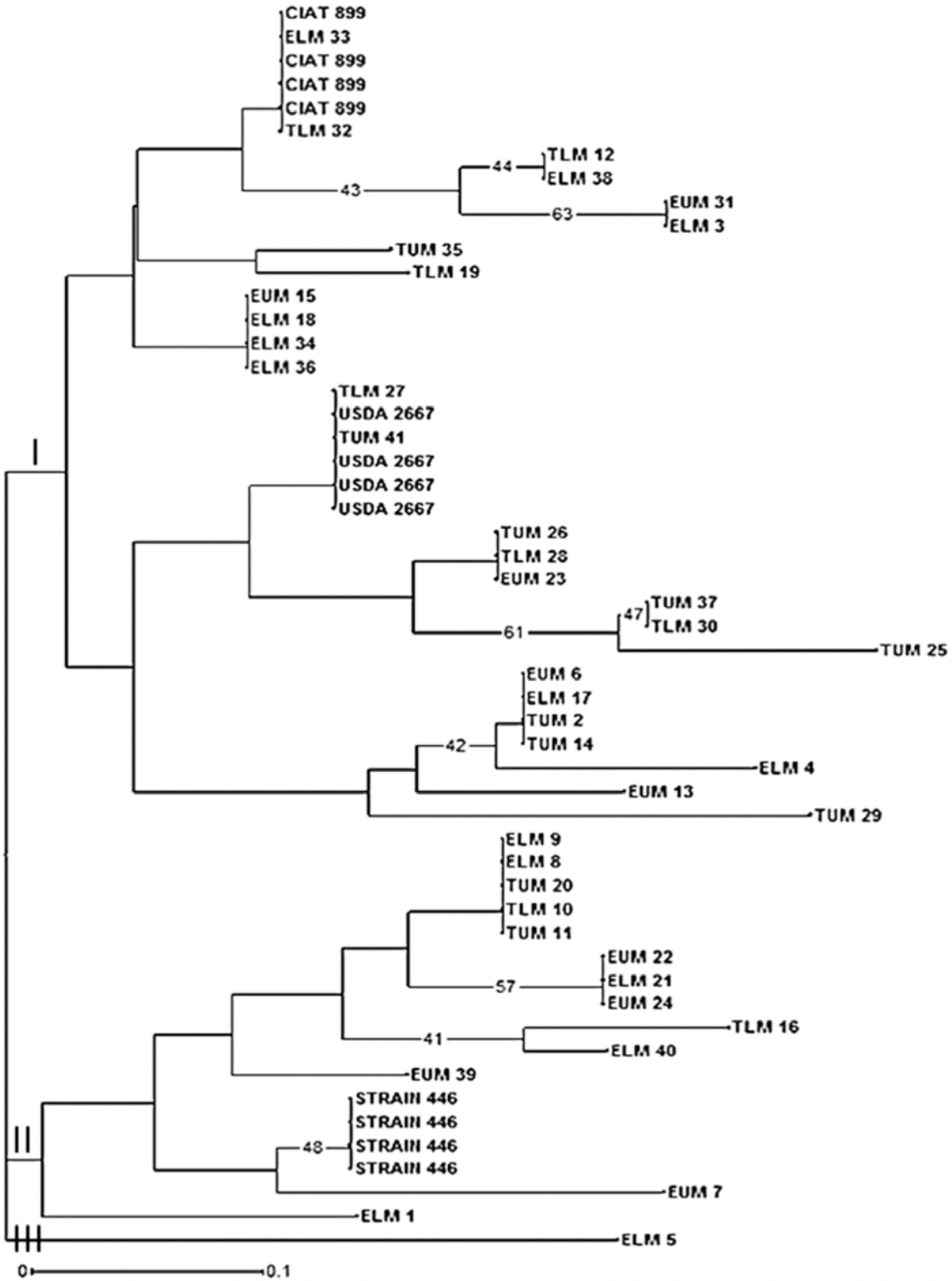


FIGURE 6 | Phylogenetic relationship of 41 native rhizobia isolates from Eastern Kenya and three reference rhizobia strains (CIAT 899, USDA 2667, and strain 446) inferred using the Neighbor-Joining method. Numbers shown at the nodes of the dendrogram indicate the percentage bootstrap support for 1000 iterations. Only bootstrap values $\geq$ 40% are shown. Scale bar indicates number of substitutions per site. ELM, Embu Lower Midland; EUM, Embu Upper Midland; TLM, Tharaka Nithi Lower Midland; TUM, Tharaka Nithi Upper Midland.

TABLE 3 | Analysis of molecular variance (AMOVA) for 41 rhizobia isolates for the four populations from Eastern Kenya based on restriction digestion of 16S rDNA.

Source	Df	SS	MS	Est. Var.	% Mol Var.	*P*-value
Among regions	1	1.010	1.010	0.005	0.5	0.412
Among pops	2	1.812	0.906	0.000	2.5	0.870
Within pops	49	81.442	1.662	1.662	97.0	0.0406
Total	52	84.264		1.667	100	

Df, degrees of freedom; SS, sum of squares; MS, mean square; Est. Var, estimated variance; % Mol. Var, percentage molecular variance; Pops, populations.

despite originating from different AEZ of Eastern Kenya. *Rhizobium etli* USDA 2667 strain was also placed in cluster I and was grouped together with TLM27 and TUM 41 isolates (**Figure 6**). The native isolate EUM7 clustered closely to *Rhizobium leguminosarum* strain 446 in cluster II but in a separate sub-branch.

DISCUSSION

Morphological Characteristics of Native Rhizobia Isolates

Gram staining results and growth on YEMA-CR and YEMA-BTB media, preliminary confirmed the standard morpho-cultural characteristics of *Rhizobium* species that nodulate with *P. vulgaris* L. as described by Vincent (1970) and Somasegaran and Hoben (1994). The Gram-negative rods observed and poor absorption of Congo red dye of the isolates on YEMA-CR further reinforced that the isolates were rhizobia (Beck et al., 1993). Their growth within 3–5 days and the color change on YEMA-BTB from deep green to yellow suggested that all isolates were fast growers and could probably fall under the genus *Rhizobium* (Al-mujahidy et al., 2013). The nine different morphotypes of rhizobia showed the diverse nature of the isolates colonizing nodules of MAC beans in Eastern Kenya. The results of this study on morphological and biochemical characterization of native rhizobia isolates nodulating common beans corresponded to the findings reported in other recent studies done in Kenya (Kawaka et al., 2014; Muthini et al., 2014) and in Ecuador (Torres-Gutiérrez et al., 2017).

Changes in temperatures, metal toxicity, pH and soil salinity are among the main factors restricting symbiotic nitrogen fixation in legume-rhizobia symbiosis and thus only those strains capable of tolerating the extreme conditions would survive (Berrada et al., 2012). The production of exopolysaccharides (EPS) by most of the isolates in this study could indicate their versatility to withstand physiological stress due to high temperatures, metal toxicity, low soil pH and salinity (Karthik et al., 2017). Soils in AEZ surrounding Mt. Kenya region are known to be slightly acidic due to excessive precipitation and soil erosion (Nyaga et al., 2014). Therefore, rhizobia strains native to Mt. Kenya and its environs are expected to have survival adaptations to counter the stressful soil and environmental conditions. Essentially, isolates exhibiting a wide adaptation to environmental stresses could be able to circumvent limiting factors and maintain a higher capacity for nitrogen fixation, thus, may be considered suitable candidates for rhizobia inoculum development (Kawaka et al., 2014; Torres-Gutiérrez et al., 2017).

Genetic Diversity of Native Rhizobia Isolates From Eastern Kenya

The restriction digest using restriction enzymes *EcoR* I, *Hae* III, and *Msp* I showed highly polymorphic and distinct DNA fragment patterns indicating the divergence of the native rhizobia isolates in Eastern Kenya. The mean Shannon–Wiener diversity (*H*) estimate of rhizobia populations from Eastern Kenya based on genetic distance of ARDRA pattern analysis showed that the isolates were genetically diverse with rhizobia population from ELM zone having the highest genetic diversity estimate compared to TLM zone, which recorded the lowest diversity. This variation in rhizobia diversity could be due to the differences in agroclimatic and soil conditions of the four sites studied in Eastern Kenya. These results reinforce the promiscuous nature of *P. vulgaris* L. to nodulate with diverse strains of *Rhizobium* (Pohajda et al., 2016). Similarly, Wasike et al. (2009) reported a relatively higher diversity of indigenous bradyrhizobia in Western Kenya compared to that of Eastern Kenya as a result of agroecological differences between the two sites. Other factors such as cropping history, land use and host genotype have been attributed to the variation of rhizobia diversity in different parts of Central highlands of Kenya (Mwenda et al., 2011), which may also be the reason of the variation in our study.

The Pairwise Population Matrix of Nei unbiased genetic distance of rhizobia populations in Eastern Kenya showed a narrow range. These findings imply that the rhizobia populations, despite originating from different AEZ of Eastern Kenya, are closely related and may have a recent common ancestral origin (Mwenda et al., 2011). The narrow genetic distance could also possibly be as a result of the conserved nature of the 16S rRNA gene, which could not discriminate between closely related rhizobia species (Berrada et al., 2012). In addition, only three restriction enzymes were used in this study and thus could not produce highly distinct and polymorphic profiles that would produce a better diversity screening potential of the ARDRA technique (Silva et al., 2012). Thus, other molecular finger printing tools that have a higher resolving power, such as third generation gene sequencing and phylogenetic analysis, should be adopted. Dai et al. (2012) noted that the phylogenetic analyses of bacteria using 16S rDNA alone may not clearly show a distinctive relationship within and among the bacteria populations involved. In addition, horizontal gene transfer and genetic recombination could have possibly contributed to the limited genetic variation of rhizobia in Eastern Kenya. Similar findings have been reported by Ismail et al. (2013) who worked on *P. vulgaris* rhizobia populations and found a narrow genetic distance among isolates obtained from different sites in Egypt.

The analyses of molecular variance (AMOVA) based on amplified 16S rDNA restriction profiles showed a highly

significant genetic variation of native rhizobia isolates within rhizobia populations and not among the four populations nor across the two regions (Counties) of Eastern Kenya. Based on ARDRA fingerprints, the low level of genetic differentiation of MAC bean rhizobia could suggest that the rhizobia population within the region is weakly structured. This could be due to the absence of physical barriers to limit gene flow (Muthini et al., 2014). Human activities such as transfer of plants, soils and the circulation of climbing bean seeds through trading within the region (Ramaekers et al., 2013) could have possibly contributed to the rhizobia genetic conservation in Eastern Kenya. Similar findings were reported by Elboutahiri et al. (2015) who observed a larger proportion of significant ($p < 0.05$) genetic variation distributed within regions (89%) than among regions (11%) in *S. meliloti* and *S. medicae* obtained from drought and salt affected regions of Morocco. Moreover, Rashid (2013) reported high variability of rhizobia isolates obtained from different geographical regions in Europe.

The principal coordinate analysis (PCA) also showed low level of genetic differentiation of native rhizobia isolates from Eastern Kenya and thus showing congruent results with the dendrogram. However, the grouping and distribution patterns of the native isolates did not correspond to the AEZ, and this is an indicative of the likelihood of a common evolutionary origin of the isolates. Similarly, Ismail et al. (2013), reported a PCA analysis of rhizobia isolates collected from different sites that did not correspond to the geographical locations in Egypt, and linked the genetic homogeneity of the isolates to the ribosomal gene recombination within and between rhizobia strains. A study by Wang et al. (2016) on biodiversity and biogeography of rhizobia associated with common beans in Shaanxi province, China, linked lateral gene transfer of symbiotic genes among different strains of nitrogen-fixing bacteria (*Agrobacterium*, *Bradyrhizobium*, *Rhizobium,* and *Ensifer*). Based on the evolutionary relationship of 41 isolates inferred using Neighbor-Joining method, some of the native isolates clustered closely with the three reference rhizobia strains used in this study. The phylogenetic tree showed a diverse genetic variation among the native rhizobia isolates as evident by the different clusters and sub-clusters.

From the phylogenetic tree, most of the native rhizobia isolates clustered together with *Rhizobium tropici* CIAT 899 and *R. etli* USDA 2667 in cluster I. For instance, native isolates TLM 27 and TUM 41 clustered together with *R. etli* USDA 2667 while isolate TLM 32 clustered with *R. tropici* CIAT 899. These indicate the close genetic relationship between some of the native rhizobia isolates to *R. tropici* CIAT 899*, R. etli* USDA 2667 and *R. leguminosarum* strain 446 (Wang et al., 2016). Other studies in Kenya have shown the dominance of these *Rhizobium* strains in Kenyan soil (Anyango et al., 1995; Mwenda et al., 2011; Onyango et al., 2015). Based on ARDRA analysis, native isolates TUM 26, TLM 28, and EUM 23 clustered together despite originating from different AEZ of Eastern Kenya. This indicates the close genetic distance between some of the rhizobia isolates native to different AEZ of Eastern Kenya. There is also a possibility of such isolates having originated from the same genetic background. However, this may not be conclusive unless other molecular tools used in studying genetic diversity such as sequencing and phylogenetic analyses are employed.

CONCLUSION

In this study, 41 rhizobia isolates were obtained from the root nodules of mid-altitude climbing (MAC) beans and based on morpho-cultural and biochemical characteristics, the isolates grouped into nine morphotypes indicating the diverse nature of rhizobia nodulating with MAC beans. Cluster analysis based on genetic characteristics obtained after PCR-ARDRA profiling, showed a larger proportion of significant ($p < 0.05$) genetic variation distributed within populations (97%) than among populations (2.5%) in four AEZ of Eastern Kenya. Despite the high diversity shown within the rhizobia population, the Pairwise Population Matrix of Nei unbiased genetic distance of rhizobia populations in the study region showed a narrow range, which is an indication of a weakly structured genetic population and a highly conserved genetic structure of rhizobia within the study area. Further molecular studies using either full or partial gene sequences of bacterial genome, which is more sensitive and has a higher resolution power, needs to be carried out to establish the true diversity of native rhizobia isolates up to the species and strain levels.

AUTHOR CONTRIBUTIONS

JK, OO, JM, and EN conceived and designed the research and data collection tools and participated in drafting the manuscript. GK and SM collected the data, participated in data analyses, and wrote the manuscript. EN performed the data analyses. All authors read and approved the final manuscript.

FUNDING

This work was supported by the Regional Universities Forum for Capacity Building in Agriculture (RUFORUM) 5th Graduate Research Grant (GRG), Grant Nos. RU 2014 GRG-102 and RU 2017 FAPA 184.

ACKNOWLEDGMENTS

The authors express their sincere gratitude to RUFORUM for supporting this study and laboratory staff from Tissue Culture, Kenyatta University for their cooperation, participation, patience, and time during the collection of molecular data and analysis.

REFERENCES

Adhikari, D., Itoh, K., and Suyama, K. (2013). Genetic diversity of common bean (*Phaseolus vulgaris* L.) nodulating rhizobia in Nepal. *Plant Soil* 368, 341–353. doi: 10.1007/s11104-012-1518-7

Al-mujahidy, S. J., Hassan, M., and Rahman, M. (2013). Isolation and characterization of *Rhizobium* spp. and determination of their potency for growth factor production. *Int. Res. J. Biotechnol.* 4, 117–123. doi: 10.14303/irjob.2013.029

Ampomah, O. Y., and Huss-Danell, K. (2016). Genetic diversity of rhizobia nodulating native Vicia spp. in Sweden. *Syst. Appl. Microbiol.* 39, 203–210. doi: 10.1016/j.syapm.2016.02.002

Anyango, B., Wilson, K. J., Beynon, J. L., and Giller, K. E. (1995). Diversity of rhizobia nodulating *Phaseolus vulgaris* L. in two Kenyan soils with contrasting pHs. *Appl. Environ. Microbiol.* 61, 4016–4021.

Baginsky, C., Brito, B., Scherson, R., Pertuzé, R., Seguel, O., Cañete, A., et al. (2015). Genetic diversity of *Rhizobium* from nodulating beans grown in a variety of Mediterranean climate soils of Chile. *Arch. Microbiol.* 197, 419–429. doi: 10.1007/s00203-014-1067-y

Baratto, C. M., Gelinski, J. M. L. N., Bernardi, A. Z., Marafon, A., and Braun, F. (2012). Potential use of molecular-typing methods for the identification and characterization of *Salmonella enterica* serotypes isolated in the poultry production chain. *Rev. Bras. Ciênc. Avíc.* 14, 173–179. doi: 10.1590/S1516-635X2012000300003

Batista, L., Irisarri, P., Rebuffo, M., Cuitiño, M. J., Sanjuán, J., and Monza, J. (2015). Nodulation competitiveness as a requisite for improved rhizobial inoculants of *Trifolium pratense*. *Biol. Fertil. Soils* 51, 11–20. doi: 10.1007/s00374-014-0946-3

Beck, D. P., Materon, L. A., and Afandi, F. (1993). *Practical Rhizobium-Legume Technology Manual*. Aleppo: International Center for Agricultural Research in the Dry Areas (ICARDA).

Berrada, H., Nouioui, I., Houssaini, M., Ghachtouli, N., Gtari, M., and Benbrahim, K. (2012). Phenotypic and genotypic characterizations of rhizobia isolated from root nodules of multiple legume species native of Fez, Morocco. *Afr. J. Microbiol. Res.* 6, 5314–5324.

Boakye, E. Y., Lawson, I. Y. D., Danso, S. K. A., and Offei, S. K. (2016). Characterization and diversity of rhizobia nodulating selected tree legumes in Ghana. *Symbiosis* 69, 89–99. doi: 10.1007/s13199-016-0383-1

Dai, J., Liu, X., and Wang, Y. (2012). Genetic diversity and phylogeny of rhizobia isolated from *Caragana microphylla* growing in desert soil in Ningxia, China. *Genet. Mol. Res.* 11, 2683–2693. doi: 10.4238/2012.June.25.5

Elboutahiri, N., Thami-Alami, I., and Udupa, S. M. (2015). Phenotypic and genetic diversity in *Sinorhizobium meliloti* and *S. medicae* from drought and salt affected regions of Morocco. *BMC Microbiol.* 10:15. doi: 10.1186/1471-2180-10-15

Grönemeyer, J. L., Kulkarni, A., Berkelmann, D., Hurek, T., and Reinhold-Hurek, B. (2014). Identification and characterization of rhizobia indigenous to the Okavango region in Sub-Saharan Africa. *Appl. Environ. Microbiol.* 4, 1–17. doi: 10.1128/AEM.02417-14

Hameed, S., Yasmin, S., Malik, K. A., Zafar, Y., and Hafeez, F. Y. (2004). *Rhizobium*, *Bradyrhizobium* and *Agrobacterium* strains isolated from cultivated legumes. *Biol. Fertil. Soils* 39, 179–185. doi: 10.1007/s00374-003-0697-z

Hammer, O., Harper, D. A. T., and Ryan, P. D. (2001). PAST: paleontological statistics software package for education and data analysis. v.2.17. *Palaeontol. Electronica* 4, 1–9.

Ismail, M., El-zanatay, A. M., Eissa, R. A., and Hewedy, O. A. (2013). Genetic diversity of *Rhizobium leguminosarum* as revealed by 16S rRNA Gene Sequence. *Am. Eurasian J. Agric. Environ. Sci.* 13, 797–801. doi: 10.5829/idosi.aejaes.2013.13.06.1969

Karthik, C., Oves, M., Sathya, K., Sri Ramkumar, V., and Arulselvi, P. I. (2017). Isolation and characterization of multi-potential *Rhizobium* strain ND2 and its plant growth-promoting activities under Cr (VI) stress. *Arch. Agron. Soil Sci.* 63, 1058–1069. doi: 10.1080/03650340.2016.126111

Kawaka, F., Dida, M. M., Opala, P. A., Ombori, O., Maingi, J., Osoro, N., et al. (2014). Symbiotic efficiency of native rhizobia nodulating common bean (*Phaseolus vulgaris* L.) in soils of Western Kenya. *Int. Sch. Res. Notices* 2014:258497. doi: 10.1155/2014/258497

Laguerre, L. E., Mavingui, P., Allard, M., Charnay, M., Louvrier, P., Mazurier, S., et al. (1996). Typing of rhizobia by PCR DNA fingerprinting and PCR-restriction fragment length polymorphism analysis of chromosomal and symbiotic gene regions?: application to *Rhizobium leguminosarum* and its different biovars. *Appl. Environ. Microbiol.* 62, 2029–2036.

Lemaire, B., Dlodlo, O., Chimphango, S., Stirton, C., Schrire, B., Boatwright, J. S., et al. (2015). Symbiotic diversity, specificity and distribution of rhizobia in native legumes of the Core Cape Sub-region (South Africa). *FEMS Microbiol. Ecol.* 91, 1–17. doi: 10.1093/femsec/fiu024

Liu, W. Y. Y. (2014). *Characterisation of Rhizobia and Studies on N Fixation of Common weed Legumes in New Zealand*. Ph.D. thesis, Lincoln University, Lincoln.

Mburu, S. W., Koskey, G., Kimiti, J. M., Ombori, O., Maingi, J. M., and Njeru, E. M. (2016). Agrobiodiversity conservation enhances food security in subsistence-based farming systems of Eastern Kenya. *Agric. Food Secur.* 5:19. doi: 10.1186/s40066-016-0068-2

Meng, L., Zhang, A., Wang, F., Han, X., Wang, D., and Li, S. (2015). Arbuscular mycorrhizal fungi and rhizobium facilitate nitrogen uptake and transfer in soybean/maize intercropping system. *Front. Plant Sci.* 6:339. doi: 10.3389/fpls.2015.00339

Muthini, M., Maingi, J. M., Muoma, J. O., Amoding, A., Mukaminega, D., Osoro, N., et al. (2014). Morphological assessment and effectiveness of indigenous rhizobia isolates that nodulate *P. vulgaris* in water hyacinth compost testing field in Lake Victoria basin. *Br. J. Appl. Sci. Technol.* 4, 718–738. doi: 10.9734/BJAST/2014/5757

Mwenda, G. M., Karanjac, N. K., Bogaa, H., Kahindib, J. H. P., Muigaia, A., and Odeed, D. (2011). Abundance and diversity of legume nodulating rhizobia in soils of Embu district, Kenya. *Trop. Subtrop. Agroecosyst.* 13, 1–10.

Nei, M., and Li, W. H. (1979). Mathematical model for studying genetic variation in terms of restriction endonucleases. *Proc. Natl. Acad. Sci. U.S.A.* 76, 5269–5273. doi: 10.1073/pnas.76.10.5269

Nyaga, J., Jefwa, J. M., Muthuri, C. W., Okoth, S. A., Matiru, V. N., and Wachira, P. (2014). Influence of soil fertility amendment practices on ex-situ utilization of indigenous arbuscular mycorrhizal fungi and performance of maize and common bean in Kenyan highlands. *Trop. Subtrop. Agroecosyst.* 17, 129–141.

Onyango, B., Beatrice, A., Regina, N., Koech, P., Skilton, R., and Francesca, S. (2015). Morphological, genetic and symbiotic characterization of root nodule bacteria isolated from Bambara groundnuts (*Vigna subterranea* L. Verdc) from soils of Lake Victoria basin, western Kenya. *J. Appl. Biol. Biotechnol.* 3, 1–10. doi: 10.7324/jabb.2015.3101

Orrell, P., and Bennett, A. E. (2013). How can we exploit above-belowground interactions to assist in addressing the challenges of food security? *Front. Plant Sci.* 4:432. doi: 10.3389/fpls.2013.00432

Park, J. M., Yang, C. Y., Park, H., and Kim, J. M. (2014). Development of a genus-specific PCR combined with ARDRA for the identification of Leuconostoc species in Kimchi. *Food Sci. Biotechnol.* 23, 511–516. doi: 10.1007/s10068-014-0070-z

Pohajda, I., Huić Babić, K., Rajnović, I., Kajić, S., and Sikora, S. (2016). Genetic diversity and symbiotic efficiency of indigenous common bean rhizobia in Croatia. *Food Technol. Biotechnol.* 54, 468–474. doi: 10.17113/ftb.54.04.16.4740

Rahmani, H. A., Räsänen, L. A., Afshari, M., and Lindström, K. (2011). Genetic diversity and symbiotic effectiveness of rhizobia isolated from root nodules of *Phaseolus vulgaris* L. grown in soils of Iran. *Appl. Soil Ecol.* 48, 287–293. doi: 10.1016/j.apsoil.2011.04.010

Ramaekers, L., Micheni, A., Mbogo, P., Vanderleyden, J., and Maertens, M. (2013). Adoption of climbing beans in the central highlands of Kenya: an empirical analysis of farmers ' adoption decisions. *Afr. J. Agric. Res.* 8, 1–19.

Rashid, M. (2013). *Genetic Diversity of Rhizobia Nodulating Lentils (Lens culinaris Medik.)*. Heidelberg, BW: Ruperto-Carola University of Heidelberg.

Saikia, S. P., and Jain, V. (2007). Biological nitrogen fixation with non-legumes: an achievable target or a dogma. *Curr. Sci.* 92, 317–322.

Silva, F. V., Simoes-Araujo, J. L., Silva Junior, J. P., Xavier, G. R., and Rumjanek, N. G. (2012). Genetic diversity of rhizobia isolates from amazon soils using cowpea (*Vigna unguiculata*) as trap plant. *Braz. J. Microbiol.* 43, 682–691. doi: 10.1017/CBO9781107415324.004

Somasegaran, P., and Hoben, H. J. (1994). *Handbook for Rhizobia Methods in Legume - Rhizobium Technology*. New York, NY: Springer Verlag. doi: 10.1007/978-1-4613-8375-8

Torres-Gutiérrez, R., Granda-Mora, K. I., Alvarado-Capó, Y., Rodriguez, A. S., Mogollón, N. G. S., and Almeida, J. R. D. (2017). Genetic and phenotypic diversity of Rhizobium isolates from Southern Ecuador. *Ciênc. Agrotecnol.* 41, 634–647. doi: 10.1590/1413-70542017416008517

Vincent, J. M. (1970). *A Manual for the Practical Study of the Root Nodule Bacteria*. Oxford: Blackwell Scientific Publications.

Wang, L., Cao, Y., Wang, E. T., Qiao, Y. J., Jiao, S., Liu, Z. S., et al. (2016). Biodiversity and biogeography of rhizobia associated with common bean (*Phaseolus vulgaris* L.) in Shaanxi Province. *Syst. Appl. Microbiol.* 39, 211–219. doi: 10.1016/j.syapm.2016.02.001

Wasike, V. W., Lesueur, D., Wachira, F. N., Mungai, N. W., Mumera, L. M., Sanginga, N., et al. (2009). Genetic diversity of indigenous *Bradyrhizobium* nodulating promiscuous soybean [*Glycine max* (L) Merr.] varieties in Kenya: impact of phosphorus and lime fertilization in two contrasting sites. *Plant Soil* 322, 151–163. doi: 10.1007/s11104-009-9902-7

Young, J. P. W., Downer, H. L., and Eardly, B. D. (1991). Phylogeny of the phototrophic *Rhizobium* strain BTAi1 by polymerase chain reaction-based sequencing of a 16S rRNA gene segment. *J. Bacteriol.* 173, 2271–2277. doi: 10.1128/jb.173.7.2271-2277.1991

Zahran, H. H. (2001). Rhizobia from wild legumes: diversity, taxonomy, ecology, nitrogen fixation and biotechnology. *J. Biotechnol.* 91, 143–153. doi: 10.1016/S0168-1656(01)00342-X

Conflict of Interest Statement: The authors declare that the research was conducted in the absence of any commercial or financial relationships that could be construed as a potential conflict of interest.

frontiers in Plant Science

ORIGINAL RESEARCH
published: 05 June 2018
doi: 10.3389/fpls.2018.00743

Trichoderma-Based Biostimulants Modulate Rhizosphere Microbial Populations and Improve N Uptake Efficiency, Yield, and Nutritional Quality of Leafy Vegetables

Nunzio Fiorentino[1,2], Valeria Ventorino[1,3], Sheridan L. Woo[3,4,5], Olimpia Pepe[1,3], Armando De Rosa[1], Laura Gioia[1], Ida Romano[1], Nadia Lombardi[5], Mauro Napolitano[1], Giuseppe Colla[6] and Youssef Rouphael[1]*

[1] Department of Agricultural Sciences, University of Naples Federico II, Portici, Italy, [2] CIRAM-Interdepartmental Center for Environmental Research, University of Naples Federico II, Naples, Italy, [3] Task Force on Microbiome Studies, University of Naples Federico II, Naples, Italy, [4] Department of Pharmacy, University of Naples Federico II, Naples, Italy, [5] National Research Council, Institute for Sustainable Plant Protection, Portici, Italy, [6] Department of Agricultural and Forestry Sciences, University of Tuscia, Viterbo, Italy

OPEN ACCESS

Edited by:
Antonio Elia, University of Foggia, Italy

Reviewed by:
Jose M. Garcia-Mina, Universidad de Navarra, Spain
José López-Bucio, Universidad Michoacana de San Nicolás de Hidalgo, Mexico

***Correspondence:**
Nunzio Fiorentino
nunzio.fiorentino@unina.it

Specialty section:
This article was submitted to Crop and Product Physiology, a section of the journal Frontiers in Plant Science

Received: *12 March 2018*
Accepted: *15 May 2018*
Published: *05 June 2018*

Citation:
Fiorentino N, Ventorino V, Woo SL, Pepe O, De Rosa A, Gioia L, Romano I, Lombardi N, Napolitano M, Colla G and Rouphael Y (2018) Trichoderma-Based Biostimulants Modulate Rhizosphere Microbial Populations and Improve N Uptake Efficiency, Yield, and Nutritional Quality of Leafy Vegetables. Front. Plant Sci. 9:743. doi: 10.3389/fpls.2018.00743

Microbial inoculants such as *Trichoderma*-based products are receiving great interest among researchers and agricultural producers for their potential to improve crop productivity, nutritional quality as well as resistance to plant pathogens/pests and numerous environmental stresses. Two greenhouse experiments were conducted to assess the effects of *Trichoderma*-based biostimulants under suboptimal, optimal and supraoptimal levels of nitrogen (N) fertilization in two leafy vegetables: Iceberg lettuce (*Lactuca sativa* L.) and rocket (*Eruca sativa* Mill.). The yield, nutritional characteristics, N uptake and mineral composition were analyzed for each vegetable crop after inoculation with *Trichoderma* strains *T. virens* (GV41) or *T. harzianum* (T22), and results were compared to non-inoculated plants. In addition, the effect of the *Trichoderma*-based biostimulants on microbes associated with the rhizosphere in terms of prokaryotic and eukaryotic composition and concentration using DGGE was also evaluated. *Trichoderma*-based biostimulants, in particular GV41, positively increased lettuce and rocket yield in the unfertilized plots. The highest marketable lettuce fresh yield was recorded with either of the biostimulant inoculations when plants were supplied with optimal levels of N. The inoculation of rocket with GV41, and to a lesser degree with T22, elicited an increase in total ascorbic acid under both optimal and high N conditions. *T. virens* GV41 increased N-use efficiency of lettuce, and favored the uptake of native N present in the soil of both lettuce and rocket. The positive effect of biostimulants on nutrient uptake and crop growth was species-dependent, being more marked with lettuce. The best biostimulation effects from the *Trichoderma* treatments were observed in both crops when grown under low N availability. The *Trichoderma* inoculation strongly influenced the composition of eukaryotic populations in the rhizosphere, in particularly exerting different effects with low N levels in comparison to the N fertilized plots. Overall,

inoculations with *Trichoderma* may be considered as a viable strategy to manage the nutrient content of leafy horticulture crops cultivated in low fertility soils, and assist vegetable growers in reducing the use of synthetic fertilizers, developing sustainable management practices to optimize N use efficiency.

Keywords: *Eruca sativa* Mill., *Lactuca sativa* L., mineral composition, N uptake, *Trichoderma,* DGGE, microbial diversity

INTRODUCTION

Italy is the European leader for leafy vegetable production with about 15,000 ha and 150 kilo-tons *per annum* in protected greenhouses[1] that are mainly produced in Campania region (southern Italy) and in northern regions of Lombardia and Veneto. Among the leafy vegetables destined as fresh cut produce, lettuce (*Lactuca sativa* L.) and rocket (*Eruca sativa* Mill.) have been gaining prominence in the national and international vegetable markets (Colonna et al., 2016).

Leafy vegetable crop production relies heavily on nitrate (NO_3^-) availability which constitutes the most important source of nitrogen (N) (Colla et al., 2010, 2011). Roughly, half of the N fertilizer applied is utilized by the vegetable crops, however, the remainder is lost by leaching to the soil, which can contribute to surface and groundwater contamination (Tilman et al., 2002). The search for strategies to improve agricultural practices, aimed at increasing uptake efficiency without affecting crop productivity, represents a strong stimulus for researchers, extension specialists as well as vegetable growers (Colla et al., 2018). Over the past three decades many efforts have been employed to enhance crop nitrogen use efficiency (NUE), through plant breeding programs and biotechnological approaches, however, with limited success and economic benefits due to the complexity of the genetic traits involved (Xu et al., 2012).

The use of plant biostimulants, which include organic and inorganic natural substances (i.e., humic acids, protein hydrolysates, seaweed extracts, and silicon) as well as beneficial microorganisms (i.e., mycorrhizal fungi, *Trichoderma* spp. and plant growth promoting rhizobacteria) to enhance nutrient uptake and crop production could be considered as a sustainable and environmentally friendly approach to secure yield stability under low-input conditions (Ventorino et al., 2014; Colla et al., 2015a, 2017; du Jardin, 2015; Lorito and Woo, 2015; Rouphael et al., 2015; Viscardi et al., 2016). According to López-Bucio et al. (2015) *Trichoderma* have gained importance as microbial plant biostimulants in horticulture. In the past, *Trichoderma*-based products were particularly noted as successful biological control agents for contrasting plant pathogens, mainly phytopathogenic fungi, as well as inducing resistance to biotic stresses (Harman, 2000; Harman et al., 2004; Hermosa et al., 2012; Woo et al., 2014; Lorito and Woo, 2015). However, in addition to their biopesticide activity, some *Trichoderma* strains have been proven to have a biostimulant activity, plant growth promotion, improved yield and nutritional quality, as well as mitigating the detrimental effect of abiotic stresses (Lorito et al., 2010; Hermosa et al., 2012; López-Bucio et al., 2015). Therefore, it is not surprising that *Trichoderma* are found as successful beneficial microbial biological agents, also present as active ingredients in over 200 agricultural products such as biopesticides, biofertilizers, bio-growth enhancers and biostimulants marketed worldwide for conventional and organic agricultural production (Woo et al., 2014).

In a recent review, López-Bucio et al. (2015) reported that the mechanism of stimulation by *Trichoderma* involves a multilevel root–shoot communication. The phytostimulation effect of *Trichoderma* applications has been attributed to several direct and indirect effects on plants, including the release of substances with auxin activity (i.e., indole-3-acetaldehyde, indole-3-carboxaldehyde, and indole-3-ethanol), small peptides as well as volatile organic compounds, which improve root system architecture (total root length, density, and branching) and assimilation/solubilization of macronutrients (P) and micronutrients (Fe, Mn, and Zn), thus boosting plant growth and crop productivity (Harman, 2000; Howell, 2003; Harman et al., 2004; Contreras-Cornejo et al., 2009, 2011; Lorito et al., 2010; Hermosa et al., 2012; Lorito and Woo, 2015; Colla et al., 2015b,c; Rouphael et al., 2017a). Furthermore, endophytic fungi including *Trichoderma* spp. interact with other members of the microbial community in the plant rhizosphere and therefore it is important to assess the ecological impact of different soil managements and microbial-based biostimulant on soil ecosystem and in particular on quantitative and qualitative microbial populations (Mar Vázquez et al., 2000; Lace et al., 2015).

Although studies on the biostimulant role of *Trichoderma* on eliciting plant growth and yield as well as on enhancing tolerance to environmental stresses such as salinity and drought (López-Bucio et al., 2015) have been found, nothing is known about the beneficial responses on *Trichoderma* application on leafy vegetables grown under different N inputs. Moreover, the application of inoculants, chemical fertilizers or a combination of organic and chemical strategies, could modulate the microbial communities in the plant rhizosphere (Mar Vázquez et al., 2000), with an ecological impact on agro-ecosystem that can be investigated by culture-independent molecular techniques (Belyaeva et al., 2012; Ventorino et al., 2013, 2016; Gupta et al., 2014, 2016).

The aim of the current study was to determine the effect of different *Trichoderma*-based biological treatments (*T. harzianum* strain T22 and *T. virens* strain GV41) on the quantitative and qualitative characteristics, including the chemical composition of two important leafy vegetables, lettuce and rocket, cultivated in different fertilization conditions corresponding to suboptimal, optimal, and supraoptimal N levels for each crop. Furthermore, analysis was conducted to evaluate the effect of the N

[1] http://agri.istat.it

fertilization and microbial-biostimulant applications on the microbes associated to the rhizosphere of the two horticultural crops in terms of prokaryotic and eukaryotic composition and concentration. The knowledge gained from this research will shed light on the mode of action of *Trichoderma* strains under different N availability and will permit the development of sustainable farming strategies to cultivate highly productive leafy vegetables using reduced amounts of synthetic N fertilizer.

MATERIALS AND METHODS

Plant Material, Experimental Conditions, and Design

Two consecutive experiments were conducted in the 2016 winter-summer growing season, the first on lettuce (*Lactuca sativa* L. var. Iceberg cv. 'Silvinas'; Rijk Zwaan, Bologna, Italy) from February 2nd to March 31st (Experiment 1), and the second on rocket (*Eruca sativa* Mill.) from June 13th to July 11th (Experiment 2). Experiments were conducted in a unheated polyethylene greenhouse located at the University of Naples Federico II, Portici (NA), south Italy (40° 49'N, 14° 15'E; 72 m a.s.l.). The soil was a sandy loam (76% sand, 17% silt, 7% clay), with a pH of 6.9, electrical conductivity of 0.6 mS cm^{-1}, organic matter of 1.25% (w/w), C:N of 10.8, total N at 0.11%, carbonates at 0.3%, NO_3-N and NH_4-N at 108 and 13 mg kg^{-1}, respectively, P at 38 mg kg^{-1}, and exchangeable K at 980 mg kg^{-1}.

The test conditions for each horticultural crop consisted of three fertilization regimes and three microbial inoculations. Suboptimal (Low N), optimal (Opt N) and supraoptimal (High N), fertilization levels were applied to the two crops corresponding to 0, 90, and 180 kg N $ha^{-1}$for lettuce, and 0, 60, and 120 kg N $ha^{-1}$for rocket. Microbial-based biostimulant treatments included *Trichoderma harzianum* strain T22 (T22) and *Trichoderma virens* strain GV41 (GV41), as well as a control (NoT).

In both experiments, nine treatments from the factorial combination of the above-described fertilization rates and microbial applications were arranged in randomized complete block split-plot design (N fertilization as the main factor and *Trichoderma* inoculation as sub-factor) with three replicates for a total of 27 experimental plots. The area of each experimental plot measured 3.5 m^2, containing an expected plant density of 11 plants m^{-2} for lettuce and 3,000 seeds m^{-2} for rocket.

No P and K fertilization was performed due to the high content of these macronutrients found in the soil samples examined. The N was applied as ammonium nitrate (34%) by fertigation, in a drip irrigation system with in-line emitters at 35 cm distances, and an emitter flow rate of 3.3 L h^{-1} was adopted for lettuce, whereby the total N amount was allocated in 3 weekly applications starting 10 days after transplanting (DAT). Instead for rocket, N fertigation was performed using a sprinkler irrigation system, with applications at 3 and 8 days after seeding (DAS), thus distributing the total N amount into two equal doses. In both experiments, the NO_3^- concentration in the water source used for the irrigation was lower than 50 mg L^{-1}; therefore the N concentration coming from irrigation water was considered negligible. Throughout the growing cycle, pathogens and pests were controlled according to standard crop protection practices adopted by commercial leafy vegetable growers in Italy.

Preparation of Fungal Biostimulant Inoculum

Trichoderma harzianum strain T22 and *T. virens* strain GV41, were obtained from the microbe collection of the Department of Agricultural Sciences, University of Naples Federico II, Portici. Fungi were grown on Potato Dextrose Agar (PDA, HiMedia Mumbai, India), at room temperature (25°C), with light (16 h day/8 h night), until sporulation. Conidial spores were collected in sterile water, then a 50 ml spore suspension (concentration of 1×10^6 spores ml^{-1}) was used as a starter inoculum for solid-state fermentation on sterile rice (500 g), in breathable bags (Microsac, Nevele, Belgium), then incubated at 25°C with light (16 h day/8 h night) in a growth chamber. After 7 days the spores were collected by washing the colonized rice with sterile water. The final concentration of the spore suspension was adjusted to 1×10^7 spores ml^{-1}, then used for the microbial-biostimulant applications in both greenhouse trials.

Lettuce seedlings were treated with the *Trichoderma* inoculum by using a root dip method. At the time of transplant, the styrofoam trays containing the seedlings were submerged in the liquid spore suspension for 10 min, in order to completely wet the roots. Plant trays were drained of excess liquid, the plant-plug was removed from the tray, then transplanted to pre-bored holes in the soil. Each plant was watered at the base with 25 ml of the spore suspension. The microbial-biostimulant treatment was repeated at 24 DAT, by watering 50 ml $plant^{-1}$ of the spore suspension.

For the rocket experiment, the *Trichoderma* inoculations were applied as a seed-coating treatment, using a 1×10^8 spores ml^{-1} spore suspension to uniformly cover the seed surface, then left to air-dry, and hand-seeded to the prepared soil at approximate concentration of 3,000 seeds m^{-2}.

Evaluation of Lettuce and Rocket Productivity

Lettuce plants were harvested 60 DAT in order to evaluate the total and marketable yields, sampling in one square meter quadrats from the center of each experimental plot. Total and marketable yield of rocket was determined at 30 DAS following the same procedure as for lettuce. Vegetative material of both lettuce and rocket was weighed at harvest to obtain the fresh weight, then dried at 80°C for about 72 h until achieving constant weight, and weighed again to determine the dry matter content of the plant biomass. A sub-sample of the dried leaf tissues was collected for the ion analyses. To determine the amount of ascorbic acid (AsA) in lettuce and rocket, fresh leaves were frozen in liquid nitrogen and stored at −80°C until used.

Analysis of Total Ascorbic Acid Content

The total ascorbic acid in lettuce and rocket leaf extracts, defined as ascorbic acid and dehydroascorbate acid, was determined by an assay based on the reduction of Fe^{3+} to Fe^{2+} by AsA and the spectrophotometric detection of Fe^{2+} complexes with

2,2-dipyridyl (Kampfenkel et al., 1995). Dehydroascorbate is reduced to ascorbic acid by pre-incubation of the sample with dithiothreitol. The absorbance of the solution was measured at 525 nm in a spectrophotometer Hach DR 2000 (Hach Co., Loveland, CO, United States), and data were expressed as mg ascorbic acid on 100 g fresh weight.

Leaf Ion Analyses

Dried lettuce or rocket leaf tissues were ground separately in a Wiley mill (IKA, MF10.1, Staufen, Germany) and passed through 0.5 mm sieve, then the homogenized plant tissues were used for ion analyses.

For the cations (K^+ and Ca^{2+}) and anions (NO_3^- and PO_4^{3-}) analysis, 250 mg of dried material was extracted in 50 ml of ultrapure water using a shaking water bath (ShakeTemp SW22, Julabo, Seelbach, Germany) at 80°C for 10 min, as described by Rouphael et al. (2017b,c). Briefly, the mixture was centrifuged at 6,000 rpm for 10 min (R-10 M, Remi Elektrotechnik Limited, India), then filtered through a 0.20 μm filter paper (Whatman International Ltd., Maidstone, United Kingdom). The monovalent and bivalent cations were separated by ion chromatography (ICS-3000, Dionex, Sunnyvale, CA, United States) and quantified with an electrical conductivity detector. A conductivity detector with IonPac CG12A (4 × 250 mm, Dionex, Corporation) guard column and IonPac CS12A (4 × 250 mm, Dionex, Corporation) analytical column were used for the analysis of the monovalent and bivalent cations, whereas for nitrate and phosphate an IonPac AG11-HC guard (4 × 50 mm) column and IonPac AS11-HC analytical column (4 × 250 mm) were used.

Analysis of Soil Minerals

Soil nitrogen (total N) concentration was assessed after mineralization with sulfuric acid (96%, Carlo Erba Reagents, Milan, Italy) in the presence of potassium sulfate and a low concentration of copper by the Kjeldahl method (Bremner, 1965). Mineral N was determined spectrophotometrically (FIAstar 5000 Analyzer, FOSS analytical Denmark) on soil extracts. Gas semi-permeable membrane method (ISO11732 procedure[2]) was carried out for NH_4-N, while the sulfanilamide-naphtylethylendiamine dihydrochloride method was carried out to analyze NO_3-N after nitrate to nitrite reduction with a copper–cadmium column (ISO 13395 procedure[3]).

Determination of Fungal Concentration in the Rhizo-Soil

At the time of harvest for experiments 1 and 2, soil samples were collected from the plant rhizosphere and the surrounding soil, from each treatment plot. Detection and quantification of fungal colony forming units (CFU) was conducted using standard soil microbial plating techniques by serial dilutions. The total number of fungal CFU were estimated on Rose Bengal-Chloramphenicol agar (HiMedia Pvt. Ltd., Mumbai, India) supplemented with 0.1% (v/v) Igepal (Sigma-Aldrich, Milan, Italy). One gram of soil was added to 100 ml of water and left in vigorous orbital agitation for 10 min; then 10-fold serial dilutions of the soil suspension were performed. A 100 μl aliquot of each soil dilution was spread on the surface of the solid media and plated in three replicates. The CFU of fungi were counted after incubation for 3–7 days at 25°C.

Molecular Characterization of Soil Microbes

Total DNA was extracted from soil samples using a Fast DNA SPIN Kit for Soil (MP Biomedicals, Illkirch, France) according to the manufacturer's instructions.

The primers V3f (5′-CCTACGGGAGGCAGCAG-3′) and V3r (5′-ATTACCGCGGCTGCTGG-3′) (Muyzer et al., 1993) were employed for prokaryotic DGGE analysis. The primers NL1 (5′-GCATATCAATAAGCGGAGGAAAAG-3′) (Kurtzman and Robnett, 1998) and LS2 (5′-ATTCCCAAACAACTCGACTC-3′) (Cocolin et al., 2000) of the 26S rRNA gene were used to analyze the eukaryotic population. As described by Muyzer et al. (1993), a GC clamp was added to forward primers. The PCR mixture and conditions for both amplifications were performed according to Ventorino et al. (2016). DGGE analyses were performed in a polyacrylamide gel [8% (wt/vol) acrylamide-bisacrylamide (37:5:1)] with a denaturing gradient of 30–60% using a Bio-Rad DCode Universal Mutation System (Bio-Rad Laboratories, Milan, Italy) as previously described (Ventorino et al., 2018).

Statistical Analysis

Experimental data were statistically analyzed by two-way analysis of variance using the SPSS 21 software package. To separate treatment means within each measured parameter, Least Significant Difference (LSD) test was performed at a significance level of $p \leq 0.05$. Phoretix 1 advanced version 3.01 software (Phoretix International Limited, Newcastle upon Tyne, England) was used to automatically detect the DGGE bands, matching bands were determined, then a cluster analysis was performed as previously indicated by Ventorino et al. (2013). The correlation matrix of the band patterns was performed by using the method described by Saitou and Nei (1987). Finally, the percentage of similarity (S) of the microbial community was estimated by analyzing the resulting matrix using the average linkage method in the cluster procedure of Systat 5.2.1.

RESULTS

Fungal Concentration in the Soil

In general, the total fungal concentration (CFU) in rhizo-soils (including also the applied *Trichoderma* inoculum) of the two leafy vegetables was species-dependent, and also influenced by the nitrogen fertilizer applications as well as by the strains of *Trichoderma* used. In the absence of *Trichoderma* inoculation, the different N fertilizations did not modify the fungal CFU in lettuce rhizo-soil, whereas a significant reduction (by 46%) was observed only with the high N fertilization in rocket rhizo-soil. The overall CFU of fungi isolated from the lettuce soils ranged from 1.5×10^6

[2]https://www.iso.org/standard/38924.html

[3]https://www.iso.org/standard/21870.html

to 1.7×10^7 CFU g^{-1} of soil. Interestingly, in the unfertilized conditions, the highest number of fungal colonies in lettuce was observed in the treatments with GV41 (1.7×10^7 CFU g^{-1} of soil), followed by NoT (1.0×10^7 CFU g^{-1} of soil) and finally with plants inoculated with T22 (3.6×10^6 CFU g^{-1} of soil). Moreover, under optimal N conditions, the fungal concentration did not differ between NoT and T22 treatments (1.3×10^7 CFU g^{-1} of soil), which were significantly higher than that of GV41 (1.8×10^6 CFU g^{-1} of soil). Under High N levels, the overall fungal presence was relatively high (1.5×10^7 CFU g^{-1} of soil in NoT), and the greatest concentration among the inoculated plots was observed with GV41 (9.2×10^6 CFU g^{-1} of soil) compared to the T22 (1.5×10^6 CFU g^{-1} of soil) treatment.

The overall fungal concentration in rocket rhizo-soils, for all N fertilizer and biostimulant treatments was 10-fold less than those observed in the experiment conducted on the lettuce, with counts ranging from 1.6×10^5 to 6.5×10^5 CFU g^{-1} of soil. The fungal CFUs in both inoculated treatments were not different from the untreated control (NoT), at both low N (3.9×10^5 CFU g^{-1} of soil) and optimal N (3.9×10^5 CFU g^{-1} of soil) levels of fertilization. Nevertheless, there was an increased concentration of fungi found with treatments of T22 in Opt N (5.3×10^5 CFU g^{-1} of soil), and with both biostimulants under the High N (average value of 5.9×10^5 CFU g^{-1} of soil) fertilization regime. Finally, the response of the fungal populations to inoculations with either *Trichoderma*, in the High N fertilization regime, were always greater than the CFU found in the uninoculated control treatment (1.6×10^5 CFU g^{-1} of soil), with GV41 exhibiting higher fungal concentrations (6.5×10^5 CFU g^{-1} of soil) than T22 (5.3×10^5 CFU g^{-1} of soil).

Crop Yield and Nutritional Quality

No visible chlorosis and/or necrosis symptoms were observed in both leafy vegetables a consequence of *Trichoderma* inoculation with T22 and GV41 (**Supplementary Figure S1**). *Trichoderma*-based biostimulants positively affected both lettuce and rocket yield in the unfertilized plots (Low N), but the effect of the fungal inocula on the production of the leafy vegetable crops was variable when N fertilizer was applied (**Figure 1**). The highest marketable lettuce fresh yield was recorded with both biostimulant inoculations when plants were supplied with the optimal N dose (90 kg N ha^{-1}), but no significant differences were noted between the two *Trichoderma* strains (496 g FW $plant^{-1}$, on the average). Lettuce grown in absence of N fertilization demonstrated a significant yield increase when inoculated with GV41 (by 34% and 24% for total and marketable weight, respectively), and a moderate increase with T22 (by 16% and 17% for total and marketable weight, respectively).

No microbial-biostimulant effect was recorded for rocket under optimal N supply (60 kg N ha^{-1}), with an average marketable yield of 4,275 g m^{-2}, while the GV41 treatment increased total yield of rocket by 33% in comparison to the non-inoculated (NoT) control in non-fertilized plots (**Figure 1**).

Crop productivity for both leafy vegetables under the excess N fertilization regime (180 kg N ha^{-1} and 120 kg ha^{-1} for lettuce and rocket, respectively) was similar to that observed under the optimal N treatments. Interestingly, in the High N condition, a reduction in lettuce yield was noted with the GV41 inoculation (by 11% and 19% for total and marketable yields, respectively) in comparison to Opt N. Similarly, with rocket grown under High N conditions, the GV41 biostimulant also significantly reduced yield in comparison to NoT and to T22 treatments.

In both leafy vegetables the total ascorbic acid content (including ascorbic and dehydroascorbic acid), which constitute and important component of functional quality of horticultural crops (Kyriacou and Rouphael, 2018), was significantly influenced by the *Trichoderma*-based biostimulants, N availability rate and their interaction (**Figure 2**). For instance, the total ascorbic acid content in lettuce was relatively low, ranging from 7.3 to 22.7 mg 100 g^{-1} FW, whereas the total ascorbic acid content in rocket was much greater, ranging from 26.4 to 72.7 mg 100 g^{-1} FW (**Figure 2**). In the lettuce experiment, the highest values of total ascorbic acid was recorded in unfertilized treatment irrespective of *Trichoderma* inoculation. In the case of rocket, the highest total ascorbic acid content was recorded in plants inoculated with GV41 at the optimal N level (**Figure 2**). Our results also demonstrated that inoculation with GV41 under both N fertilization treatments incurred a significant increase in total ascorbic acid compared with T22 and NoT plants, whereas an opposite trend was observed in unfertilized treatment (**Figure 2**).

Nitrate Content and Total N Uptake

Neither N fertilization rate nor microbial-based biostimulant had a significant effect on nitrate content in either lettuce (average 1,614.2 mg kg^{-1} fw) or rocket (average 3,251.2 mg kg^{-1} fw). The nitrate content found in both leafy vegetables was below the maximum limit established by the European Commission regulations N° 1881/2006 and 1258/2011 for these two horticultural commodities: 2,000 mg kg^{-1} fw for lettuce and 6,000–7,000 mg kg^{-1} fw for rocket.

N-uptake in plants from unfertilized treatments was significantly greater in rocket (101 kg N ha^{-1}) than in lettuce (68 kg N ha^{-1}), and the microbial-based biostimulant effect under different N fertilization rates exhibited different responses in the two tested leafy vegetables. In absence of external N inputs, inoculation with GV41 permitted a higher uptake of native mineral N (by 51% and 59% in lettuce and rocket, respectively), whereas the inoculation with T22 treatment increased N uptake (by 33%) only in lettuce (**Figure 3**). Furthermore, inoculation with GV41 and T22 strains under optimal N conditions incurred a significant increase in lettuce N uptake by 32% and 12%, respectively, whereas the influence of *Trichoderma* inoculation in rocket was minimal or not significant (**Figure 3**). Finally, under High N fertilization the effect of microbial-based biostimulants on N uptake of lettuce and rocket was strongly limited (**Figure 3**). Soil mineral N content (in the 0–20 cm top layer) measured at lettuce harvest was not significantly affected by either biostimulant or fertilizer applications. Nevertheless, a tendency to higher soil mineral N content in the fertilized treatments (24 and 25 ppm for Opt N and High N, respectively) was recorded in comparison to the unfertilized control (20 ppm on the average). No differences were detected between Low N

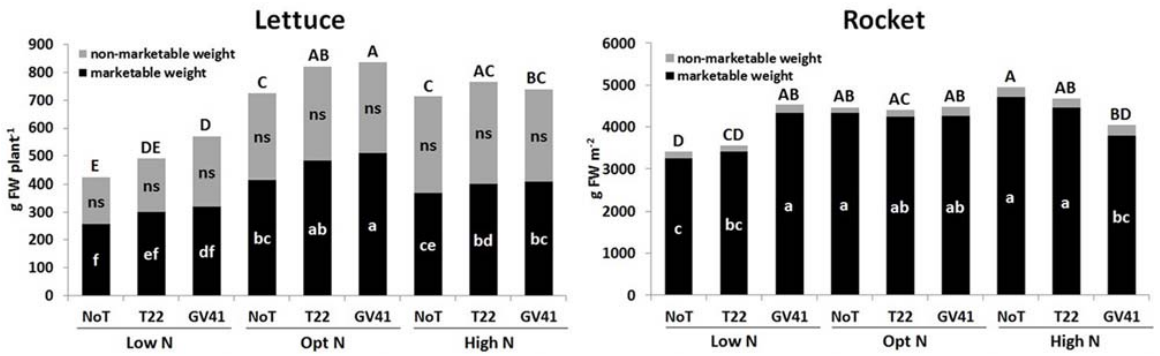


FIGURE 1 | Total, marketable and non-marketable yield of lettuce and rocket as affected by N fertilization doses and *Trichoderma* inoculation. Low N, non-fertilized conditions (low N availability) for both crops; Opt N, optimal N fertilization, amounting to 90 kg N ha^{-1} and 60 kg N ha^{-1} for lettuce and rocket, respectively; High N, supraoptimal N fertilization, amounting to 180 and 120 kg N ha^{-1} for lettuce and rocket, respectively; NoT, non-inoculated control; T22, *T. harzianum* strain T22; GV41, *T. virens* strain GV41. Different letters indicate different means according to LSD test ($P < 0.05$). Lower cases are referred to the marketable weight, while upper cases are referred to the total weight production.

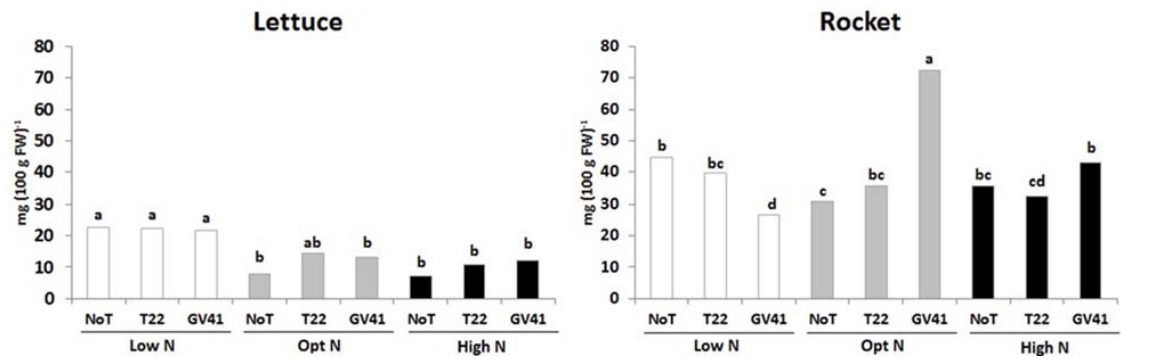


FIGURE 2 | Ascorbic acid content in aboveground lettuce and rocket tissues as affected by N fertilization doses and *Trichoderma* inoculation. Low N, non-fertilized conditions (low N availability) for both crops; Opt N, optimal N fertilization, amounting to 90 kg N ha^{-1} and 60 kg N ha^{-1} for lettuce and rocket, respectively; High N, supraoptimal N fertilization, amounting to 180 and 120 kg N ha^{-1} for lettuce and rocket, respectively; NoT, non-inoculated control; T22, *T. harzianum* strain T22; GV41, *T. virens* strain GV41. Different letters indicate different means according to LSD test ($P < 0.05$).

and fertilized treatments for rocket, with an average soil mineral N content of 12 ppm in the soil.

Ion Content in Leaves

The K^+ and Ca^{2+} and in particular, the PO_4^{3-} concentrations in lettuce were highly influenced by N fertilization, *Trichoderma*-based biostimulant and the interaction (**Table 1**). The highest phosphate and calcium concentrations were recorded on lettuce plants inoculated with GV41 under Opt N (**Table 1**). Independent to N fertilization level, the greatest mineral accumulation (phosphate, potassium, and calcium) was observed in leaves of lettuce plants inoculated with GV41, then T22 (for PO_4^{3-}). Furthermore, in lettuce, the concentration of phosphate and potassium significantly increased in correspondence to an increase in the fertilization dose (from 0 to 180 kg N ha^{-1}), with no significant differences noted between the Opt N and High N doses.

Neither *Trichoderma* inoculation nor N fertilization dose had a significant effect on PO_4^{3-} concentration in rocket leaves (**Table 1**). The K^+ concentration in rocket leaves was only affected by the interaction of the biostimulants with the N fertilization level, with the highest values recorded in the unfertilized treatment. Finally, the *Trichoderma* inoculation, averaged over N fertilization dose, affected the Ca^{2+} concentration in rocket leaf tissue which was 14% greater than non-inoculated and T22 inoculated plants (**Table 1**).

Prokaryotic and Eukaryotic Populations in Soil

In general, the DGGE profiles of prokaryotes in the lettuce soil samples produced 21–27 bands (**Figure 4A**). Statistical analysis on the position and intensity of the bands allowed the classification of three major clusters clearly associated to the three N doses applied in the experiment: Cluster 1: Low N; Cluster 2: Opt N; Cluster 3: High N (**Figure 4A**). Clusters 2 and 3 demonstrated a similarity of 66%, while Cluster 1 was only 57% similar to the assembly of these two groups. It was interesting

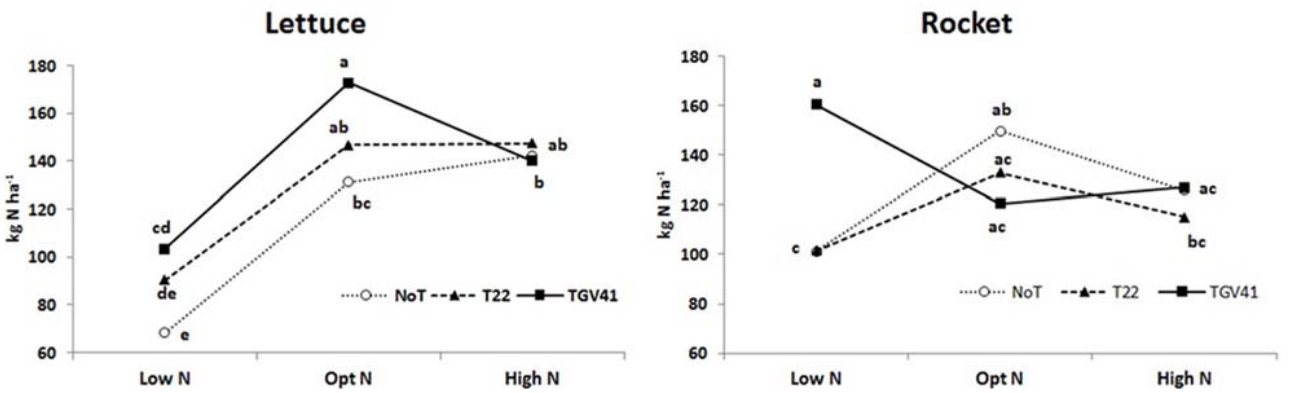


FIGURE 3 | Total N uptake for lettuce and rocket as affected by different N doses and *Trichoderma* inoculation. Low N, non-fertilized conditions (low N availability) for both crops; Opt N, optimal N fertilization, amounting to 90 kg N ha^{-1} and 60 kg N ha^{-1} for lettuce and rocket, respectively; High N, supraoptimal N fertilization, amounting to 180 and 120 kg N ha^{-1} for lettuce and rocket, respectively; NoT, non-inoculated control; T22, *T. harzianum* strain T22; GV41, *T. virens* strain GV41. Different letters indicate different means according to LSD test ($P < 0.05$).

TABLE 1 | Analysis of variance and mean comparisons for mineral concentration of lettuce and rocket leaves grown with different N fertilization dose-sand inoculated with *Trichoderma*-based biostimulants.

Treatments	Lettuce			Rocket		
	PO_4^{3-}	K^+	Ca^{2+}	PO_4^{3-}	K^+	Ca^{2+}
	g kg^{-1} (d.w.)			g kg^{-1} (d.w.)		
NoT	2.9 b	67.6 b	8.3 b	11.0	60.1	24.9 b
T22	4.1 a	75.5 ab	9.5 ab	10.0	53.6	23.8 b
GV41	4.8 a	79.2 a	10.0 a	10.7	57.3	27.7 a
Significance	**	*	*	n.s.	n.s.	*
Low N	2.9 b	69.0 b	8.5	10.0	60.7	24.3
Opt N	4.7 a	80.5 a	10.8	10.4	55.4	26.6
High N	4.2 a	72.7 ab	8.5	11.3	55.0	25.4
Significance	**	*	n.s.	n.s.	n.s.	n.s.
Low N-NoT	2.2 d	56.3 b	6.7 c	9.6	57.3 bc	21.8 b
Low N -T22	3.1 cd	71.9 a	9.1 ac	9.6	55.7 bc	23.3 b
Low N -GV41	3.4 cd	78.8 a	9.7 ab	10.7	69.0 a	27.7 a
Opt N-NoT	3.4 c	75.1 a	10.0 ab	10.8	61.7 ab	27.4 a
Opt N-T22	4.8 b	81.6 a	10.9 ab	9.4	50.6 c	24.6 ab
Opt N-GV41	5.9 a	84.8 a	11.5 a	11.0	53.7 bc	27.8 a
High N-NoT	3.0 cd	71.4 a	8.4 bc	12.4	61.2 ab	25.4 ab
High N-T22	4.4 b	73.0 a	8.5 bc	11.0	54.5 bc	23.4 b
High N-GV41	5.1 ab	73.8 a	8.7 bc	10.5	49.2 c	27.5 a
Significance	**	*	*	n.s.	*	*

*Different letters within each column indicate significant differences between treatments according to LSD test ($P < 0.05$). n.s., non-significant differences, *significant differences at $P \leq 0.05$ and ** at $P \leq 0.01$.*
Low N, non-fertilized conditions (low N availability) for both crops; Opt N, optimal N fertilization, amounting to 90 kg N ha^{-1} and 60 kg N ha^{-1} for lettuce and rocket, respectively; High N, supraoptimal N fertilization, amounting to 180 and 120 kg N ha^{-1} for lettuce and rocket, respectively; NoT, non-inoculated control; T22, T. harzianum strain T22; GV41, T. virens strain GV41.

to note that within each of the major clusters delineated by the N levels, the subgroupings of the prokaryotes were always similar and determined by the *Trichoderma* applications. In fact, the GV41 inoculation greatly influenced the composition of prokaryotes in the lettuce rhizosphere, always indicating a separation of the populations from this treatment as a distinct group. In all nitrogen fertilization conditions, the prokaryotes from NoT and T22 inoculations paired to form sub-clusters at high similarity levels of 90%, 84%, and 86% (for Low N, Opt N, and High N, respectively). The NoT and T22 sub-clusters were less related to GV41, differentiating at 72%, 83%, and 79% similarity in Low N, Opt N, and High N conditions, respectively.

DGGE of the eukaryotic populations in the lettuce rhizosphere showed a diverse profile, that did not clearly correspond to the fertilization, or was not consistently associated to the *Trichoderma* treatments in the groupings formed (**Figure 4B**). Cluster analysis identified two major groups, with a similarity of 40%: Cluster 1 included Low N-T22 and Low N-GV41; whereas, Cluster 2 was comprised of Low N-NoT, Opt N-NoT, Opt N-T22, Opt N-GV41, High N-NoT, High N -T22, High N-GV41. The major Cluster 1, included the non-fertilized soils inoculated with T22 and GV41, that contained the lowest eukaryote biodiversity (eight bands); whereas the Cluster 2 grouped eukaryotes from the N-fertilized soils and Low N-NoT sample produced a number of bands ranging from 17 to 23.

The *Trichoderma* inoculations strongly influenced the composition of eukaryotic populations in the absence of nitrogen fertilization in lettuce whereby inoculations with T22 and GV41 formed a sub-cluster that was not highly comparable (50% of similarity). Instead, under both Opt N and High N a sub-cluster was observed that included NoT and T22 with a high level of similarity (85–86%), that was divergent from GV41 (**Figure 4B**).

The prokaryotic populations in rocket rhizo-soil samples exhibited 29–43 bands in DGGE profiles (**Figure 5A**). The cluster analysis of the DGGE identified three major clusters that grouped on the basis of nitrogen fertilization applied, similar to results observed in the rhizosphere of lettuce Clusters 2 and 3, with nitrogen treatments, grouping at a similarity level of 67%, while the Cluster 1 was only 56% similar to these groups. The effect of *Trichoderma* inoculations to the prokaryote

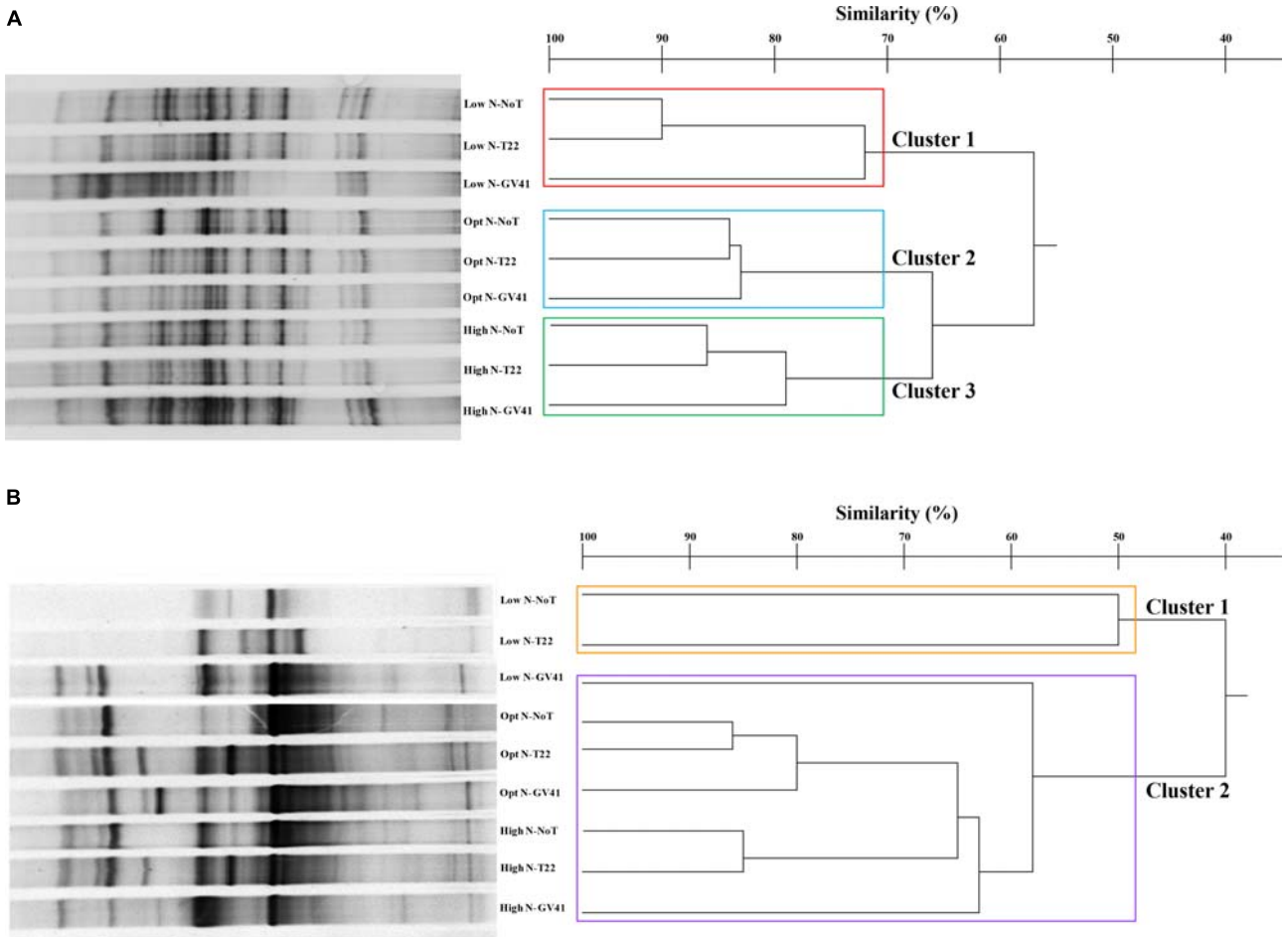


FIGURE 4 | DGGE profiles and dendrogram showing the degree of similarity (%) of the PCR-DGGE profiles of the prokaryotes **(A)** and eukaryotes **(B)** in rhizo-soil samples from lettuce. Low N, non-fertilized conditions (low N availability); Opt N, optimal N fertilization, amounting to 90 kg N ha^{-1}; High N, supraoptimal N fertilization, amounting to 180 kg N ha^{-1}; NoT, non-inoculated control; T22, *T. harzianum* strain T22; GV41, *T. virens* strain GV41.

populations was variable depending upon the N inputs. T22 and GV41 inoculations showed a similarity of 84% under optimal fertilization and 75% under no fertilization, always separating distinctively from the no biostimulant treatment (**Figure 5A**). Under High N, T22 and NoT formed a sub-cluster with a 86% similarity that was 80% similar to GV41.

Once again, the eukaryotic populations showed a different complex of DGGE patterns (11–26 bands) compared to prokaryotes, that was similar to that noted with lettuce (**Figure 5B**). Statistical analysis indicated two major clusters that separated at a similarity level of 50% into a group with low N conditions (Cluster 1: Low N-NoT, Low N-T22, Low N-GV41), and a grouping with the fertilized conditions (Cluster 2: Opt N-NoT, Opt N-T22, Opt N-GV41, High N-NoT, High N-T22, High N-GV41) (**Figure 5B**).

The effect of *Trichoderma* inoculation on eukaryotic populations of rocket rhizosphere was more marked than that observed with the prokaryotes. Both strains T22 and GV41 formed a sub-cluster at a similarity level of 72% under non-fertilized conditions, that was 56% similar to NoT. On the contrary, in both fertilization levels, a sub-cluster including non-inoculated and T22, grouped at 89% and 80% similarity for Opt N and High N, respectively, that separated them from GV41 at a similarities of 73% and 62% for Opt N and High N, respectively.

DISCUSSION

Implications of *Trichoderma*-Based Biostimulant for Crop Productivity and Leaf Quality

The application of *Trichoderma*-based agricultural products in vegetable cropping systems is on the rise, stimulated by the increasing interest of growers, extension specialists, and scientists to improve crop productivity in a sustainable manner (Woo et al., 2014; Colla et al., 2015c; Lorito and Woo, 2015). A biostimulant effect was noted in the greenhouse experiment with lettuce, demonstrating that inoculations of *Trichoderma* significantly

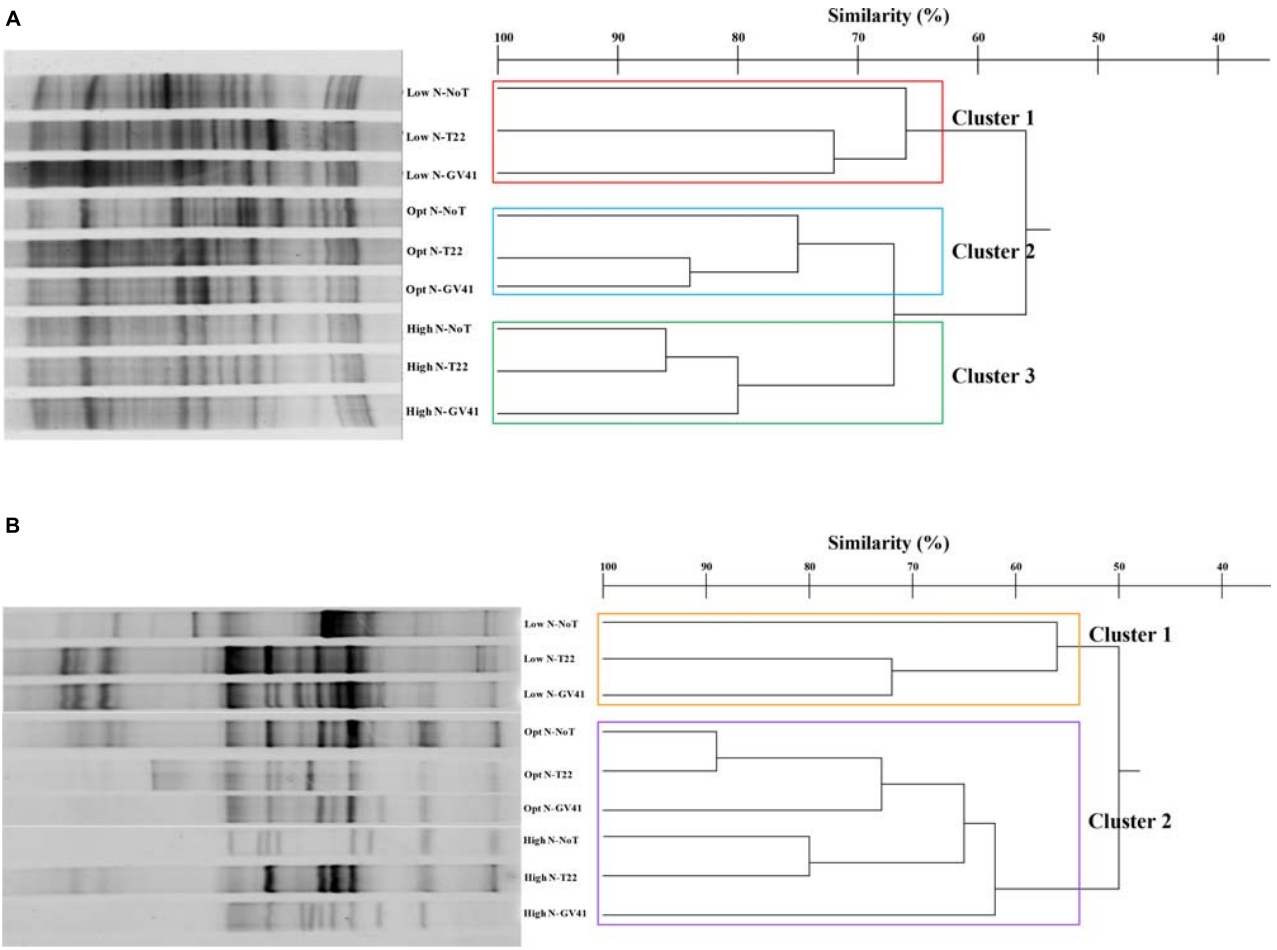


FIGURE 5 | DGGE profiles and dendrogram showing the degree of similarity (%) of the PCR-DGGE profiles of the prokaryotes **(A)** and eukaryotes **(B)** in rhizo-soil samples from rocket. Low N, non-fertilized conditions (low N availability); Opt N, optimal N fertilization, amounting to 60 kg N ha^{-1}; High N, supraoptimal N fertilization, amounting to 120 kg N ha^{-1}; NoT, non-inoculated control; T22, *T. harzianum* strain T22; GV41, *T. virens* strain GV41.

increased the yield in all fertilization conditions. In particular, the application of strain GV41 had the most positive effect on production under both low and optimal N availability.

On the other hand, in rocket, the inoculations with the same *Trichoderma* did not produce a generalized advantage in yield that was comparable to that observed in lettuce. The treatment with GV41 produced a significant increase in yield, but only under low N input fertilization. This demonstrates that the response of the two tested leafy vegetables to *Trichoderma*-based biostimulants is not global, but complex, depending upon the crop botanical family (*Asteraceae* versus *Brassicaceae*), as well as the *Trichoderma* strains. Our findings are in agreement with the results of several research groups who have demonstrated that the increase in plant growth after applications of *Trichoderma* depends on the plant species, and also the genotypes (varieties) (Baker, 1988; Ousley et al., 1994). For instance, Ousley et al. (1994) demonstrated that the inoculation with six strains of *Trichoderma* improved significantly the growth parameters of lettuce, whereas Baker (1988) who tested the same strains on radish and pea, reported a limited increase in crop performance. Furthermore, Tucci et al. (2011) showed that the inoculation of cultivated and wild tomato varieties with *T. atroviride* and *T. harzianum* improved crop performance in a genotype-dependent manner.

The increased plant growth and productivity of lettuce or rocket grown in varying nutrient conditions may involve several biological and chemical processes in the rhizosphere that are attributable to *Trichoderma* mechanisms of action, for example, the modification of soil nutrient availability or the modulation of root growth. Some *Trichoderma* species-strains are producers of secondary metabolites that are hormone-like, and the exudation of molecules with auxin-similar activity, small peptides or volatile organic compounds in the rhizosphere are known to have plant growth promotion effects (Vinale et al., 2008; Sofo et al., 2012; Pelagio-Flores et al., 2017). Specifically, in regards to the present investigations, *T. harzianum* strain T22 is not a noted producer of bioactive compounds that stimulate plant growth, however, applications of T22 in the rhizosphere is able to activate plant metabolic processes involving phytohormones (auxins/cytokinins) in treated plants, i.e., in cherries GiSeLa6,

that improve above-ground plant and below-ground root system development (Harman, 2000; Sofo et al., 2012). Some *T. virens* are known to produce auxinic compounds that effect plant growth (Pelagio-Flores et al., 2017), but in the case of *T. virens* GV41 (used in the present study), this has not been observed, and this strain has been more noted as a producer of gliotoxin, a compound that may protect the plant due to its inhibitory effects on microbes that damage plant health (Vargas et al., 2014).

Interestingly, another factor that appears to have an important role on the *Trichoderma* stimulation effect of the plant is the regulation of pH in the rhizosphere by this fungus. *Trichoderma* can modify the acidity of the root environment that influences the plant response in the interaction with the fungus, relative to the production of active hormonal compounds in diverse genomic and functional processes (Sofo et al., 2012; Pelagio-Flores et al., 2017). Furthermore, recent work has demonstrated that root exudates produced by plants in response to biotic and abiotic stresses can act as chemoattractants to *Trichoderma*, thus regulating interactions with this microbe that result in beneficial plant effects (Lombardi et al., 2018).

Other mechanisms that *Trichoderma* uses that produce positive effects on plant growth include: plant disease control and induced resistance to pathogen attack; modulation of the root system architecture (total root length, density and branching) to increase area and absorption capacity for water and nutrient uptake; plus the assimilation/solubilization of macronutrients (P) and micronutrients (Fe, Mn, and Zn), possibly influenced by pH changes moderated by *Trichoderma* that can alter nutrient availability and uptake, thus boosting plant growth and yield (Altomare et al., 1999; Vinale et al., 2008; Hermosa et al., 2012; Sofo et al., 2012; Li et al., 2015; Pelagio-Flores et al., 2017). In our experiments, no effect on soil pH in the rhizosphere was recorded with the *Trichoderma* treatments whereby no significant difference was noted in soil samples analyzed at the beginning of the trial (average pH = 6.9 $\pm$ 0.2) and those analyzed at time of harvest for either crops (average pH = 7.1 $\pm$ 0.3). However, it is also possible that there was a temporal change in soil pH initially during the first weeks of crop growth that favored plant nutrient uptake, however, the measurements conducted during the experiment were not sufficient enough to detect this variation. Finally, *Trichoderma*, as a member of soil microbe community, has a role in the multitude complex of physical, chemical, and biological interactions that occur and greatly influence the agro-environment, and consequently effects crop health and productivity.

It is well established that *Brassicaceae* species, such as rocket, are noted for their negative effects on many soil microbes, bacteria, and fungi, due to the production of numerous inhibitory compounds such as glucosinolates that are released in the soil of the rhizosphere (Majchrzak et al., 2010; Ackroyd and Ngouajio, 2011; Szczygłowska et al., 2011). Further, the limited effect of *Trichoderma*-based biostimulants on rocket could be associated to several intrinsic preharvest factors, such as crop rotation, the N availability conditions, as well as the method of application of the inoculum, that may influence the establishment and colonization of these beneficial microbes in the rhizosphere. *Brassicaceae* are well known for their high phytoextraction ability, as proven by their frequent use as N catch-crops in cropping systems to limit nitrate leaching during the rainy season (Justes et al., 2012). Due to its high N uptake, rocket can be also used for biomonitoring of mineral N flush after fertilizer application (León Castro and Whalen, 2016). The better nutritional status (higher K^+, Ca^{2+}, PO_4^{3-}), as well as the higher N uptake recorded for rocket in our greenhouse trial supports this characteristic. It is possible that the high root-uptake capacity of rocket buffered the *Trichoderma* biostimulation in fertilized plots, a positive effect that was only noted in the non-fertilized treatment.

The differential beneficial effects of *Trichoderma* on the two tested crops could also be attributed to the length of the growing cycle (57 and 28 days for lettuce and rocket, respectively) in which the influence of the fungus on the plant is limited by the time of its interaction with the treated crop. The longer duration of the lettuce crop cycle also provides more time for root system development and diversification, influencing the ability of the microbial-based biostimulant to better colonize the crop rhizosphere (Alonso-Ramírez et al., 2014), thus increasing the efficiency of both *Trichoderma* biostimulants under different N availability conditions. On the contrary, the shorter growing cycle, such as that of rocket, may only result in biostimulation if the beneficial microbe has a higher performance (i.e., GV41), due to an ability to more rapidly colonize the rhizosphere under N limiting conditions. Furthermore, successful results with biostimulant depend not only on the crop rotation, strains and length of the growing cycle, but also on the method of application. In the present study, different modes of biostimulant application were used due to the different methods of initial crop establishment in the field, lettuce by transplant of seedlings and rocket by direct seeding. *Trichoderma* was applied directly to the roots of lettuce, thus permitting immediate contact of the fungal inoculum with the roots, whereas the application to rocket was by seed treatment, which is a reduced inoculant load and requires the development of a rhizosphere competent strain in order to colonize the developing root system. The different response of lettuce and rocket in terms of crop productivity to the *Trichoderma* inoculations in the present study confirmed the importance of the application method (Kleifeld and Chet, 1992; Brotman et al., 2013; Gupta et al., 2014; Mahmood et al., 2016).

Although the overall increase in leafy vegetables yield with *Trichoderma*-treatments is positive for the producers, enhancing product quality by microbial inoculation is a challenging and important issue to address in light of the growing interest of vegetable consumers, in particular those of fresh produce, to obtain products with a higher nutrient content (Kyriacou et al., 2016; Kyriacou and Rouphael, 2018). In the current study, the improvement in plant quality characteristics with *Trichoderma* inoculation was species-dependent since the beneficial effect was only observed on rocket. Interestingly, rocket plants treated with GV41 under optimal N and to a lesser extent under high N conditions, showed enhanced biosynthesis and accumulation of plant compounds, i.e., ascorbic acid, indicating that *Trichoderma*-based biostimulants can positively modulate plant secondary metabolism, as well as increase the phytochemicals that provide health benefits to the consumer (López-Bucio et al., 2015). Additional research is required

in order to enhance understanding on the molecular and physiological mechanisms behind the capability of these plant beneficial microbes to increase phytochemicals that contribute to our well-being, and to select promising *Trichoderma* strains that are able to enhance the nutraceutical properties of horticultural commodities.

Implications of *Trichoderma*-Based Biostimulant for Minimizing N-Input in Horticultural Systems

Nitrogen fertility management plays a key role in sustaining crop growth and ensuring food safety in horticultural-cropping systems by increasing N availability to both crops and soil microflora (Geisseler and Scow, 2014). N inputs must be adjusted accordingly, not only to crop requirements, but also to native soil fertility (Sanz-Cobena et al., 2017) in order to avoid an excess in N accumulation in vegetable produce such as nitrates. In addition, the N surplus unavailable to crops is prone to leaching to ground and surface water, that represents a serious environmental concern, especially in coarse soils with a high vulnerability to this phenomenon (Nitrates Directive 91/676/EEC). The soil in our experiment demonstrated a good fertility level due to its coarse texture, very low carbonate content and medium total C and N content. According to the empirical equation proposed by Remy and Marin-Lafleche (1974), this soil has a medium annual mineralization rate of 1.9% $year^{-1}$ corresponding to an annual availability of 100 kg N ha^{-1} $year^{-1}$ (calculated on a 0.40 m layer with a soil density of 1.2 Mg m^{-3}). This calculation does not discriminate between different climatic conditions, nevertheless it is well known that in the Mediterranean region, the N mineralization peak under irrigated greenhouse conditions occurs during the spring–summer period (the same period of our experimental trial) when soil temperature and moisture are optimal for soil microbiota involved in the nitrification process. Analysis of crop N uptake in our trials confirmed this base level of nitrogen in the native soils, i.e., unfertilized plots, with values ranging from 70 to 100 kg N ha^{-1} for lettuce and rocket, respectively. In the absence of microbial-based biostimulants, application of fertilizers at the optimal (recommended) level increased crop yields in comparison to the unfertilized control, while excess fertilization did not modify crop growth and N uptake pattern. The nitrate content was not significantly influenced by either the biostimulant or the N applications, having values below the maximum threshold of nitrates (2,500 mg NO_3^- kg^{-1} FW) imposed by Commission Regulation (EC) No 1881/2006 for lettuce as well as for rocket (6,000 mg NO_3^- kg^{-1} FW in summer-grown rocket or 7,000 mg NO_3^- kg^{-1} FW in winter-grown rocket).

According to these results, excessive N availability (due to fertilizer doses and soil native fertility) does not represent a problem to food safety for lettuce or rocket production in our study area, since the nitrate content in both leafy vegetables was below the maximum limit established by the European Commission. This result could be associated to the high nitrate-reductase activity of leafy vegetables grown in Mediterranean area during the spring–summer growing period, usually coupled with a high photosynthetic activity (Larios et al., 2001).

As shown by several authors the effect of mineral fertilization can be significantly lowered in soils with a high native fertility, leading to slight variations in plant response and nutrient uptake (Montemurro et al., 2006; Alluvione et al., 2013). This was also observed in the present experiments where no difference in soil mineral N availability was recorded between the unfertilized control (Low N plot) and fertilized treatments to the harvest of both lettuce and rocket. Our hypothesis is that the high soil organic matter mineralization rates occurring under greenhouse conditions increased native soil mineral N availability, buffering the effect of N fertilization, as the difference in N root uptake activity of the two crops and with *Trichoderma* biostimulation emerges especially in the unfertilized conditions. This was true for both lettuce and rocket whose N uptake significantly increased with GV41-based biostimulant in unfertilized plots, pushing up values to the same level of non-inoculated plants in fertilized plots. In addition, GV41 significantly increased N uptake in lettuce under Opt N fertilization, as well as in rocket in unfertilized conditions, highlighting that specific strains, i.e., *T. virens* strain GV41, can be applied to increase NUE and reduce N surplus in horticultural systems. From these results stems a very interesting opportunity for designing a lettuce-rocket crop rotation program aimed at managing N input–output resources. This objective could be achieved by performing an optimal N fertilization only on the first crop, in this case lettuce, combined with applications of the GV41 biostimulant to both crops. In this manner, the second vegetable production (non-fertilized rocket) could take advantage of the residual N fertility remaining in the soil from the previous crop, thus maintaining vegetable yields close to those of fertilized systems and minimizing the N leaching risk due to soil N surplus. It must be pointed out that, in order to be sustainable, this approach should be applied in high fertility soils, similar to that tested in our trial, or when a long-term strategy to maintain soil fertility is adopted (i.e., organic farming). Finally, the marked positive effect of *Trichoderma*-based biostimulants under Low N availability conditions highlighted that GV41 inoculation can be a viable strategy to increase yield and nutrient uptake of leafy crops in low fertility soils.

Implications of *Trichoderma*-Based Biostimulants for Modulating Soil Microbial Communities

A culture-independent approach was employed to obtain a qualitative picture of the effect of the chemical and biological amendments on resident soil microbial community in the rhizosphere of lettuce and rocket. The cluster analysis demonstrated that prokaryotic populations were affected more by nitrogen fertilization levels than by the *Trichoderma* inoculations. Different nitrogen treatments can determine different plant mediated effects that induce changes in the associated microbial communities of the rhizosphere (Giagnoni et al., 2016). Li et al. (2016) reported that different levels of N significantly influenced the distribution and composition of the bacterial

community in a long-term monoculture of lettuce, as well, N inputs can significantly alter microbial diversity in vegetable production systems. This is true for the regulation of N-fixing activity of soil microbiota, particularly at a transcriptional level, that is affected by factors such as the application of different nitrogen sources (Saraf et al., 2011). The metabolically expensive nitrogenase system of free-living diazotrophs is more sensitive to levels of ammonium rather than to nitrate, since the process is repressed when the cellular level of fixed nitrogen is sufficiently high (Ruppel and Merbach, 1995; Saraf et al., 2011). Application of N fertilizers can stimulate plant root exudation allowing a better utilization of nutrients by microbiota (Sørensen, 1997). The microbiota in agro-ecosystems may respond differently to N fertilization and this could lead to unpredictable results on N-fixing activity in the rhizosphere (Saraf et al., 2011). Recently the disturbance of some bacterial populations in response to chemical fertilization, especially those involved in the nitrogen cycling, is a phenomenon that has been well documented (Gupta et al., 2015; Fiorentino et al., 2016).

Trichoderma inoculations also affected the bacterial community structure in the rhizosphere of both lettuce and rocket, although the effect of the two microbial-based biostimulants differed according to vegetable crops and/or to N inputs. The composition of prokaryotic populations was more influenced by *T. virens* GV41 than by *T. harzianum* T22 in the lettuce rhizosphere, whereas a clear separation was observed in the GV41 treated soils, in all nitrogen fertilization conditions. However, in the rocket rhizosphere, the same distribution of prokaryotes was detected only under High N-fertilization with GV41; while at both Low N and Opt N inputs, soils were strongly affected by both microbial biostimulants. Gupta et al. (2016) observed significant changes of the qualitative profiling of microbial communities in biological-treated rhizosphere samples, indicating a noticeable impact of bacterial and fungal inocula on native microbial populations.

The combination of chemical and biological amendments exerted different effects on the native eukaryote communities, depending upon the plant species. It is known that diverse crops and plant compounds can strongly affect soil microbial communities, and these factors are the primary players that have a direct impact on soil microbial dynamics (Larkin, 2008). Similarly, the main driver for fungal population diversity was the fertilization, regardless of N levels, since a clear separation was observed among non-treated and N treated soils for both leafy vegetables, indicating that fungi were less sensitive to the N inputs than bacteria. Moreover, *Trichoderma* inoculation influenced the eukaryotic population of the rhizosphere in the non-fertilized treatment, and this effect was more pronounced especially in lettuce since the fungal biodiversity was strongly reduced. Overall, results emphasized that the eukaryotic communities were affected differently by soil fertilization than the prokaryotic populations. The differences in native bacterial and fungal rhizosphere populations could be due to the fact that bacteria are generally more sensitive to any environmental changes since they have a much shorter generation time than fungi, and therefore can respond more quickly to soil amendments (Lazcano et al., 2013).

The highly complex prokaryotic DGGE patterns compared to eukaryotic profiles in rocket soil samples highlighted that the rhizosphere was mainly dominated by bacteria, as supported by previous studies (Bardgett, 2005; Gupta et al., 2016). However, under optimal fertilization, an increase in fungal biodiversity was observed probably due to the fact that the optimal N fertilization exerted a growth and activity stimulation effect on resident fungal populations.

CONCLUSION

The increased pressure on vegetable farmers to maximize crop productivity, but at the same time limit the use of synthetic N fertilizers, represents an important challenge for plant scientists in research to develop novel strategies to secure agricultural production in a sustainable manner (by increasing NUE). *T. virens* GV41 was proven to be the best performing microbial-biostimulant in terms of crop growth and nutrient uptake on both lettuce and rocket, demonstrating the differential effect of different fungal inoculants on crop production. This biostimulant increased fertilizer N use efficiency of lettuce, favored the uptake of native soil N by both lettuce and rocket, and also improved N uptake by roots under low N availability conditions. The application of *Trichoderma*-based products to leafy vegetable cropping systems can be considered to increase crop yield, nutrient use efficiency and crop quality (i.e., mineral and ascorbic acid content). The effect of the biostimulants can significantly vary depending on the duration of the crop cycle, method of inoculant application, and the specific root uptake efficiency of the plant species. Additional experiments should be performed in diverse pedoclimatic conditions in order to further investigate the important issues that need to be addressed for developing efficient crop management practices. Moreover, it is important to determine the impact that such inoculants may have on the native microbial populations and investigate the manner that these applications may act, not only as direct biostimulants of crops, but also as beneficial components of the plant microbiome to improve productivity. The findings of this study can have a positive impact in the design of horticultural crop rotation systems aimed at minimizing N inputs, reducing the consequent risks to the environment and establishing sustainable agriculture.

AUTHOR CONTRIBUTIONS

NF defined the experimental protocol, carried out soil and vegetable samplings, coordinated crop N analyses, performed the data elaboration and statistical analyses of crop performance (yield and N uptake), and was significantly involved in writing the whole manuscript. YR defined the experimental protocol, coordinated the ion and ascorbic acid analyses, performed the data elaboration and statistical analyses of crop quality, and

was significantly involved with GC (for the agronomic part) in writing the whole manuscript. VV and IR carried out the molecular characterization (DGGE) of soil microorganisms, carried out data elaboration, and drafted the manuscript for this part. OP coordinated microbiological (DGGE) analyses and drafted the manuscript for this part. SW contributed to the setting-up of the experiment, was involved in results interpretation of microbiological data, and in writing of the manuscript for this part. ADR and LG carried out the experiments as well as soil and vegetable samplings, performed soil and plant analyses, and the first elaborations of agronomic data. NL prepared *Trichoderma*-biostimulants and carried out plant inoculations, and she collaborated in manuscript preparation for the microbiological section. MN performed the microbial counts and statistical analysis, and he collaborated in manuscript preparation.

FUNDING

Research was supported by funding from the following projects: Regione Campania cod. id. 07-C00001819, B.U.R.C. n. 23 del 14 aprile 2009, L.R. 28/03/2002 N. 5. ADF Project (#20090291, MIDAS #000850), Subcontract agreement PYT YR 2010 and MAReA Project – MIUR, PON3PE_00106_1, Prot. N. 732 - 04/03/2014.

ACKNOWLEDGMENTS

We would like to thank Mauro Senatore, Vincenzo Cenvinzo, and Eugenio Cozzolino for their technical assistance in the field. Moreover, we would also like to thank Roberto Maiello, Maria Giordano, Christophe El-Nakhel, and Sabrina Nocerino for their support in managing soil and plant chemical analyses.

SUPPLEMENTARY MATERIAL

The Supplementary Material for this article can be found online at: https://www.frontiersin.org/articles/10.3389/fpls.2018.00743/full#supplementary-material

FIGURE S1 | Photos showing representative lettuce and rocket plants as affected by different N doses and *Trichoderma* inoculation. Low N, non-fertilized conditions (low N availability) for both crops; Opt N, optimal N fertilization, amounting to 90 kg N ha^{-1} and 60 kg N ha^{-1} for lettuce and rocket, respectively; High N, supraoptimal N fertilization, amounting to 180 and 120 kg N ha^{-1} for lettuce and rocket, respectively; NoT, non-inoculated control; T22, *T. harzianum* strain T22; GV41, *T. virens* strain GV41.

REFERENCES

Ackroyd, V. J., and Ngouajio, M. (2011). Brassicaceae cover crops affect seed germination and seedling establishment in cucurbit crops. *Horttechnology* 21, 525–532.

Alluvione, F., Fiorentino, N., Bertora, C., Zavattaro, L., Fagnano, M., Chiarandà, F. Q., et al. (2013). Short-term crop and soil response to C-friendly strategies in two contrasting environments. *Eur. J. Agron.* 45, 114–123. doi: 10.1016/j.eja.2012.09.003

Alonso-Ramírez, A., Poveda, J., Martín, I., Hermosa, R., Monte, E., and Nicolás, C. (2014). Salicylic acid prevents *Trichoderma harzianum* from entering the vascular system of roots. *Mol. Plant Pathol.* 15, 823–831. doi: 10.1111/mpp.12141

Altomare, C., Norvell, W. A., and Harman, G. E. (1999). Solubilization of phosphates and micronutrients by the plant-growth-promoting and biocontrol fungus *Trichoderma harzianum* Rifai. *Appl. Environ. Microbiol.* 65, 2926–2933.

Baker, R. (1988). *Trzchoderma* spp. as plant-growth stimulants. *Crit. Rev. Microbiol.* 7, 97–106. doi: 10.3109/07388558809150724

Bardgett, R. D. (2005). *The Biology of Soil: A Community and Ecosystem Approach*, eds S. U. M. J. Crawley, C. Little, T. R. E. Southwood, and S. Ulfstrand. Oxford: Oxford University Press. doi: 10.1093/acprof:oso/9780198525035.001.0001

Belyaeva, O. N., Haynes, R. J., and Sturm, E. C. (2012). Chemical, physical and microbial properties and microbial diversity in manufactured soils produced from co-composting green waste and biosolids. *Waste Manage.* 32, 2248–2257. doi: 10.1016/j.wasman.2012.05.034

Bremner, J. M. (1965). "Total nitrogen," in *Methods of Soil Analysis*, eds C. A. Black, D. D. Evans, I. L. White, and L. E. Ensminger (Madison, WI: American Society of Agronomy), 1149–1178.

Brotman, Y., Landau, U., Cuadros-Inostroza, Á., Takayuki, T., Fernie, A. R., Chet, I., et al. (2013). *Trichoderma*-plant root colonization: escaping early plant defense responses and activation of the antioxidant machinery for saline stress tolerance. *PLoS Pathog.* 9:e1003221. doi: 10.1371/journal.ppat.1003221

Cocolin, L., Bisson, L. F., and Mills, D. A. (2000). Direct profiling of the yeast dynamics in wine fermentations. *FEMS Microbiol. Lett.* 189, 81–87. doi: 10.1016/S0378-1097(00)00257-3

Colla, G., Hoagland, L., Ruzzi, M., Cardarelli, M., Bonini, P., Canaguier, R., et al. (2017). Biostimulant action of protein hydrolysates: unraveling their effects on plant physiology and microbiome. *Front. Plant Sci.* 8:2202. doi: 10.3389/fpls.2017.02202

Colla, G., Hye-Ji, K., Kyriacou, M. C., and Rouphael, Y. (2018). Nitrates in fruits and vegetables. *Sci. Hortic.* 237, 221–238. doi: 10.1016/j.scienta.2018.04.016

Colla, G., Nardi, S., Cardarelli, M., Ertani, A., Lucini, L., Canaguier, R., et al. (2015a). Protein hydrolysates as biostimulants in horticulture. *Sci. Hortic.* 196, 28–38. doi: 10.1016/j.scienta.2015.08.037

Colla, G., Rouphael, Y., Bonini, P., and Cardarelli, M. (2015b). Coating seeds with endophytic fungi enhances growth, nutrient uptake, yield and grain quality of winter wheat. *Int. J. Plant Prod.* 9, 171–189.

Colla, G., Rouphael, Y., Di Mattia, E., El-Nakhel, C., and Cardarelli, M. (2015c). Co-inoculation of *Glomus intraradices* and *Trichoderma atroviride* acts as a biostimulant to promote growth, yield and nutrient uptake of vegetable crops. *J. Sci. Food Agric.* 95, 1706–1715. doi: 10.1002/jsfa.6875

Colla, G., Rouphael, Y., Mirabelli, C., and Cardarelli, M. (2011). Nitrogen-use efficiency traits of mini-watermelon in response to grafting and nitrogen-fertilization doses. *J. Plant Nutr. Soil Sci.* 174, 933–941. doi: 10.1002/jpln.201000325

Colla, G., Suãrez, C. M. C., Cardarelli, M., and Rouphael, Y. (2010). Improving nitrogen use efficiency in melon by grafting. *HortScience* 45, 559–565.

Colonna, E., Rouphael, Y., Barbieri, G., and De Pascale, S. (2016). Nutritional quality of ten leafy vegetables harvested at two light intensities. *Food Chem.* 199, 702–710. doi: 10.1016/j.foodchem.2015.12.068

Contreras-Cornejo, H. A., Macías-Rodríguez, L., Beltrán-Peña, E., Herrera-Estrella, A., and López-Bucio, J. (2011). *Trichoderma*-induced plant immunity likely involves both hormonal- and camalexin-dependent mechanisms in *Arabidopsis thaliana* and confers resistance against necrotrophic fungus *Botrytis cinerea*. *Plant Signal. Behav.* 6, 1554–1563. doi: 10.4161/psb.6.10.17443

Contreras-Cornejo, H. A., Macias-Rodriguez, L., Cortes-Penagos, C., and López-Bucio, J. (2009). *Trichoderma virens*, a plant beneficial fungus, enhances biomass production and promotes lateral root growth through an auxin-dependent mechanism in *Arabidopsis*. *Plant Physiol.* 149, 1579–1592. doi: 10.1104/pp.108.130369

du Jardin, P. (2015). Plant biostimulants: definition, concept, main categories and regulation. *Sci. Hortic.* 196, 3–14. doi: 10.1016/j.scienta.2015.09.021

Fiorentino, N., Ventorino, V., Bertora, C., Pepe, O., Moschetti, G., Grignani, C., et al. (2016). Changes in soil mineral N content and abundances of bacterial

communities involved in N reactions under laboratory conditions as predictors of soil N availability to maize under field conditions. *Biol. Fertil. Soils* 52, 523–537. doi: 10.1007/s00374-016-1095-7

Geisseler, D., and Scow, K. M. (2014). Long-term effects of mineral fertilizers on soil microorganisms - A review. *Soil Biol. Biochem.* 75, 54–63. doi: 10.1016/j.soilbio.2014.03.023

Giagnoni, L., Pastorelli, R., Mocali, S., Arenella, M., Nannipieri, P., and Renella, G. (2016). Availability of different nitrogen forms changes the microbial communities and enzyme activities in the rhizosphere of maize lines with different nitrogen use efficiency. *Appl. Soil Ecol.* 98, 30–38. doi: 10.1016/j.apsoil.2015.09.004

Gupta, R., Bisaria, V. S., and Sharma, S. (2015). Effect of agricultural amendments on *Cajanus cajan* (pigeon pea) and its rhizospheric microbial communities - A comparison between chemical fertilizers and bioinoculants. *PLoS One* 10:e0132770. doi: 10.1371/journal.pone.0132770

Gupta, R., Bisaria, V. S., and Sharma, S. (2016). Response of rhizospheric bacterial communities of *Cajanus cajan* to application of bioinoculants and chemical fertilizers: a comparative study. *Eur. J. Soil Biol.* 75, 107–114. doi: 10.1016/j.ejsobi.2016.02.008

Gupta, V. K., Schmoll, M., Herrera-Estrella, A., Upadhyay, R. S., Druzhinina, I., and Tuohy, M. G. (2014). *Biotechnology and Biology of Trichoderma*. Oxford: Elsevier Science and Technology. doi: 10.1016/C2012-0-00434-6

Harman, G. E. (2000). Myths and dogmas of biocontrol: changes in perceptions derived from research on *Trichoderma harzianum* T-22. *Plant Dis.* 84, 377–393. doi: 10.1094/PDIS.2000.84.4.377

Harman, G. E., Howell, C. R., Viterbo, A., Chet, I., and Lorito, M. (2004). *Trichoderma* species - Opportunistic, avirulent plant symbionts. *Nat. Rev. Microbiol.* 2, 43–56. doi: 10.1038/nrmicro797

Hermosa, R., Viterbo, A., Chet, I., and Monte, E. (2012). Plant-beneficial effects of *Trichoderma* and of its genes. *Microbiology* 158, 17–25. doi: 10.1099/mic.0.052274-0

Howell, C. R. (2003). Mechanisms employed by *Trichoderma* species in the biological control of plant diseases: USDA/ARS south. *Plains Agric. Res. Cent.* 87:7. doi: 10.1094/PDIS.2003.87.1.4

Justes, E., Beaudoin, N., Bertuzzi, P., Charles, R., Constantin, J., Dürr, C., et al. (2012). "The use of cover crops to reduce nitrate leaching: effect on the water and nitrogen balance and other ecosystem services," in *Proceedings of the International Fertiliser Society Meeting*, Cambridge, 4–43.

Kampfenkel, K., Van Montagu, M., and Inzé, D. (1995). Extraction and determination of ascorbate and dehydroascorbate from plant tissue. *Anal. Biochem.* 225, 165–167. doi: 10.1006/abio.1995.1127

Kleifeld, O., and Chet, I. (1992). *Trichoderma harzianum* - interaction with plants and effect on growth response increased number of blooms in Periwinkles. *Plant Soil* 144, 267–272. doi: 10.1007/BF00012884

Kurtzman, C. P., and Robnett, C. J. (1998). Identification and phylogeny of ascomycetous yeasts from analysis of nuclear large subunit (26S) ribosomal DNA partial sequences. *Antonie Van Leeuwenhoek* 73, 331–371. doi: 10.1023/A:1001761008817

Kyriacou, M. C., and Rouphael, Y. (2018). Towards a new definition of quality for fresh fruits and vegetables. *Sci. Hortic.* 234, 463–469. doi: 10.1016/j.scienta.2017.09.046

Kyriacou, M. C., Rouphael, Y., Di Gioia, F., Kyratzis, A., Serio, F., Renna, M., et al. (2016). Micro-scale vegetable production and the rise of microgreens. *Trends Food Sci. Technol.* 57, 103–115. doi: 10.1016/j.tifs.2016.09.005

Lace, B., Genre, A., Woo, S., Faccio, A., Lorito, M., and Bonfante, P. (2015). Gate crashing arbuscular mycorrhizas: *in vivo* imaging shows the extensive colonization of both symbionts by *Trichoderma atroviride*. *Environ. Microbiol. Rep.* 7, 64–77. doi: 10.1111/1758-2229.12221

Larios, B., Aguera, E., De la Haba, P., Perez-Vicente, R., and Maldonado, J. M. (2001). A short-term exposure of cucumber plants to rising atmospheric CO_2 increases leaf carbohydrate content and enhances nitrate reductase expression and activity. *Planta* 212, 305–312. doi: 10.1007/s004250000395

Larkin, R. P. (2008). Relative effects of biological amendments and crop rotations on soil microbial communities and soilborne diseases of potato. *Soil Biol. Biochem.* 40, 1341–1351. doi: 10.1016/j.soilbio.2007.03.005

Lazcano, C., Gómez-Brandón, M., Revilla, P., and Domínguez, J. (2013). Short-term effects of organic and inorganic fertilizers on soil microbial community structure and function: a field study with sweet corn. *Biol. Fertil. Soils* 49, 723–733. doi: 10.1007/s00374-012-0761-7

León Castro, L., and Whalen, J. K. (2016). Ion exchange membranes as an indicator of soil mineral nitrogen availability and nitrogen uptake by arugula (*Eruca sativa* L.) in soils amended with green manure. *Biol. Agric. Hortic.* 32, 206–220. doi: 10.1080/01448765.2016.1163293

Li, J. G., Shen, M. C., Hou, J. F., Li, L., Wu, J. X., and Dong, Y. H. (2016). Effect of different levels of nitrogen on rhizosphere bacterial community structure in intensive monoculture of greenhouse lettuce. *Sci. Rep.* 6:25305. doi: 10.1038/srep25305

Li, R. X., Cai, F., Pang, G., Shen, Q. R., Li, R., and Chen, W. (2015). Solubilisation of phosphate and micronutrients by *Trichoderma harzianum* and its relationship with the promotion of tomato plant growth. *PLoS One* 10:e0130081. doi: 10.1371/journal.pone.0130081

Lombardi, N., Vitale, S., Turrà, D., Reverberi, M., Fanelli, C., Vinale, F., et al. (2018). Root exudates of stressed plants stimulate and attract *Trichoderma* soil fungi. *Mol. Plant Microbe Interact.* doi: 10.1094/MPMI-12-17-0310-R [Epub ahead of print].

López-Bucio, J., Pelagio-Flores, R., and Herrera-Estrella, A. (2015). *Trichoderma* as biostimulant: exploiting the multilevel properties of a plant beneficial fungus. *Sci. Hortic.* 196, 109–123. doi: 10.1016/j.scienta.2015.08.043

Lorito, M., and Woo, S. L. (2015). "Discussion agronomic," in *Principles of Plant-Microbe Interactions*, ed. B. Lugtenberg (Berlin: Springer International Publishing), 345–353. doi: 10.1007/978-3-319-08575-3_36

Lorito, M., Woo, S. L., Harman, G. E., and Monte, E. (2010). Translational research on *Trichoderma*: from 'omics to the field. *Annu. Rev. Phytopathol.* 48, 395–417. doi: 10.1146/annurev-phyto-073009-114314

Mahmood, A., Turgay, O. C., Farooq, M., and Hayat, R. (2016). Seed biopriming with plant growth promoting rhizobacteria: a review. *FEMS Microbiol. Ecol.* 92, 1–14. doi: 10.1093/femsec/fiw112

Majchrzak, B., Kurowski, T. P., Wachowska, U., and Jaźwińska, E. (2010). Changes in soil microbial communities as a result of growing *Brassicaceae* crops. *Acta Agrobot.* 63, 161–169. doi: 10.5586/aa.2010.018

Mar Vázquez, M., César, S., Azcón, R., and Barea, J. M. (2000). Interactions between arbuscular mycorrhizal fungi and other microbial inoculants (*Azospirillum*, *Pseudomonas*, *Trichoderma*) and their effects on microbial population and enzyme activities in the rhizosphere of maize plants. *Appl. Soil Ecol.* 15, 261–272. doi: 10.1016/S0929-1393(00)00075-5

Montemurro, F., Maiorana, M., Ferri, D., and Convertini, G. (2006). Nitrogen indicators, uptake and utilization efficiency in a maize and barley rotation cropped at different levels and sources of N fertilization. *Field Crops Res.* 99, 114–124. doi: 10.1016/j.fcr.2006.04.001

Muyzer, G., De Waal, E., and Uitterlinden, A. (1993). Profiling of complex microbial populations by denaturing gradient gel electrophoresis analysis of polymerase chain reaction-amplified genes coding for 16S rRNA. *Appl. Environ. Microbiol.* 59, 695–700.

Ousley, M. A., Lynch, J. M., and Whipps, J. M. (1994). Potential of *Trichoderma* spp. as consistent plant growth stimulators. *Biol. Fertil. Soils* 17, 85–90. doi: 10.1007/BF00337738

Pelagio-Flores, R., Esparza-reynoso, S., Garnica-vergara, A., López-Bucio, J., and Herrera-estrella, A. (2017). *Trichoderma*-induced acidification is an early trigger for changes in *Arabidopsis* root growth and determines fungal phytostimulation. *Front. Plant Sci.* 8:822. doi: 10.3389/fpls.2017.00822

Remy, J. C., and Marin-Lafleche, A. (1974). L'analyse de terre: realisation d'un programme d'interpretation automatique. *Ann. Agron.* 25, 607–632.

Rouphael, Y., Cardarelli, M., Bonini, P., and Colla, G. (2017a). Synergistic action of a microbial-based biostimulant and a plant derived-protein hydrolysate enhances lettuce tolerance to alkalinity and salinity. *Front. Plant Sci.* 8:131. doi: 10.3389/fpls.2017.00131

Rouphael, Y., Colla, G., Giordano, M., El-Nakhel, C., Kyriacou, M. C., and De Pascale, S. (2017b). Foliar applications of a legume-derived protein hydrolysate elicit dose-dependent increases of growth, leaf mineral composition, yield and fruit quality in two greenhouse tomato cultivars. *Sci. Hortic.* 226, 353–360. doi: 10.1016/j.scienta.2017.09.007

Rouphael, Y., De Micco, V., Arena, C., Raimondi, G., Colla, G., and De Pascale, S. (2017c). Effect of *Ecklonia maxima* seaweed extract on yield, mineral composition, gas exchange, and leaf anatomy of zucchini squash grown under saline conditions. *J. Appl. Phycol.* 29, 459–470. doi: 10.1007/s10811-016-0937-x

Rouphael, Y., Franken, P., Schneider, C., Schwarz, D., Giovannetti, M., Agnolucci, M., et al. (2015). Arbuscular mycorrhizal fungi act as biostimulants in horticultural crops. *Sci. Hortic.* 196, 91–108. doi: 10.1016/j.scienta.2015.09.002

Ruppel, S., and Merbach, W. (1995). Effects of different nitrogen sources on nitrogen fixation and bacterial growth of *Pantoea agglomerans* and *Azospirillum* sp. in bacterial pure culture: an investigation using 15N2 incorporation and acetylene reduction measures. *Microbiol. Res.* 150, 409–418. doi: 10.1016/S0944-5013(11)80023-6

Saitou, N., and Nei, M. (1987). The neighbor-joining method: a new method for reconstructing phylogenetic trees. *Mol. Biol. Evol.* 4, 406–425. doi: 10.1093/oxfordjournals.molbev.a040454

Sanz-Cobena, A., Lassaletta, L., Aguilera, E., del Prado, A., Garnier, J., Billen, G., et al. (2017). Strategies for greenhouse gas emissions mitigation in Mediterranean agriculture: a review. *Agric. Ecosyst. Environ.* 238, 5–24. doi: 10.1016/j.agee.2016.09.038

Saraf, M., Rajkumar, S., and Saha, T. (2011). "Perspective of PGPR in agro-ecosystems," in *Bacteria in Agrobiology: Crop Ecosystems*, ed. D. K. Maheshwari (Berlin: Springer-Verlag), 361–385. doi: 10.1007/978-3-642-18357-7s_13

Sofo, A., Tataranni, G., Xiloyannis, C., Dichio, B., and Scopa, A. (2012). Direct effects of *Trichoderma harzianum* strain T-22 on micropropagated shoots of GiSeLa6® (*Prunus cerasus*× *Prunus canescens*) rootstock. *Environ. Exp. Bot.* 76, 33–38. doi: 10.1016/j.envexpbot.2011.10.006

Sørensen, J. (1997). "The rhizosphere as a habitat for soil microorganisms," in *Modern Soil Microbiology*, eds J. D. van Elsas, J. T. Trevors, and E. M. H. Wellington (New York, NY: Marcel Dekker, Inc.), 21–45.

Szczygłowska, M., Piekarska, A., Konieczka, P., and Namieśnik, J. (2011). Use of *Brassica* plants in the phytoremediation and biofumigation processes. *Int. J. Mol. Sci.* 12, 7760–7771. doi: 10.3390/ijms12117760

Tilman, D., Cassman, K. G., Matson, P. A., Naylor, R., and Polasky, S. (2002). Agricultural sustainability and intensive production practices. *Nature* 418, 671–677. doi: 10.1038/nature01014

Tucci, M., Ruocco, M., De Masi, L., De Palma, M., and Lorito, M. (2011). The beneficial effect of *Trichoderma* spp. on tomato is modulated by the plant genotype. *Mol. Plant Pathol.* 12, 341–354. doi: 10.1111/j.1364-3703.2010.00674.x

Vargas, W. A., Mukherjee, P. K., Laughlin, D., Wiest, A., Moran-diez, M. E., and Kenerley, C. M. (2014). Role of gliotoxin in the symbiotic and pathogenic interactions of *Trichoderma virens*. *Microbiology* 4, 2319–2330. doi: 10.1099/mic.0.079210-0

Ventorino, V., Parillo, R., Testa, A., Aliberti, A., and Pepe, O. (2013). Chestnut biomass biodegradation for sustainable agriculture. *BioResources* 8, 4647–4658. doi: 10.15376/biores.8.3.4647-4658

Ventorino, V., Parillo, R., Testa, A., Viscardi, S., Espresso, F., and Pepe, O. (2016). Chestnut green waste composting for sustainable forest management: microbiota dynamics and impact on plant disease control. *J. Environ. Manage.* 166, 168–177. doi: 10.1016/j.jenvman.2015.10.018

Ventorino, V., Romano, I., Pagliano, G., Robertiello, A., and Pepe, O. (2018). Pre-treatment and inoculum affect the microbial community structure and enhance the biogas reactor performance in a pilot-scale biodigestion of municipal solid waste. *Waste Manage.* 73, 69–77. doi: 10.1016/j.wasman.2017.12.005

Ventorino, V., Sannino, F., Piccolo, A., Cafaro, V., Carotenuto, R., and Pepe, O. (2014). *Methylobacterium populi* VP2: plant growth-promoting bacterium isolated from a highly polluted environment for polycyclic aromatic hydrocarbon (PAH) biodegradation. *Sci. World J.* 2014:931793. doi: 10.1155/2014/931793

Vinale, F., Sivasithamparam, K., Ghisalberti, E. L., Marra, R., Barbetti, M. J., Li, H., et al. (2008). A novel role for *Trichoderma* secondary metabolites in the interactions with plants. *Physiol. Mol. Plant Pathol.* 72, 80–86. doi: 10.1016/j.pmpp.2008.05.005

Viscardi, S., Ventorino, V., Duran, P., Maggio, A., De Pascale, S., Mora, M. L., et al. (2016). Assessment of plant growth promoting activities and abiotic stress tolerance of *Azotobacter chroococcum* strains for a potential use in sustainable agriculture. *J. Soil Sci. Plant Nutr.* 16, 848–863. doi: 10.4067/S0718-95162016005000060

Woo, S. L., Ruocco, M., Vinale, F., Nigro, M., Marra, R., Lombardi, N., et al. (2014). *Trichoderma*-based products and their widespread use in agriculture. *Open Mycol. J.* 8, 71–126. doi: 10.2174/1874437001408010071

Xu, G., Fan, X., and Miller, A. J. (2012). Plant nitrogen assimilation and use efficiency. *Annu. Rev. Plant Biol.* 63, 153–182. doi: 10.1146/annurev-arplant-042811-105532

Conflict of Interest Statement: The authors declare that the research was conducted in the absence of any commercial or financial relationships that could be construed as a potential conflict of interest.

frontiers in Plant Science

ORIGINAL RESEARCH
published: 20 June 2018
doi: 10.3389/fpls.2018.00861

Effects of an Animal-Derived Biostimulant on the Growth and Physiological Parameters of Potted Snapdragon (*Antirrhinum majus* L.)

Giuseppe Cristiano[1], *Emanuele Pallozzi*[2], *Giulia Conversa*[3], *Vincenzo Tufarelli*[4] and *Barbara De Lucia*[1]*

[1] Department of Agricultural and Environmental Sciences, University of Bari Aldo Moro, Bari, Italy, [2] Institute of Agro-Environmental & Forest Biology, National Research Council (CNR), Rome, Italy, [3] Department of the Science of Agriculture, Food and Environment, University of Foggia, Foggia, Italy, [4] Section of Veterinary Science and Animal Production, Department of DETO, University of Bari Aldo Moro, Bari, Italy

To assess the effect a new animal-derived biostimulant on the growth, root morphology, nitrogen content, leaf gas exchange of greenhouse potted snapdragon, three treatments were compared: (a) three doses of biostimulant (D): 0 (D_0 or control), 0.1 ($D_{0.1}$), and 0.2 g L^{-1} ($D_{0.2}$); (b) two biostimulant application methods (M): foliar spray and root drenching; (c) two F_1 *Antirrhinum majus* L. hybrids (CV): "Yellow floral showers" and "Red sonnet." The treatments were arranged in a randomized complete-block design with four replicates, with a total of 48 experimental units. Plant height (+11%), number of shoots (+20%), total shoot length (+10%), number of leaves (+33%), total leaf area (+29%), and number of flowers (+59%) and total aboveground dry weight (+13%) were significantly increased by the biostimulant application compared to the control, regardless of the dose. The lowest dose resulted in the best effect on the ground plant dry weight (+38%) and, in order to the root system, on total length (+55%), average diameter (+36%), volume (+66%), tips (+49%), crossings (+88%), forks (+68%), projected (+62%), and total surface area (+28%). Compared to the control, plants treated with the biostimulant significantly enhanced leaf (+16%) and root (+8%) nitrogen content, photosynthetic rate (+52%), transpiration rate (+55%), and stomatal conductance (+81%), although there were no changes in dark-adapted chlorophyll fluorescence. Differences in the application method were not evident in the aboveground morphological traits, except in the plant shoot number (root drenching: +10%). The foliar spray compared to root drenching had a significant effect only on flower dry weight (3.8 vs. 3.0 g $plant^{-1}$). On the other hand, root drenching had a positive effect on ground dry weight (2.7 vs. 2.3 g $plant^{-1}$), root morphology, leaf-N and root-N content (+3%), transpiration rate (+21%), stomatal conductance (+40%), concentration of CO_2 in intracellular spaces (+11%), as well as on the efficiency of Photosystem II (+11%). A higher pot quality was obtained in "Red sonnet" compared to "Yellow floral shower." Based on our findings, applying the biostimulant to potted snapdragon at the lowest dose, as part of a fertilizing regime, improves the crop quality in an agro-environmental sustainable way.

Keywords: biomass, gas exchange, protein hydrolysates, root architecture, sustainable production

OPEN ACCESS

Edited by:
Youssef Rouphael,
Università degli Studi di Napoli Federico II, Italy

Reviewed by:
Chiara Cirillo,
Università degli Studi di Napoli Federico II, Italy
Jan Graefe,
Leibniz-Institut für Gemüse- und Zierpflanzenbau (IGZ), Germany

***Correspondence:**
Barbara De Lucia
barbara.delucia@uniba.it

Specialty section:
This article was submitted to Crop and Product Physiology, a section of the journal Frontiers in Plant Science

Received: *09 February 2018*
Accepted: *04 June 2018*
Published: *20 June 2018*

Citation:
Cristiano G, Pallozzi E, Conversa G, Tufarelli V and De Lucia B (2018) Effects of an Animal-Derived Biostimulant on the Growth and Physiological Parameters of Potted Snapdragon (Antirrhinum majus L.). Front. Plant Sci. 9:861. doi: 10.3389/fpls.2018.00861

INTRODUCTION

Biostimulants are environmental-friendly substances that can increase crop yield by acting on plant metabolism (Yakhin et al., 2017), thus improving nutrient use efficiency (Vernieri et al., 2006; De Pascale et al., 2017) and affecting both root growth (Zeljkovic et al., 2010; De Lucia and Vecchietti, 2012) and root architecture (Yazdani et al., 2014). They can have both a direct or indirect effect on plants. They can alter the biological, biochemical, and physical properties of the soil (Rouphael et al., 2017a,c), enhance the performance of plants under abiotic stress (Van Oosten et al., 2017) and they can also impact on the overall transcriptome profile by modifying the plant metabolome (Battacharyya et al., 2015). Biostimulants are used with root drenching or foliar spray application (Kunicki et al., 2010), in addition to fertilizers to boost their action (Mugnai et al., 2008).

Biostimulants are composed of bioactive compounds (Calvo et al., 2014; Du Jardin, 2015) such as amino acids, peptides, humic substances, seaweed extracts, and other beneficial elements (Colla et al., 2014; Nardi et al., 2016). Protein hydrolysates (PHs) are an important group of biostimulants, with a high content of peptides and amino acids, and therefore they display a positive effect on crop performance (Colla et al., 2017). Both PHs from animal- and plant-derived raw materials (Colla et al., 2015), act when applied at low rates (Zhang et al., 2003; Kauffman et al., 2007; Kunicki et al., 2010; Ertani et al., 2016).

Compared to plant PH biostimulants, animal PH biostimulants have a higher nitrogen content ranging from 9 to 16% of total dry matter, and they are released more gradually (Polo et al., 2006). Polo and Mata (2018), evaluating the effects of different doses of an enzymatically hydrolyzed PH biostimulant (Pepton) compared to a seaweed biostimulant (Acadian) on cherry tomatoes, showed that both biostimulants provide amino acids (much more in case of Pepton) and minerals (mainly iron in case of Pepton and sulfur and boron in case of Acadian) that enhanced growth and yield. Glycine and proline are the most abundant amino acids in collagen-based biostimulants, while glutamic acid is dominant in vegetal-based biostimulants (Baroccio et al., 2017). The production process is extremely important in determining the final PHs composition too: chemical hydrolysis lowers the tryptophan content; on the other hand enzymatic hydrolysis, combined with a temperature of 60°C, reduces such amino acid losses (Tuomisto and Teixeira de Mattos, 2011). A few cases of phytotoxicity and plant growth depression have been found after using commercial animal PH biostimulants, which could be due to incorrect product concentration and/or sub-optimal field conditions (Ruiz et al., 2000; Cerdán et al., 2009; Lisiecka et al., 2011). The correct application of animal PH biostimulants could be a way of both decreasing the use of mineral fertilizers and reducing the disposal of animal-processing wastes.

Bulgari et al. (2015) reported that biostimulants enhance both vegetative and blooming performance in the greenhouse cultivation of bedding plants. Among ornamental bedding plants, snapdragon (*Antirrhinum majus* L., *Plantaginaceae* family), known also for its medical properties (Bulír, 2009), is one of the most important floricultural plants used as cut flowers, pot plants and landscaping purposes (Carter and Grieve, 2008; Asrar et al., 2012). Thus, the fine tuning of agronomical protocols aimed to improve both the plant growth and quality is of great interest in the cultivation of snapdragon.

To the best our knowledge, the use of PH-based biostimulants in ornamental potted plant production is still poorly studied, despite their important contribution to the sustainability of ornamental production.

The aim of this research was to assess the effects of animal-derived PH biostimulant on the growth and blooming parameters, nitrogen plant content, root morphology, leaf gas exchange, and chlorophyll fluorescence in greenhouse potted snapdragon plants.

MATERIALS AND METHODS

Experimental Conditions

The experiment was carried out from 1 December 2015 to 14 May 2016 (166 days), in a heated greenhouse, covered with ethyl vinyl acetate (EVA) plastic film, located in Terlizzi (Bari, Southern Italy, 41° 07′ 55″ N, 16° 32′ 45″ E, 180 m a.s.l.), and equipped with environmental control software (Clima control/Pro, Ragusa, Italy). Natural photoperiod, mean air temperature of 20/13°C day/night, and 65% relative humidity inside the greenhouse were maintained throughout the growth stages.

Treatments and Experimental Design

Three treatments were compared: (a) three doses of biostimulant (D): 0 (D_0 or control), 0.1 ($D_{0.1}$), and 0.2 g L^{-1} ($D_{0.2}$); (b) two biostimulant application methods (M): foliar spray and root drenching; (c) two F_1 *Antirrhinum majus* L. hybrids (CV): "Yellow floral showers" (Sakata seeds) and "Red sonnet" (Sakata seeds). The two hybrids, both ideal for spring production, are characterized by a different growth behavior: the first is a *nanum pumilum* with a dwarf habit, while the second has a sturdy branching.

Thirty-day-old healthy and uniform size seedlings with three pairs of leaves were produced in plug trays by a specialized

TABLE 1 | Amino acid content of the animal protein hydrolysates used as a biostimulant on snapdragon plants ("Yellow floral shower" and "Red sonnet").

Amino acid	Content (mg L^{-1})	Amino acid	Content (mg L^{-1})
Glycine	10.90	Serine	1.62
Glutamic acid	6.52	Phenylalanine	1.24
Proline	6.50	Isoleucine	0.86
Hydroxyproline	5.28	Cysteine + Cystine	0.46
Alanine	4.94	Methionine	0.38
Aspartic acid	3.45	Tyrosine	0.34
Arginine	2.98	Threonine	0.34
Leucine	2.21	Valine	0.15
Betaine	2.02	Histidine	<DL
Lysine	1.85	Tryptophan	<DL

DL, detection limit.

TABLE 2 | Main effects of biostimulant dose, application method and cultivar on plant height, shoot number, total shoot length, leaves number, total leaf area, and flowers number in snapdragon plants.

Treatments	Plant height (cm)	Shoots (n/plant)	Total shoot length (cm /plant)	Leaves (n/plant)	Total leaf area (cm^2 /plant)	Flowers (n/plant)
DOSE (g L^{-1}) (D)						
0	33b	5.3b	168.7b	500a	619b	59b
0.1	36a	6.5a	183.3a	670a	789a	88a
0.2	37a	6.2a	190.6a	656a	805a	100a
METHOD (M)						
Foliar spray	36a	5.7b	183.0a	592a	784a	86
Root drenching	35a	6.3a	178.4a	626a	755a	80a
CULTIVAR (CV)						
Yellow floral showers	24b	5.3b	95.6b	416b	496b	53b
Red sonnet	48a	6.7a	266.1a	802a	1044a	112a
SIGNIFICANCE						
D	*	**	*	***	*	***
M	ns	*	ns	*	ns	*
CV	**	*	**	**	***	**
D × M	ns	*	ns	ns	ns	*
D × CV	ns	**	ns	*	ns	*
M × CV	ns	ns	**	**	ns	ns
D × M × CV	ns	ns	*	ns	**	ns

Different letters within each column indicate significant differences according to Duncan's multiple-range test ($P \leq 0.05$).
*ns, non significant, *$P < 0.05$, **0.01, and ***0.001, indicate level of significance.*

nursery. On 1 December 2015 the seedlings were individually transplanted into 2.0 L plastic pots filled with a mixture of potting substrate (Plantaflor®, Germany) and perlite (4:1 v/v). The final substrate mixture was healthy and well drained. It had a pH of 5.7 and an EC of 1.5 dS m^{-1}. Pots were arranged on the ground, covered with mulching film, at a density of 15 plants m^{-2}.

Each experimental unit consisted of eight plants. The treatments were arranged in a randomized complete-block design with four replicates, with a total of 48 experimental units. The biostimulant used was an animal-derived PH product (Hydrostim®, Hydrofert, Italy) obtained through the enzymatic hydrolysis of proteins from erythrocytes (red blood cells) under alkaline conditions (enzymatic kit with producer details), containing 38% organic matter, 10.2% total nitrogen, and 52% amino acids and soluble peptides (**Table 1**).

Biostimulant treatments started 45 days after transplanting (on 14 January) and were applied weekly eight times, until flower bud differentiation (on 3 March).

Foliar spray treatments were applied on the leaves of snapdragon plants at the dose of 150 mL/plant using a hand sprayer. Care was taken to ensure no dripping occurred onto the substrate. Root drenching treatments were performed with the same volume (150 mL/plant) which was applied directly on top of the growing media. The same volume of tap water was applied to the foliar spray and root drenching control plants.

Plants were fertigated starting 1 month after transplantation by a micro-irrigation system with a nutrient solution containing 40 mg L^{-1} N, 8 mg L^{-1} P, 60 mg L^{-1} K, 44 mgL^{-1} Ca, and 8 mg L^{-1} Mg, plus microelements (Fe: 3 mg L^{-1}, Mn: 2 mg L^{-1}, Cu: 0.1 mg L^{-1}, Bo: 0.5 mg L^{-1}) with an electrical conductivity (EC) of 1.2 dS m^{-1} at 25°C and with a pH 6.0 ± 0.1. Apart from the fertilization, cultivation was conducted following the grower's standard practices.

Growth Measurements, Root Morphology, and Ornamental Characteristics

At the end of the cultivation period (166 days after transplantation), the growing medium was gently washed from the roots, and the plants were divided into shoots, leaves, flowers, and roots. These were then oven dried at 70°C until they reached a constant dry weight.

Five plants per treatment were harvested and their height, total shoot length, number of shoots, leaves, and flowers were measured. Total leaf area per plant was also determined by a leaf area meter (Delta-T, Decagon Devices, Pullman, Washington, USA). Total above-ground (shoot+leaves+flowers) and ground fresh and dry weight were calculated.

Fresh root systems were carefully washed with tap water after harvest, spread out on a transparent tray, and scanned at 400 dpi with a scanner (Epson Expression © 10000 XL, Japan). The captured images were then processed using image analysis software (WinRHIZO v. 2005b ©, Regent Instruments Inc., Québec, Canada) to determine total root length, average diameter, volume, tips, forks, crossings, projected, and surface area. For each replicate and treatment, roots of three plants were scanned.

Total leaf and root nitrogen content was measured using 1 g samples of foliar and root tissues, using the Kjeldahl method after 96% H_2SO_4 hot digestion.

Gas Exchange and Chlorophyll Fluorescence Measurements

At the phenological stage of full flowering of plants, leaf gas exchange was measured using an IRGA (LI-6400XT portable gas exchange system, Li-COR, Lincoln, NE, USA), equipped with a 2 cm^2 leaf chamber with a built-in fluorescence system (LI-6400-40, Li-COR, Lincoln, NE, USA).

The chamber air flow and CO_2 concentration were set at 300 $\mu mol\ s^{-1}$ and 400 ppm, respectively. Measurements were performed at the same time of the day (9:00–11:00 and 13:00–15:00 p.m. CET Time) to minimize the physiological changes driven by environmental factors on fully expanded mature leaves of the same age. The fluorescence measurements were performed on the plants with a different order each day and no shift in parameters was noted during the day as we avoided the early and late hours. The plants were never under water stress.

Leaves were exposed to a saturating photosynthetic photon flux density of 1000 $\mu mol\ m^{-2}\ s^{-1}$, at a temperature of 25°C and with the relative humidity within the leaf cuvette ranging between 40 and 60%. The parameters were recorded when the leaves inside the chamber reached a steady-state status. The instrument provides a continuous display of gas exchange parameters. Steady-state was reached when the first decimal number of photosynthesis was stable (and therefore the other parameters). This usually happened after 2–3 min, The internal chamber fan was set to maximum speed producing a fast air turnover inside the small fluorescence chamber.

Photosynthesis (A), stomatal conductance (g_s), and internal concentration of CO_2 (C_i) were calculated by Li-COR software. The electron transport rate (ETR), maximum quantum efficiency of PSII (F_v/F_m) and the actual quantum yield of PSII in illuminated leaves (F'_v/F'_m) were measured following a saturating pulse of light (10,000 $\mu mol\ m^{-2}\ s^{-1}$). The gas exchanges and fluorescence data presented are means from at least eight leaves per replication. F_v/F_m determinations were performed after adapting the leaves to the dark for 30 min. Shading clips were used on the measured leaves, and the plant to be measured was also placed in a dark room.

Statistical Analysis

The data were analyzed by three-way ANOVA using CoStat—Statistics Software. Treatment means were separated with Duncan's multiple range test ($P \leq 0.05$).

RESULTS

Morphological and Qualitative Traits

The main effects of the biostimulant dose, application method and cultivar on morphological characteristics are reported in **Table 2**. The snapdragon height was significantly increased by the biostimulant application compared to untreated plants (+11%), A similar trend was observed in terms of the number of shoots per plant (+20%), total shoot length (+10%), number of leaves per plant (+33%), total leaf area (+29%), and number of flowers per plant (+59%). No significant differences were found by comparing the effects of the two doses of biostimulant. The root application method only had an effect on the plant shoot number (+10%). The cultivar factor also had a significant effect on all morphological characteristics. However, data analysis showed a significant interaction between the application method and cultivar regarding total shoots length and leaves per plant. The interaction between the dose and cultivar was also significant in terms of the number of shoots, leaves, and flowers per plant. The interaction-effect between the dose and application method was significant only in terms of the number of both shoots and flowers per plant (**Table S1**, Supplementary materials), whereas the interaction dose * application method * cultivar was found to be significant for total shoot length and total leaf area (**Table S2**, Supplementary materials).

TABLE 3 | Main effects of biostimulant dose, application method and cultivar on dry weights in various parts of snapdragon plants.

Treatments	Dry weight (g/ plant)				
	Above ground parts				Underground parts
	Shoot	Leaf	Flower	Total	
DOSE (g L^{-1}) (D)					
0	22.7c	8.0b	2.7b	33.4b	2.1c
0.1	25.3a	9.2a	3.8a	38.4 a	2.9a
0.2	23.6b	9.4a	3.7a	36.7a	2.5b
METHOD (M)					
Foliar spray	23.9a	8.7a	3.8a	36.4 a	2.3b
Root drenching	23.9a	9.1a	3.0b	35.9 a	2.7a
CULTIVAR (CV)					
Yellow floral showers	15.8b	6.3b	2.3b	24.4 b	2.0b
Red sonnet	32.0a	11.4a	4.5a	48.1 a	3.1a
SIGNIFICANCE					
D	*	**	**	**	***
M	ns	ns	***	ns	***
CV	***	**	**	**	**
D × M	ns	ns	***	ns	**
D × CV	ns	ns	*	***	*
M × CV	ns	ns	***	ns	*
D × M × CV	ns	ns	**	*	ns

Different letters within each column indicate significant differences according to Duncan's multiple-range test ($P \leq 0.05$).
*ns, non significant, *$P < 0.05$, **0.01, and ***0.001, indicate level of significance.*

Plant Biomass

In the present experiment, snapdragons grown under the biostimulant treatment had a greater content of dry weight in terms of the different above-ground plant parts (shoots, leaves, and flowers) in comparison to the control (**Table 3**). The lowest dose increased the shoot dry weight (+11%) compared to the control, both leaf and flower dry weights were significantly

TABLE 4 | Main effects of biostimulant dose, application method and cultivar on total root length, root diameter, root volume, root tips, root crossings, and root forks in snapdragon plants.

Treatments	Total root length (m 10^3/plant)	Root diameter (mm)	Root volume (cm^3/plant)	Root tips (n 10^3/plant)	Root crossings (n 10^3/plant)	Root forks (n 10^3/plant)
DOSE (g L^{-1}) (D)						
0	3.3c	1.04b	4.60c	21.6c	4.3c	28.3c
0.1	5.1a	1.42a	7.65a	32.1a	8.1a	47.5a
0.2	4.2b	1.02b	5.66b	30.9b	7.3b	41.3b
METHOD (M)						
Foliar spray	3.8b	1.04b	5.29b	26.8b	5.9b	35.0b
Root drenching	4.6a	1.28a	6.65a	29.4a	7.2a	43.0a
CULTIVAR (CV)						
Yellow floral showers	3.4b	1.11b	5.15b	22.8b	5.2b	31.4b
Red sonnet	4.9a	1.21a	6.79a	33.6a	7.9a	46.7a
SIGNIFICANCE						
D	**	**	**	**	**	**
M	**	*	*	*	*	*
CV	**	**	**	**	**	**
D × M	**	**	**	**	**	**
D × CV	**	*	**	**	*	*
M × CV	**	**	*	ns	**	**
D × M × CV	**	**	*	*	**	**

Different letters within each column indicate significant differences according to Duncan's multiple-range test ($P \leq 0.05$).
ns, non significant, $^{*}P < 0.05$ and $^{**}0.01$ indicate level of significance.

increased by the biostimulant application (respectively +16 and 41%).

Applying biostimulant as a foliar method significantly increased the dry weight of the snapdragon flowers compared to the root application method (+27%). The differences between cultivars were highly significant for all the three plant parts, where "Red sonnet" was characterized by the greater dry weight (shoots +102%, leaves +81%, and flowers +96%) than "Yellow flower showers."

Plant total aboveground dry weight was significantly higher in both doses compared to the control: from 0 to 0.1 g L^{-1} dose dry weight increased by 15%, whereas from 0 to 0.2 g L^{-1} by 10%.

Applying the biostimulant at the lowest dose resulted in the best effect on the ground plant dry weight (+38%) compared to control plants. The application method had a highly significant effect only on ground plant dry weight (root vs. foliar application: +17%), whereas the cultivar factor greatly affected both above-ground and ground dry weights, where "Red sonnet" recorded the highest values, 48.1 and 3.1 g, respectively. Examining the different interactions among factors, all interactions were significant for ground dry weight (**Tables S1**, **S3**, **S4**, Supplementary materials); conversely, the only highly significant interaction was detected between the dose and cultivar for above-ground plant dry weight (**Table S2**, Supplementary materials).

The interactions M * CV were highly significant only for flower dry weight (**Table 3**), the same was found for the interaction between dose and application method (**Table S1**, Supplementary materials).

Root Morphology

The application of an animal-derived PH biostimulant to snapdragon plants positively influenced the root morphology compared to the untreated plants (**Tables 4**, **5**, **Figures 1–3**). Applying biostimulant at the dose of 0.1 g L^{-1} resulted in a significant improvement in total root length, average root diameter, and root volume in comparison to control plants, by 55, 36, and 66%, respectively. These significant increases were also seen in terms of root tips, crossings, and forks per plant, with the 0.1 g L^{-1} dose increasing the number of tips by 49%, crossings by 88%, and forks 68%.

Using biostimulants as a drench method significantly enhanced the plant root morphology compared to foliar method; in addition, the "Red sonnet" resulted in the best root system response compared to "Yellow floral showers." The interactions among factors were significant for all the plant traits related to the root morphology, except for root tips in Method * Cultivar (**Table 4**). Regarding the Dose * Method interaction, plants grown in both 0.1 and 0.2 g L^{-1} and treated with the drenching method, resulted in the highest values in root length ($D_{0.1}$: 5.6 m, $D_{0.2}$: 4.8 m; **Figure 1A**), root tip number ($D_{0.1}$: 33.4 10^3, $D_{0.2}$: 33.7 10^3; **Figure 1C**), and crossing number ($D_{0.1}$: 9.6 10^3, $D_{0.2}$: 7.8 10^3; **Figure 1D**). Conversely, for the root average diameter, the best value was recorded only in 0.1 g L^{-1} * Root drenching interaction (1.55 mm, **Figure 1B**).

Significant interactions were detected between biostimulant Dose * Cultivar (**Figure 2**): highest length values were recorded in plants treated with 0.1 g L^{-1} * "Red sonnet" (5.4 m, **Figure 2A**); in addition both 0.1 g L^{-1} * "Yellow floral showers" and 0.1 g

TABLE 5 | Main and interaction effects of biostimulant dose, application method and cultivar for projected and total root surface area in snapdragon plants.

Treatments	Projected root area (cm^2 /plant)	Total root surface area (cm^2 /plant)
DOSE (g L^{-1}) (D)		
0	136.4c	414.2c
0.1	221.0a	697.6a
0.2	172.3b	545.3b
METHOD (M)		
Foliar spray	157.9b	493.0b
Root drenching	195.3a	611.7a
CULTIVAR (CV)		
Yellow Floral Showers	148.2a	468.0b
Red Sonnet	204.9b	636.7a
D × M		
D0 × Foliar spray	132.5d	401.9c
D0 × Root drenching	140.3d	426.5c
D0.1 × Foliar spray	193.4bc	611.1b
D0.1 × Root drenching	248.7a	784.2a
D0.2 × Foliar spray	147.9cd	466.1c
D0.2 × Root drenching	196.8b	624.6b
D × CV		
D0 × Yellow floral showers	95.7d	305.5c
D0 × Red sonnet	177.1b	522.9b
D0.1 × Yellow floral showers	210.8ab	665.6a
D0.1 × Red sonnet	231.3a	729.7a
D0.2 × Yellow floral showers	138.2c	433.1b
D0.2 × Red sonnet	206.5ab	657.6a
M × CV		
Foliar spray × Yellow floral showers	137.9b	432.7b
Root drenching × Red sonnet	158.4b	553.3b
Foliar spray × Yellow floral showers	177.4b	503.3b
Root drenching × Red sonnet	232.1a	720.1a
SIGNIFICANCE		
D	***	***
M	***	***
CV	***	***
D × M	**	***
D × CV	***	***
M × CV	**	***
D × M × CV	ns	***

*Different letters within each column indicate significant differences according to Duncan's multiple-range test ($P \leq 0.05$). ns, non significant, **$P < 0.01$ and ***0.001, indicate level of significance.*

L^{-1} * "Red sonnet" had the highest values in root average diameter (respectively 1.35 and 1.50 mm, **Figure 2B**), for tips: 0.1 g L^{-1}* "Red sonnet" with 39.2 10^3 (**Figure 2C**). Crossing number resulted in the highest values in 0.1 g L^{-1}, irrespective of the cultivars, and in 0.2 g L^{-1}* "Red sonnet" (**Figure 2D**). Between the Method * Cultivar interaction, the same significant trend was found in root drenching * "Red sonnet," in terms of root length (**Figure 3A**), root' diameter (**Figure 3B**), and crossing number (**Figure 3C**).

Table 5 shows that a 0.1 g L^{-1} dose of biostimulant resulted in the best effect on both the projected and total surface root area, compared to the other treatments. The root drenching method significantly increased these traits compared to the foliar spray (respectively 195.3 vs. 157.9 cm^2 and 636.7 vs. 468 cm^2); "Red sonnet" responded more efficiently than "Yellow floral showers."

Significant D * M, D * CV, and M * CV interactions were found for the projected and surface root area. 0.1 g L^{-1}* root drenching plants produced the highest values of both projected and total surface root area (248.7 and 784.2 cm^2 respectively). In both cultivars, 0.1 g L^{-1} treatment showed the highest projected and total surface root area values; the same trend was recorded in 0.2 g L^{-1}* "Red sonnet." Projected and total surface root area were highest in "Red sonnet" plants at 0.2 g L^{-1} rate (respectively 232.1 and 720.1 cm^2).

The interaction D * M * CV was found to be significant for all parameters (**Table S5**, Supplementary materials).

Nitrogen Content

The animal-derived PH biostimulant had a significant effect on total leaf and root nitrogen content in the snapdragon (**Table 6**). Applying the biostimulant improved the nitrogen plant content compared to the control, although no significant differences were found comparing the effects of the two doses of biostimulant applied (foliar-N +16% and root-N +8%). Compared to spraying, drenching led to higher nitrogen values (both foliar-N and root-N +3%). The cultivar factor had a significant effect on plant nitrogen content, where "Red sonnet" recorded the highest values (foliar-N: 406.5 mg kg^{-1} and root-N: 295.8 mg kg^{-1}).

Both the interactions D * M and D * CV were found to be significant only for total leaf-N content (**Tables S1**, **S6**, Supplementary materials).

Leaf Gas Exchange and Chlorophylls Fluorescence

The biostimulant use in snapdragon had a positive influence on the parameters related to the leaf gas exchange (**Table 7**). Irrespectively of the two doses applied, the biostimulant significantly influenced the leaf net photosynthesis (+52%), transpiration rate (+55%), stomatal conductance (0.8%), and concentration of CO_2 in intracellular spaces (+9%), compared to control plants. On the other hand, the electronic transport was significantly higher at 0.1 g L^{-1} of animal-derived biostimulant (+129.1) compared to the other treatments.

The biostimulant method did not affect the plant photosynthetic rate, however it had a significant effect on the transpiration rate (+21%), stomatal conductance (+40%), and concentration of CO_2 in intracellular spaces (+11%), where the highest values occurred using the root application method. With reference to leaf gas exchange parameters, "Yellow floral showers" had significantly higher values in terms of concentration of CO_2 in intracellular spaces, transpiration rate, and electronic transport, whereas a higher water use efficiency was found in "Red sonnet."

The interactions between the dose and application method (**Table S1**, Supplementary materials) and between method *

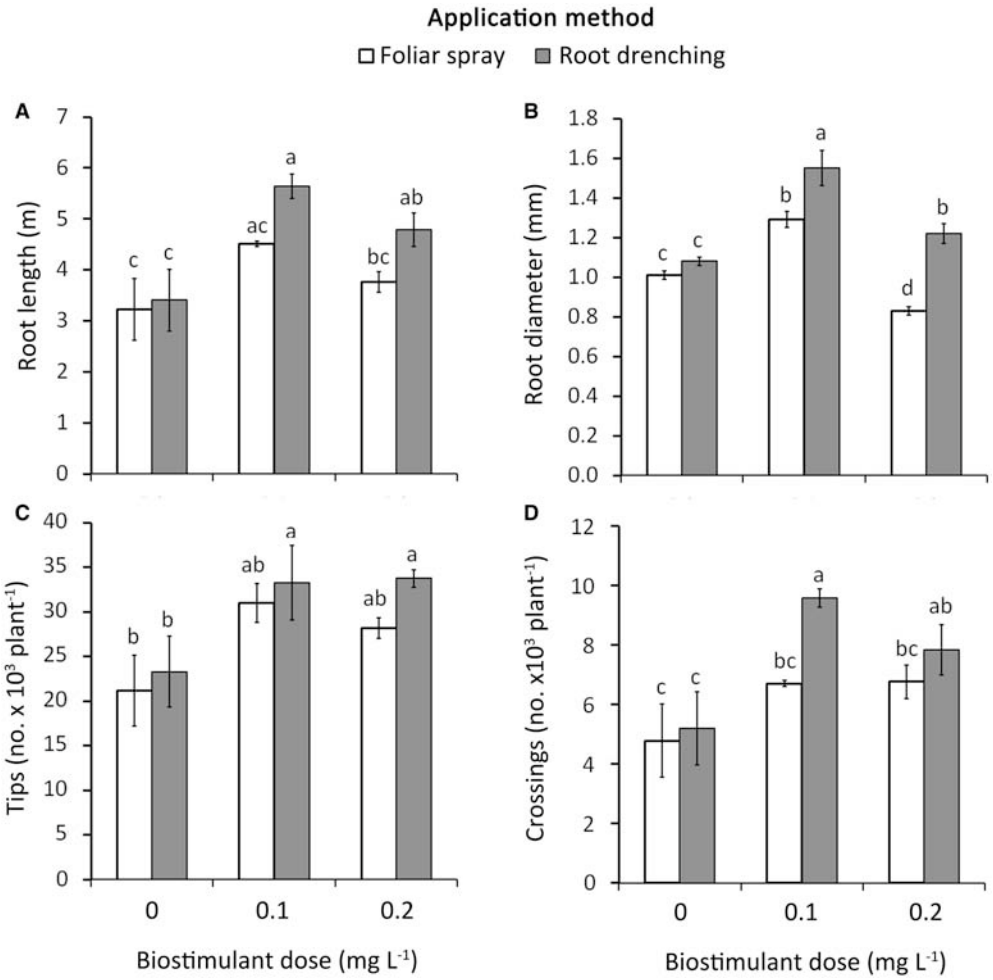


FIGURE 1 | Effect of biostimulant dose (D_0, $D_{0.1}$, and $D_{0.2}$) and application method (F, foliar spray; R, root drenching) on root length **(A)**, root diameter **(B)**, tip number **(C)**, and crossing number **(D)** at the end of the experiment (166 DAT). Vertical bars (standard error) ($n = 6$) with different letters are significantly different according to Duncan's test ($P = 0.05$).

cultivar (**Table S4**, Supplementary materials) were significant only for the CO_2 concentration in the intracellular spaces. In addition, the interaction between the dose and cultivar for transpiration rate and CO_2 concentration were significant only in the intracellular spaces (**Table S6**, Supplementary materials).

The effects of the biostimulant on the fluorescence parameters of snapdragon are shown in **Table 7**. The application of a biostimulant dose of 0.1 g L^{-1} resulted in a significant increase in the efficiency of Photosystem II compared to the other treatments. The values of this parameter increased significantly when the biostimulant was applied through root application (+11%). In addition, the efficiency of Photosystem II was significantly higher in the "Yellow floral showers" compared to "Red sonnet." A highly significant interaction was found between the biostimulant dose and application method (**Table 7**) as well as among the dose, application method and cultivar (**Table S2**, Supplementary materials). No difference was found when applying biostimulant in relation to the chlorophylls fluorescence, with the exception of the cultivar factor where chlorophylls fluorescence was higher in "Red sonnet."

DISCUSSION

To the best of our knowledge, the effects of using an animal-derived PH biostimulant on the growth parameters and physiological behavior of potted snapdragon plants have not previously been reported. Therefore, the cross-referencing in the discussion of the findings in this study will be based on the results available from other plant species.

Biostimulants have been found to increase the growth traits in many horticultural crops, in terms of increased shoot, root biomass, nutrient uptake, and plant yield (Ertani et al., 2009; Kunicki et al., 2010; Colla et al., 2014, 2015; Santi et al., 2017). In the present study, the application of an animal-derived biostimulant resulted in an improvement in morphological and qualitative traits (**Table 2**). Ertani et al. (2009) reported an

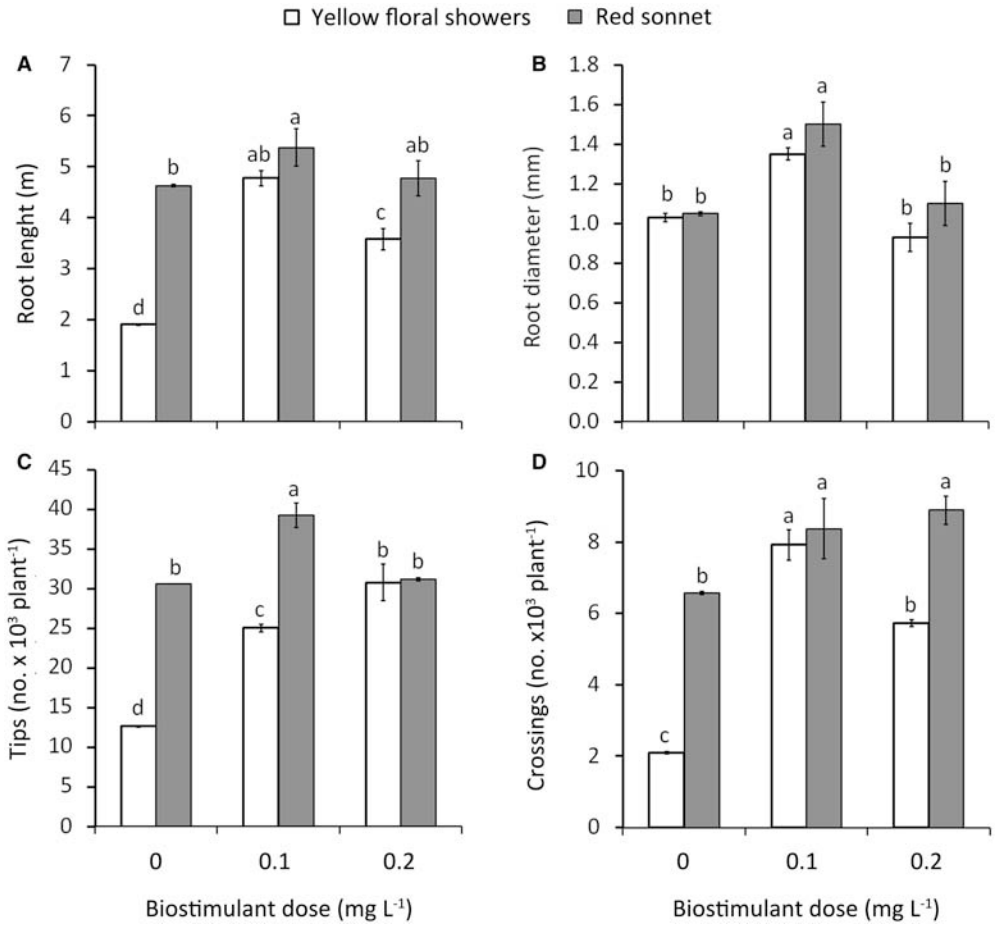


FIGURE 2 | Effect of biostimulant dose (D_0, $D_{0.1}$, and $D_{0.2}$) and cultivar ("Yellow floral shower" and "Red sonnet") on root length **(A)**, root diameter **(B)**, tip number **(C)**, and crossing number **(D)** at the end of the experiment (166 DAT). Vertical bars (standard error) ($n = 6$) with different letters are significantly different according to Duncan's test ($P = 0.05$).

increase in root and leaf growth in maize treated with an animal-derived biostimulant, which also induced morphological changes in the root system increasing the root dry weight of plants (**Table 3**). In agreement with our findings, the same authors found that the most evident plant increments were observed when the biostimulant was applied in the range of 0.01–0.1 g L^{-1}. In another study, Quartieri et al. (2002) tested the effect of an animal-derived biostimulant as a foliar application in potted kiwifruit plants and found an improvement in hypogeal plant dry weight. In nursery-grown passionfruit, Morales-Pajan and Stall (2004) observed that foliar applications with an animal-derived biostimulant increased the seedling growth.

Regarding ornamental flower crops, the present work is in line with (**Tables 2**, **4**, **5**) reports by De Lucia and Vecchietti (2012), who investigated the effects of three different agricultural biostimulants based on hydrolyzed proteins from algae, animal derived-protein hydrolysate and alfalfa origin on L.A. lily hybrids (*Lilium longiflorum* x *L. asiaticum*) grown in a soilless system. The three biostimulants, applied as foliar spray or soil drenching, led to similar performances, reducing the crop cycle of plants and increasing the leaf area and flower buds; the plant root system was also more developed compared to the control.

Applying an animal-derived biostimulant to tomato grown under greenhouse conditions, led to an increase in plant height and number of flowers per plant compared to untreated plants (Parrado et al., 2008). Botta (2013) conducted a cold stress trial on lettuce under controlled environmental conditions using an animal-derived biostimulant. They found that the biostimulant application led to higher shoot and root fresh weights and stomatal conductance compared to untreated control-plants.

When investigating the effects of fish-derived biostimulants on the growth of lettuce, Xu and Mou (2017) found that biostimulants significantly increased the lettuce leaf number per plant, shoot and root dry weight, but had no effect on leaf area. In contrast to the general trend of the results available, studying spinach plants from different cultivars, Kunicki et al. (2010) observed that applying an animal-derived biostimulant as a foliar method had no effect on plant yield, however the cultivar factor significantly influenced the spinach dry weight. Ruiz et al. (2000) reported that the foliar application of an animal-derived

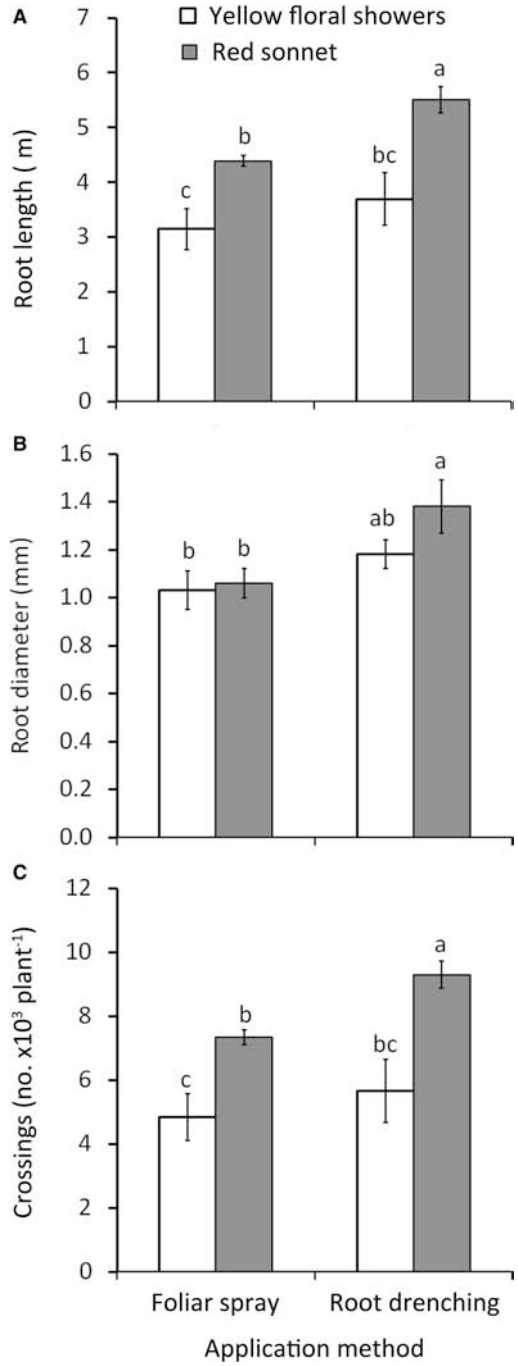


FIGURE 3 | Effect of biostimulant application method (F, foliar spray; R, root drenching) and cultivar ("Yellow floral shower" and "Red sonnet") on root length **(A)**, root diameter **(B),** and crossing number **(C)** at the end of the experiment (166 DAT). Vertical bars (standard error) ($n = 9$) with different letters are significantly different according to Duncan's test ($P = 0.05$).

TABLE 6 | Main effects of biostimulant dose, application method and cultivar on leaf and root total N content of snapdragon plants.

Treatments	Total N (mg kg^{-1})	
	Leaf	**Root**
DOSE (g L^{-1}) (D)		
0	361.1b	277.5b
0.1	422.5a	300.6a
0.2	417.8a	299.1a
METHOD (M)		
Foliar spray	389.3b	281.4b
Root drenching	401.9a	290.8a
CULTIVAR (CV)		
Yellow floral showers	395.7b	288.9b
Red sonnet	406.5a	295.8a
Significance		
D	***	***
M	***	***
CV	*	**
D × M	*	ns
D × CV	*	ns
M × CV	ns	ns
D × M × CV	ns	ns

Different letters within each column indicate significant differences according to Duncan's multiple-range test ($P \leq 0.05$); ns, non significant, $^{*}P < 0.05$, $^{**}0.01$, and $^{***}0.001$, indicate level of significance.

biostimulant reduced growth and yield as well as the root nitrate uptake and nitrogen efficiency in pepper.

Our results (**Table 6**), instead, suggest that snapdragon plants treated with a biostimulant by drenching, increased foliar and root nitrogen content, although no differences were found comparing the two doses applied. Root length and surface area are an integrative indicator of the plant response to water and nutrient uptake (Clothier and Green, 1997; Ryser, 2006). Colla et al. (2014) showed that in tomato the increase in root apparatus resulting from protein hydrolysate applications may also have contributed to increasing the nitrogen uptake by plants.

Other authors have highlighted the positive effects of biostimulants in plant nutrition. Studying cucumber development, Rauthan and Schnitzer (1981) reported the growth of above and below ground plant parts. In addition the use of a biostimulant on bermudagrass was found to enhance the root surface area (Tucker et al., 2006). In agreement with previous studies, it was also found that animal-derived biostimulants stimulate N metabolism and assimilation (Baglieri et al., 2014; Calvo et al., 2014; Colla et al., 2015; Rouphael et al., 2017b). In a review of the literature, Maini (2006) reported that the components of an animal-derived biostimulant preparation penetrated rapidly into treated leaves, and were subsequently distributed to other leaves. Schiavon et al. (2008) also reported that the enzyme activity in N reduction and assimilation was stimulated by an animal-derived biostimulant applied to maize plants.

In agreement with our findings, Halpern et al. (2015) and Santi et al. (2017) also demonstrated the positive effects of biostimulant application on plant nutrient uptake including nitrogen. In the present study, no case of snapdragon plant death was observed, thus demonstrating that the application of the

TABLE 7 | Main effects of biostimulant dose, application method and cultivar on net photosynthesis, transpiration rate, stomatal conductance, concentration of CO_2, electronic transport rate, efficiency of Photosystem II and fluorescence parameters in snapdragon plants.

Treatments	Net photosynthesis (μmol CO_2 m^{-2} s^{-1})	Transpiration rate (mmol H_2O m^{-2} s^{-1})	Stomatal conductance (mmol H_2O m^{-2} s^{-1})	Concentration of CO_2 (ppm)	Electronic transport rate (μmol e^- m^{-2} s^{-1})	Efficiency of photosystem II (F'v/F'm)	Chlorophylls fluorescence (Fv/Fm)
DOSE (g L^{-1}) (D)							
0	9.43 b	1.83b	0.08b	175.8b	112.1c	0.26b	0.83a
0.1	14.51a	2.88a	0.15a	198.9a	129.1a	0.29a	0.84a
0.2	14.21a	2.82a	0.14a	209.1a	120.7b	0.27b	0.84a
METHOD (M)							
Foliar spray	11.96a	2.28b	0.10b	184.6b	117.7a	0.26b	0.84a
Root drenching	12.85a	2.75a	0.14a	204.6a	124.0a	0.29a	0.84a
CULTIVAR (CV)							
Yellow floral showers	12.18a	2.82a	0.13a	205.8a	132.9a	0.30a	0.81b
Red sonnet	12.62a	2.19b	0.11a	183.3b	108.8b	0.25b	0.87a
SIGNIFICANCE							
D	***	*	**	**	*	**	ns
M	ns	*	*	**	ns	**	ns
CV	ns	*	ns	ns	*	*	*
D × M	ns	ns	ns	*	ns	**	ns
D × CV	ns	*	ns	**	ns	*	ns
M × CV	ns	ns	ns	*	ns	*	ns
D × M × CV	ns	ns	ns	ns	ns	**	ns

Different letters within each column indicate significant differences according to Duncan's multiple-range test ($P \leq 0.05$).
ns, non significant, $^{*}P < 0.05$, $^{**}0.01$, and $^{***}0.001$, indicate level of significance.

animal-derived biostimulant maintained crop uniformity, which was also demonstrated by Tsouvaltzis et al. (2014) in greenhouse lettuce crops.

According to findings by Ferrini and Nicese (2002) for English oak and by Xu and Mou (2017) for lettuce, our use of a plant biostimulant enhanced many physiological parameters such as photosynthetic rate, stomatal conductance and transpiration rate, thus ensuring a higher carbon assimilation efficiency (**Table 7**).

As suggested by Yakhin et al. (2017), amino acid based biostimulants are readily absorbed and translocated by plant tissues. They also function as modulators of stomatal opening once absorbed, acting on the stimulation of photosynthesis or down regulating the plant stress signaling pathway. A high photosynthetic rate of shoots secures high root activity by supplying a sufficient amount of photosynthates to the roots (Yang et al., 2004). The biostimulant used in this study did not alter chlorophyll fluorescence (**Table 7**) as also found by Xu and Mou (2017) in lettuce, suggesting that there were no stress-induced perturbations in the photosynthetic apparatus (Baker and Rosenqvist, 2004).

CONCLUSIONS

Based on our data, the biostimulant enhanced the ornamental quality in potted snapdragon. Plant morphological and qualitative traits, leaf and root-N content, photosynthetic rate, transpiration rate, and stomatal conductance were significantly increased by the biostimulant application compared to the control, regardless of the dose. The lowest dose also resulted in the best effect on both the dry weight of above-ground plant and the root system.

The root drenching method enhanced the plant shoot number, ground dry weight, root morphology, leaf, and root-N content and gas exchange. A higher pot quality was obtained in "Red sonnet" compared to "Yellow floral shower."

Based on these findings, applying the biostimulant at the lowest dose to potted snapdragon, as part of a fertilizing regime, improves the crop quality in an agro-environmental sustainable way.

AUTHOR CONTRIBUTIONS

GC carried out the experiment, processed the experimental data, performed the analysis, designed the figures, and wrote part of the research dealing with animal-derived PHs biostimulant effects on plant growth; EP conducted the physiological measurements and wrote part of the research dealing with animal-derived PHs biostimulant effects on gas exchange and fluorescence; GCo gave support in the data analysis and interpretation; VT provided background information on PHs based biostimulants; BD developed the concept of this experiment, designed the study, wrote part of the research dealing with animal-derived PHs biostimulant effects on root system. All authors provided critical feedback, made contributions to analysis and interpretation of data, discussed the results, contributed to the writing of the manuscript and gave final approval of the version to be published.

ACKNOWLEDGMENTS

This work was supported by the Apulia Region Special Grant PIF Florovivaismo Misura 124 (2015-2016).

SUPPLEMENTARY MATERIAL

The Supplementary Material for this article can be found online at: https://www.frontiersin.org/articles/10.3389/fpls.2018.00861/full#supplementary-material

Table S1 | The effects of the interaction between biostimulant dose and application methods on snapdragon plants characteristics: shoots (n /plant), flower (n /plant), flower dry weight (g/ plant), ground dry weight (g/plant), projected root area (cm^2 /plant), total leaf-N (mg kg^{-1}), concentration of CO_2 (ppm).

Table S2 | The effects of the interaction between biostimulant dose application methods and cultivar on snapdragon plants characteristics: total shoot length (cm /plant), total leaf area (cm^2/plant), flower dry weight (g/ plant), total above dry weight (g/plant), projected root area (cm^2/plant), and efficiency of Photosystem II (F'v/F'm).

Table S3 | The effects of the interaction between biostimulant dose and cultivar on snapdragon plants characteristics: shoots (n /plant), flower (n /plant), leaf (n/plant), flower dry weight (g/ plant), total above dry weight (g/plant), ground dry weight (g/plant).

Table S4 | The effects of the interaction between biostimulant application methods and cultivar on snapdragon plants characteristics: leaf (n/plant), ground dry weight (g/plant), projected root area (cm^2 /plant), and concentration of CO_2 (ppm).

Table S5 | The effects of the interaction between biostimulant dose application methods and cultivar on snapdragon plants characteristics: total root length, root diameter, root volume, root tips, root crossings, and root forks.

Table S6 | The effects of the interaction between biostimulant dose and cultivar on snapdragon plants characteristics: projected root area (cm_2 /plant), total leaf-N content (mg kg^{-1}), transpiration rate (mmol H_2O m^{-2} s^{-1}), and concentration of CO_2 (ppm).

REFERENCES

Asrar, A. A., Abdel-Fattah, G. M., and Elhindi, K. M. (2012). Improving growth, flower yield, and water relations of snapdragon (*Antirhinum majus* L.) plants grown under well-watered and water-stress conditions using arbuscular mycorrhizal fungi. *Photosynthetica* 50, 305–316. doi: 10.1007/s11099-012-0024-8

Baglieri, A., Cadili, V., Mozzetti Monterumici, C., Gennari, M., Tabasso, S., Montoneri, E., et al. (2014). Fertilization of bean plants with tomato plants hydrolysates. Effect on biomass production, chlorophyll content and N assimilation. *Sci. Hortic.* 176, 194–199. doi: 10.1016/j.scienta.2014.07.002

Baker, N. R., and Rosenqvist, E. (2004). Applications of chlorophyll fluorescence can improve crop production strategies: an examination of future possibilities. *J. Exp. Bot.* 55, 1607–1621. doi: 10.1093/jxb/erh196.

Baroccio, F., Barilaro, N., and Tolomei, P. (2017). Classification of biostimulants origin using amino acids composition of hydrolyzed proteins. *J. Hortic. Sci. Res.* 1, 30–35.

Battacharyya, D., Babgohari, M. Z., Rathor, P., and Prithiviraj, B. (2015). Seaweed extracts as biostimulants in horticulture. *Sci. Hortic.* 196, 39–48. doi: 10.1016/j.scienta.2015.09.012

Botta, A. (2013). Enhancing plant tolerance to temperature stress with amino acids: an approach to their mode of action. *Acta Hortic.* 1009, 29–35. doi: 10.17660/ActaHortic.2013.1009.1

Bulír, P. (2009). Testing of Koch method applied for evaluation of ornamental trees in the Czech Republic. *Hortic. Sci.* 36, 154–161.

Bulgari, R., Cocetta, G., Trivellini, A., Vernieri, P., and Ferrante, A. (2015). Biostimulants and crop responses: a review. *Biol. Agric. Hortic.* 31, 1–17. doi: 10.1080/01448765.2014.964649

Calvo, P., Nelson, L., and Kloepper, J. W. (2014). Agricultural uses of plant biostimulants. *Plant Soil* 383, 3–41. doi: 10.1007/s11104-014-2131-8

Carter, C. T., and Grieve, C. M. (2008). Mineral nutrition, growth, and germination of *Antirrhinum majus* L. (snapdragon) when produced under increasingly saline conditions. *Hortscience* 43, 710–718.

Cerdán, M., Sánchez-Sánchez, A., Oliver, M., Juárez, M., and Sánchez-Andreu, J. J. (2009). Effect of foliar and root applications of amino acids on iron uptake by tomato plants. *Acta Hortic.* 830, 481–488. doi: 10.17660/ActaHortic.2009.830.68

Clothier, B. E., and Green, S. R. (1997). Roots: the big movers of water and chemical in soil. *Soil Sci.* 162, 534–543. doi: 10.1097/00010694-199708000-00002

Colla, G., Hoagland, L., Ruzzi, M., Cardarelli, M., Bonini, P., Canaguier, R., et al. (2017). Biostimulant action of protein hydrolysates: unraveling their effects on plant physiology and microbiome. *Front. Plant Sci.* 8:2202. doi: 10.3389/fpls.2017.02202

Colla, G., Nardi, S., Cardarelli, M., Ertani, A., Lucini, L., Canaguier, R., et al. (2015). Protein hydrolysates as biostimulants in horticulture. *Sci. Hortic.* 196, 28–38. doi: 10.1016/j.scienta.2015.08.037

Colla, G., Rouphael, Y., Canaguier, R., Svecova, E., and Cardarelli, M. (2014). Biostimulant action of a plant-derived protein hydrolysate produced through enzymatic hydrolysis. *Front. Plant Sci.* 5:448. doi: 10.3389/fpls.2014.00448

De Lucia, B., and Vecchietti, L. (2012). Type of biostimulant and application method effects on stem quality and root system growth in LA Lily. *Eur. J. Hortic. Sci.* 77, 1–10.

De Pascale, S., Rouphael, Y., and Colla, G. (2017). Plant biostimulants: innovative tool for enhancing plant nutrition in organic farming. *Eur. J. Hortic. Sci.* 82, 277–285 doi: 10.17660/eJHS.2017/82.6.2

Du Jardin, P. (2015). Plant biostimulants: definition, concept, main categories and regulation. *Sci. Hortic.* 196, 3–14. doi: 10.1016/j.scienta.2015.09.021

Ertani, A., Cavani, L., Pizzeghello, D., Brandellero, E., Altissimo, A., and Ciavatta, Cet al. (2009). Biostimulant activity of two protein hydrolyzates in the growth and nitrogen metabolism of maize seedlings. *J. Plant Nutr. Soil Sci.* 172, 237–244. doi: 10.1002/jpln.200800174

Ertani, A., Pizzeghello, D., Francioso, O., Tinti, A., and Nardi, S. (2016). Biological activity of vegetal extracts containing phenols on plant metabolism. *Molecules* 21:205. doi: 10.3390/molecules21020205

Ferrini, F., and Nicese, F. (2002). Response of English Oak (*Quercus robur* L.) Trees to biostimulants application in the urban environment. *J. Arboric.* 28, 70–75.

Halpern, M., Bar-Tal, A., Ofek, M., Minz, D., Muller, T., and Yermiyahu, U. (2015). The use of biostimulants for enhancing nutrient uptake. *Adv. Agron.* 130, 141–174. doi: 10.1016/bs.agron.2014.10.001

Kauffman, G. L., Kneivel, D. P., and Watschke, T. L. (2007). Effects of a biostimulant on the heat tolerance associated with photosynthetic capacity, membrane thermostability, and polyphenol production of perennial ryegrass. *Crop Sci.* 47, 261–267. doi: 10.2135/cropsci2006.03.0171

Kunicki, E., Grabowska, A., Sekara, A., and Wojciechowska, R. (2010). The effect of cultivar type, time of cultivation, and biostimulant treatment on the yield of spinach (*Spinacia oleracea* L.). *Folia Hortic.* 22, 9–13. doi: 10.2478/fhort-2013-0153

Lisiecka, J., Knaflewski, M., Spizewski, T., Fraszczak, B., Kaluzewicz, A., and Krzesinski, W. (2011). The effect of animal protein hydrolysate on quantity and quality of strawberry daughter plants cv 'Elsanta'. *Acta Sci. Pol. Hortoru* 10, 31–40.

Maini, P. (2006). The experience of the first biostimulant, based on amino acids and peptides: a short retrospective review on the lçaboratory researches and the practical results. *Fertil. Agrorum* 1, 29–43.

Morales-Pajan, J. P., and Stall, W. (2004). Passion fruit (*Passiflora edulis*) transplant production i affected by selected biostimulants. *Proc. Fla. State Hortic. Soc.* 117, 224–227.

Mugnai, S., Azzarello, E., Pandolfi, C., Salamagne, S., Briand, X., and Mancuso, S. (2008). Enhancement of ammonium and potassium root influxes by the application of marine bioactive substances positively affects *Vitis vinifera* plant growth. *J. Appl. Phycol.* 20, 177–182. doi: 10.1007/s10811-007-9203-6

Nardi, S., Pizzeghello, D., Schiavon, M., and Ertani, A. (2016). Plant biostimulants: physiological responses induced by protein hydrolyzed-based products and humic substances in plant metabolism. *Sci. Agric.* 73, 18–23. doi: 10.1590/0103-9016-2015-0006

Parrado, J., Bautista, J., Romero, E. J., Garcia-Martinez, A. M., Friaza, V., and Tejada, M. (2008). Production of a carob enzymatic extract: potential use as a biofertilizer. *Bioresour. Technol.* 99, 2312–2318. doi: 10.1016/j.biortech.2007.05.029

Polo, J., Barroso, R., Ródenas, J., Azcón-Bieto, J., Cáceres, R., and Marf,à, O. (2006). Porcine hemoglobin hydrolysate as a biostimulant for lettuce plants subjected to conditions of thermal stress. *Horttechnology* 16, 483–487.

Polo, J., and Mata, P. (2018). Evaluation of a biostimulant (Pepton) based in enzymatic hydrolyzed animal protein in comparison to seaweed extracts on root development, vegetative growth, flowering, and yield of gold cherry tomatoes grown under low stress ambient field conditions. *Front. Plant Sci.* 8:2261. doi: 10.3389/fpls.2017.02261

Quartieri, M., Cavani, L., Lucchi, A., Marangoni, B., and Tagliavini, M. (2002). Effects of the rate of protein hydrolysis spray concentration on growth of potted kiwifruit (*Actinidia deliciosa*) plants. *Acta Hortic.* 594, 341–347. doi: 10.17660/ActaHortic.2002.594.42

Rauthan, B. S., and Schnitzer, M. (1981). Effects of a soil fulvic acid on the growth and nutrient content of cucumber (*Cucumis sativus*) plants. *Plant Soil* 63, 491 495.

Rouphael, Y., Cardarelli, M., Bonini, P., and Colla, G. (2017a). Synergistic action of a microbial-based biostimulant and a plant derived-protein hydrolysate enhances lettuce tolerance to alkalinity and salinity. *Front. Plant Sci.* 8:131. doi: 10.3389/fpls.2017.00131

Rouphael, Y., Colla, G., Giordano, M., El-Nakhel, C., Kyriacou, M. C., and De Pascale, S. (2017b). Foliar applications of a legume-derived protein hydrolysate elicit dose dependent increases of growth, leaf mineral composition, yield and fruit quality in two greenhouse tomato cultivars. *Sci. Hortic.* 22, 353–360. doi: 10.1016/j.scienta.2017.09.007

Rouphael, Y., De Micco, V., Arena, C., Raimondi, G., Colla, G., and De Pascale, S. (2017c). Effect of Ecklonia maxima seaweed extract on yield, mineral composition, gas exchange and leaf anatomy of zucchini squash grown under saline conditions. *J. Appl. Phycol.* 29, 459–470. doi: 10.1007/s10811-016-0937-x

Ruiz, J. M., Castilla, N., and Romero, L. (2000). Nitrogen metabolism in pepper plants applied with different *bioregulators. J. Agric. Food Chem.* 48, 2925–2929. doi: 10.1021/jf990394h

Ryser, A. (2006). The mysterious root length. *Plant Soil* 286, 1–6. doi: 10.1007/s11104-006-9096-1

Santi, C., Zamboni, A., Varanini, Z., and Pandolfini, T. (2017). Growth Stimulatory effects and genome-wide transcriptional changes produced by protein hydrolysates in maize seedlings. *Front. Plant Sci.* 8:433. doi: 10.3389/fpls.2017.00433

Schiavon, M., Ertani, A., and Nardi, S. (2008). Effects of an alfaalfa protein hydrolysate on the gene expression and activity of enzymes of TCA cycle and N metabolism in *Zea mays* L. *J. Agric. Food Chem.* 56, 11800–11808. doi: 10.1021/jf802362g

Tsouvaltzis, P., Koukounaras, A., and Siomos, S. A. (2014). Application of amino acids improves lettuce crop uniformity and inhibits nitrate accumulation induced by the supplemental inorganic nitrogen fertilization. *Int. J. Agric. Biol.* 16, 951–955.

Tucker, B. J., McCarty, L. B., Liu, H., Wells, C. E., and Rieck, J. R. (2006). Mowing height, nitrogen rate, and biostimulant influence root development of field-grown 'TifEagle' Bermudagrass. *Hortscience* 41, 805–807.

Tuomisto, H. L., and Teixeira de Mattos, M. J. (2011). Environmental impacts of cultured meat production. *Environ. Sci. Technol.* 45, 6117–6123. doi: 10.1021/es200130u

Van Oosten, M. J., Pepe, O., De Pascale, S., Silletti, S., and Maggio, A. (2017). The role of biostimulants and bioeffectors as alleviators of abiotic stress in crop plants. *Chem. Biol. Technol. Agric.* 4, 1–12. doi: 10.1186/s40538-017-0089-5

Vernieri, P., Borghesi, E., Tognoni, F., Serra, G., Ferrante, A., and Piagessi, A. (2006). Use of biostimulants for reducing nutrient solution concentration in floating system. *Acta Hortic.* 718, 477–484. doi: 10.17660/ActaHortic.2006.718.55

Xu, C., and Mou, B. (2017). Drench application of fish-derived protein hydrolysates affects lettuce growth, chlorophyll content, and gas exchange. *Horttechnology* 27, 539–543. doi: 10.21273/HORTTECH03723-17.

Yakhin, O. I., Lubyanov, A. A., Yakhin, I. A., and Brown, P. H. (2017). Biostimulants in plant science: a global perspective. *Front. Plant Sci.* 7:2049. doi: 10.3389/fpls.2016.02049

Yang, C., Yang, L., Yang, Y., and Ouyang, Z. (2004). Rice root growth and nutrient uptake as influenced by organic manure in continuously and alternately flooded paddy soils. *Agric. Water Manage.* 70, 67–81. doi: 10.1016/j.agwat.2004.05.003

Yazdani, B., Nikbakht, A., and Etemadi, N. (2014). Physiological effects of different combinations of humic and fulvic acid on Gerbera. *Commun. Soil Sci. Plant Anal.* 45, 1357–1368. doi: 10.1080/00103624.2013.875200

Zeljkovic, S. B., Paradikovic, N. A., Babic, T. S., Duric, G. D., Oljaca, R. M., Vinkovic, T. M., et al. (2010). Influence of biostimulant and substrate volume on root growth and development of scarlet sage (*Salvia splendens* L.) transplants. *J. Agric. Sci.* 55, 29–36. doi: 10.2298/JAS1001029Z

Zhang, X., Ervin, E. M., and Schimdt, R. E. (2003). Effects of liquid application of a seaweed extract and a humic acid on creeping bent grass (*Agrostis palustris* Huds, A.). *J. Am. Soc. Hortic. Sci.* 128, 492–496.

Conflict of Interest Statement: The authors declare that the research was conducted in the absence of any commercial or financial relationships that could be construed as a potential conflict of interest.

The reviewer CC and the handling editor declared their shared affiliation.

frontiers in Microbiology

ORIGINAL RESEARCH
published: 26 June 2018
doi: 10.3389/fmicb.2018.01293

Characterization and Screening of Thermophilic *Bacillus* Strains for Developing Plant Growth Promoting Consortium From Hot Spring of Leh and Ladakh Region of India

***Jay Prakash Verma*[1,2]*, *Durgesh Kumar Jaiswal*[1], *Ram Krishna*[1], *Satya Prakash*[1], *Janardan Yadav*[3] and *Vijai Singh*[4]**

[1] *Institute of Environment and Sustainable Development, Banaras Hindu University, Varanasi, India,* [2] *Hawkesbury Institute for the Environment, Western Sydney University, Sydney, NSW, Australia,* [3] *Department of Soil Science and Agricultural Chemistry, Institute of Agricultural Sciences, Banaras Hindu University, Varanasi, India,* [4] *Synthetic Biology Laboratory, Department of Microbiology, School of Biological Sciences and Biotechnology, Institute of Advanced Research, Gandhinagar, India*

OPEN ACCESS

Edited by:
Giuseppe Colla,
Università degli Studi della Tuscia, Italy

Reviewed by:
Ömür Baysal,
Muğla University, Turkey
Giridhar Anam Babu,
Chonbuk National University, South Korea

***Correspondence:**
Jay Prakash Verma
verma_bhu@yahoo.co.in;
jpv.iesd@bhu.ac.in

Specialty section:
This article was submitted to Plant Microbe Interactions, a section of the journal Frontiers in Microbiology

Received: *03 August 2017*
Accepted: *28 May 2018*
Published: *26 June 2018*

Citation:
Verma JP, Jaiswal DK, Krishna R, Prakash S, Yadav J and Singh V (2018) Characterization and Screening of Thermophilic Bacillus Strains for Developing Plant Growth Promoting Consortium From Hot Spring of Leh and Ladakh Region of India. Front. Microbiol. 9:1293. doi: 10.3389/fmicb.2018.01293

In the present investigation, the main aim is to identify and characterize the potential drought tolerant plant growth promoting consortium for agricultural productivity. Three bacterial isolates were isolated from hot spring of Chumathang area of Leh district. *Bacillus* species (BHUJP-H1, BHUJP-H2, and BHUJP-H3) were done some biochemical tests including catalase, cellulase, amylase, indole-3-acetic acid, phosphate solubilisation, production of ammonia, siderophore, and hydrogen cyanide. Molecular characterization of isolates was done by 16S rDNA sequencing, e.g., *Bacillus subtilis* BHUJP-H1 (KU312403), *Bacillus* sp. BHUJP-H2 (KU312404) and *B. licheniformis* BHUJP-H3 (KU312405). The genetic diversity of the isolates was assessed by seven inter simple sequence repeat, all primer shows high polymorphism. The highest polymorphism efficiency and polymorphism information content showed by UBC-809 and UBC-836 which were 100% and 0.44 respectively, the lowest is by UBC-807 75% and 0.28 respectively. On an average 90.69% polymorphism efficiency and 0.40 polymorphism information contents obtained by used markers. The highest, 11.08 and the lowest, 4.50 effective multiplex ratios obtained for primer UBC-823 and UBC-807, on an average 7.99 effective multiplex ratio obtained. The highest, 4.89 and the lowest, 1.25 marker indexes obtained by UBC-836 and UBC-807 respectively and on an average 3.24 obtained. The UPGMA cluster analysis divided a population into two clusters I and II, in which BHUJP-H1 and BHUJP-H2 grouped under same while BHUJP-H3 grouped under another cluster. The treatment combination of *Bacillus subtilis* BHUJP-H1, *B. subtilis* BHUJP-H1+ *B. licheniformis* BHUJP-H3 and *B. subtilis* BHUJP-H1+ *Bacillus* sp. BHUJP-H2+ *B. licheniformis* BHUJP-H3 were recorded better combination for enhancing plant growth attributes of *Vigna radiata* as compared to control and others. The plant growth promoting consortium, e.g., *Bacillus subtilis* BHUJP-H1, *Bacillus subtilis* BHUJP-H1+ *B. licheniformis* BHUJP-H3 and *B. subtilis* BHUJP-H1+ *Bacillus* sp. BHUJP-H2+ *B. licheniformis* BHUJP-H3 can be further used

as effective microbial inoculant for enhancing the production of mungbean in field conditions. *Bacillus* sp. BHUJP-H1 and *Bacillus* sp. BHUJP-H2 may use as drought tolerant plant growth promoting consortium for enhancing the sustainable agricultural productivity.

Keywords: thermophile, extremophile, hot spring, *Bacillus*, microbial consortium, genetic diversity, ISSR, plant growth attributes

INTRODUCTION

Chumathang area of Ladakh region of Indian subcontinent forms a part of the tectonically active belt having many hot springs (Siangbood and Ramanujam, 2011). In general, hot springs are of utmost importance because of the unique characteristic thermophilic biocatalysts produce by thermophilic microorganisms thriving under the extreme temperature condition. Thermostable enzymes such as protease, lipase, amylase, cellulase, phosphatase, asperginase, esters, and carboxylase from thermophiles are gaining more attention due to the medical, industrial and agriculture benefits. Lele and Deshmukh (2016) and Mohammad et al. (2017) reported that the various *Bacillus* spp. has been isolated from hot springs which has ability to produce thermostable enzymes. Therefore, the hot springs are recently gained more interest toward the production and enhancement of enzymes, sugars, compatible solutes and antibiotics (Satyanarayana et al., 2005). However, biodiversity of such extreme environments, the microbes have received several attentions because of their diverse and unique ecology, chemistry and the opportunity to identify a novel molecule (Kuddus and Ramtekke, 2012). Although, the possibility of the presence of new microbes with high economic and industrial values, there are limited reports available on the microbial diversity of hot springs from India (Panda et al., 2015). As the culture dependent studies are valuable for isolating microorganisms and exploring their properties, the culture independent methods offer a more comprehensive assessment of microbial diversity (Tringe and Hugenholtz, 2008). *Bacillus licheniformis* was isolated and molecularly characterized by Saharan and Verma (2014) which show the ability of multiple plant growth promoting characteristics such as ammonia production, indole acetic acid production, phosphate solubilization, catalase production, heavy metal tolerance and ACC deaminase activity. isolate is a potential PGPR candidate for enhancing sustainable agriculture. Gutiérrez-Manero et al. (2001) reported that the *B. pumilus* and *B. licheniformis* have been documented to produce gibberellins. Other scientist reported that *B. licheniformis, B. cereus, B. circulans, B. subtilis,* and *B. thuringiensis* found as potential biocontrol agents having chitinolytic activities (Sadfi et al., 2001). The strains *Bacillus licheniformis* SB3086 secreted the Novozymes by their spores which play an important role as phosphate solubilizer strain and it is also effective against Dollar spot disease of plants (Saharan and Verma, 2014). Other scientist Kayasth et al. (2013) reported that the *Bacillus licheniformis* identified and explored as the potential PGPR strain to be developed as multifunctional biofertilizer for multiple crop production.

Thermophilic microorganisms have been recognized for its abundant significance in the industry owing their capability to function at the extreme environmental condition. The microbial diversity in various hot springs from United States, Russia, Iceland, Algeria, New Zealand, and India have recently been investigated and identified by using 16S rRNA sequence from culture-independent or culture-dependent methods (Claus and Fritz, 1989; Reysenbach et al., 1994, 2000; Huber et al., 1998; Ghosh et al., 2003; Van den Burg, 2003; Nazina et al., 2004; Schaffer et al., 2004; Belkova et al., 2007; Kecha et al., 2007; Ghati et al., 2013). Hot spring metagenomics suggests that the dominant phylum and the dominant taxa within each phylum in distinct hot spring which depends on temperature, pH, and geochemistry of waters. The need of more depth analysis requires understanding the total bacterial diversity of hot springs. In a recent study, the *Bacillus* strains have been isolated and tested by biochemical and molecular levels. These strains were recovered from the wide range of extreme environments of Atri and Taptapani hot springs of Odisha (Cihan et al., 2012). In India, the hot spring of Bakreshwar, Balarampur, Chumathang, Panamic, Manikaran, and Vashisht have been characterized as rare places which can be potential sources of novel genes and microorganisms with a unique characteristic (Sharma et al., 2009; Kumar et al., 2013).

ISSR-PCR tools and techniques is very good and effective for genetic diversity analysis. ISSR (inter simple sequence repeat) is a quick and cheap molecular marker techniques for multiple application regarding the categorization of genetic similarity among populations and species (Baysal et al., 2011). The complementary sequences between two neighboring microsatellite is applied as PCR primers; the variable region among them converts amplified. The choice of ISSR was depended on their comparative practical easiness, level of polymorphisms, cheaper technology, simply relevant for prokaryote and eukaryote for amplifying which sequences that are more copious during evolution (Kumar et al., 2013).

There are recent advancement of tools and technologies, the extremophilic microorganisms identified as a potential sources of novel pigments (as food additives), enzymes like cellulases which can be valuable in agriculture as inoculants (plant growth-promoting bacteria) or bio control agents in extreme habitats (Khan and Patel, 2007; Srinivas et al., 2009). Another importance of extremophilic strains application can be used as the enhancement of soil productivity and fertility where the soil pH is extreme, it may be more saline or acidic. In this circumstances, extremophiles microbial consortium will be better and novel aspect for sustainable agricultural production. Therefore, the stress resistant microbial diversity of Bacillaceae

and Paenibacillaceae can be identified and characterized in these extreme environments. In acidic soils, the availability of essential nutrients like, phosphorus, calcium, magnesium, and molybdenum are affected. Some studies are reported on the dissemination and multiplicity of bacteria in acidic soils (Perez et al., 2007; Yadav et al., 2011; Verma et al., 2013). The arid deserts have different types of microbial communities that can persist extreme environment including hot temperature and low moisture. Such environments encompass typically poor soils quality with low organic content and limited amounts of bioavailable inorganic nutrients. The microbiota of desert ecosystems is not only responsible for enhancing the productivity, biogeochemical cycling of elements and ecosystem balance, but also for soil neogenesis and improvement of soil structure. The balance of soil carbon storage is depended upon microbial activities in response to the climate change which will partially control and loss under future temperature and precipitation conditions. The aim of present study is to isolate and characterize bacterial species to develop plant growth promoting consortium for enhancing sustainable agriculture.

MATERIALS AND METHODS

Isolation of Microorganisms

The soil and water samples were collected from hot spring of Chumathang of Leh district, Ladakh region of Jammu and Kashmir, India. Isolation of microorganisms was done by serial dilution and plating methods. We took 1 g of moist soil in a test tube that contains 9 ml of sterile saline water (0.85% NaCl) and mixed properly then serially diluted up to 10–7. Total 100 μl aliquots of each dilution were transferred and spread aseptically on different agar plates such as Nutrient agar, Kenknight and Munaiers agar, Potato Dextrose Agar, Tryptone Soya agar, Pikovskaya agar, and King's B Base. The plates were incubated for 2–5 days at 45°C. After incubation, the different microbial colonies were found on plates. We counted and calculated the total numbers of colonies forming unit (CFU). Subsequently, the different types of colonies were picked up and streaked on respective plates for further purifying a single and pure colony. The presumptive isolates of phosphate solubilizers were screened and selected on the basis of halo zone produced in Pikovskaya agar. All microbial isolate was sub-cultured on their respective medium by the streaking method to get pure colonies and stored on slant media at 4°C and also glycerol stock in −80°C for further use. We have isolated three microbes which showed more effective and fast growth on high temperatures like 45, 50, and 60°C.

Morphological and Biochemical Properties of Microbes

Microorganisms were characterized according to morphological characteristics such as bacterial isolates colony margin, shape and color (Kloepper et al., 1992; Gilbert and Jack, 1993) and biochemical assays including, Gram staining, amylase, catalase and cellulase test (Cappuccino and Sherman, 1992; Aneja, 2003) (**Tables 1**, **2**).

Plant Growth Promoting Properties of Thermophilic *Bacillus* Strains

Plant growth promoting properties such as Indole-3-acetic acid (IAA) was estimated in unit μg/ml of broth culture and performed by methods of Bric et al. (1991) (**Table 3**). The phosphate solubilization activity was estimated in unit μg/ml of broth culture and performed on Pikovskaya agar medium containing tricalcium phosphate (Pikovskaya, 1948) followed by Ammonium bicarborate diethylene triamine penta acetic acid (AB-DTPA) method (Soltanpour and Workman, 1981) and soluble phosphorus was determined by Ascorbic acid method (Watanabe and Olsen, 1965) (**Table 4**). HCN production test was done by adapting the method of Lorck (1948). Additionally, the ammonia production was evaluated by the methods of Cappuccino and Sherman (1992).

TABLE 1 | Morphological characters of isolated bacterial strains from hot spring.

Strains	Cell morphology		Colony morphology			
	Gram staining	Shape	Form	Elevation	Margin	Color
B. subtilis BHUJP-H1	Positive	Rod	Circular	Flat	Curried	Creamy white
Bacillus sp. BHUJP-H2	Positive	Rod	Spindle	Flat	Undulate	Whitish
B. licheniformis BHU-H3	Positive	Rod	Filamentous	Flat	Curried	Whitish

TABLE 2 | Biochemical characterization of isolated bacterial strains.

Strains	Biochemical characterization			Growth at different temperature				
	Amylase	Catalase	Cellulase	30°C	40°C	50°C	60°C	80°C
B. subtilis BHUJP-H1	+	++	++	+	+	+	++	−
Bacillus sp. BHUJP-H2	−	++	+	+	+	+	+	−
B. licheniformis BHU-H3	+	++	++	+	+	+	+	−

Single positive sign (+): normal activity showed; double positive sign (++): more activity showed of particular enzyme and growth by bacterial strains; negative sign (–): no enzyme activity and no growth.

TABLE 3 | Estimation of IAA production in thermophilic *Bacillus* strains in broth cultures at different concentration of tryptophan concentrations.

Bacterial strains	IAA production (μg/ml) at different incubation time					
	150 μg/ml tryptophan concentration			300 μg/ml tryptophan concentration		
	24 h	48 h	72 h	24 h	48 h	72 h
B. subtilis BHUJP-H1	14.65 ± 0.66[a]	17.12 ± 0.13[a]	18.35 ± 0.33[a]	21.36 ± 1.29[a]	19.08 ± 1.15[a]	24.85 ± 0.36[b]
Bacillus sp. BHUJP-H2	25.41 ± 0.52[c]	26.36 ± 1.15[c]	24.06 ± 0.12[b]	25.74 ± 1.31[b]	22.94 ± 1.91[b]	22.27 ± 0.30[a]
B. licheniformis BHUJP-H3	20.93 ± 1.26[b]	24.34 ± 0.63[b]	31.79 ± 1.57[c]	24.55 ± 0.88[b]	32.52 ± 0.63[c]	34.76 ± 1.12[c]

IAA, Indole-3-acetic acid (μg/ml) at 150 and 300 μg/ml tryptophan as precursor; [a]Values are the mean ± SD (standard deviation), Mean values in each column with the same superscript (s) do not differ significantly but different superscript is showed significantly different between each treatments by Duncan post hoc multiple comparison tests ($P \leq 0.05$).

TABLE 4 | PGPR activities of isolated thermophilic *Bacillus* species.

Bacterial strains	Phosphate solubilisation (μg/ml)		NH_3	HCN	Siderphore
	3 days	6 days			
B. subtilis BHUJP-H1	12.09 ± 0.89[a]	22.48 ± 1.47[a]	+	++	+
Bacillus sp. BHUJP-H2	12.76 ± 1.15[a]	63.14 ± 0.97[c]	+	+	+
B. licheniformis BHU-H3	33.01 ± 0.91[b]	42.14 ± 1.45[b]	+	–	–

[a]Values are the mean ± SD (standard deviation), Mean values in each column with the same superscript (s) do not differ significantly but different superscript is showed significantly different between each treatment by Duncan post hoc multiple comparison tests ($P \leq 0.05$); Single positive sign (+): normal activity showed; double positive sign (++): more activity showed of particular properties like HCN (hydrogen cyanide) by bacterial strains; negative sign (–): no activity.

Effect of Organophosphate Insecticide on Growth of *Bacillus* Strains and Their Interaction

Disk diffusion method was used for growth of microbes with insecticide. Tolerance levels of microbial strains with different concentration of insecticide were determined using the filter paper disk technique. These techniques also used for test of antibiotics resistance of microbes (Bauer et al., 1966) and later used to test the effect of insecticide on microbial growth (Mallik and Tesfai, 1983; Martensson, 1992). Monocrotophos insecticide commercial name Monocrown 36% SL was obtained from the market. The recommended dose of Monocrotophos is 0.8 mL/L (0.8 μL/mL) of water. The recommended dose of Monocrotophos was diluted to 1X (0.8 μL/mL), 2X (1.6 μL/mL) and 10X (80 μL/mL) using the same solvent (water). Others, chlorpyrifos insecticide commercial name Messban 20% EC was obtained from the market. The recommended dose of chlorpyrifos is 2 mL/L of water. The recommended dose of chlorpyrifos was diluted to 1X (2 μL/mL), 2X (4 μL/mL), 3X (6 μL/mL), and 10X (20 μL/mL) using the same solvent (water). Sterile filter paper disk was used for insecticide test. Sterile filter paper disk was used for insecticide test. The sterilized disks were dipped in different concentration of insecticides and put on respective microbial inoculated plate of nutrient agar under laminar air flow. Control disk was dipped with sterile distilled water and put on respective media with inoculated strains. Insecticide disks were put on the nutrient agar plate which is uniformly spread with a pure culture of different microbial strains. The plates were then incubated at 30°C for 48 h. Thus, each plate contained four disks of different concentrations of monocrotophos insecticide (Control, 1X, 2X, and 10X) were prepared for the experiment. While, four disks of Control, 1X, 2X, 3X, and 10X concentrations of chlorpyrifos was prepared for the experiment. After 48 h plates were observed for the zone of inhibition around the disks (**Table 5**).

Molecular Identification of Isolated Microbial Strains

Genomic DNA Extraction From Microorganisms

The single colony was grown in nutrient broth at 28 ± 2°C in shaker incubator with 120 rpm for overnight. Genomic DNA extraction was isolated by using methods described by Sambrook

TABLE 5 | Inhibition zone of monocrotophos (monocrown 36% SL) and chlorpyrifos (Messban 20% EC) on growth of microbes6.

Strain	Monocrotophos after 48 h					Chlorpyrifos after 48 h				
	1x	2x	3x	10x	Response	1x	2x	3x	10x	Response
BHUJP-H1	–	–	–	–	Tolerant	+	+	+	+	Susceptible
BHUJP-H2	–	–	–	–	Tolerant	+	+	+	–	Susceptible
BHUJP-H3	–	–	–	–	Tolerant	–	–	–	–	Tolerant

Single positive sign (+): showed growth inhibition zone against insecticide concentration; negative sign (–): no inhibition zone that means bacteria grow on media plate against insecticide concentration; 1x, 2x, 3x, and 10x mean insecticide concentration increasing with respect of recommended doge in agricultural field for pest control.

and Russel (2001). Genomic DNA was checked on a 0.8% (w/v) agarose gel electrophoresis containing ethidium bromide and it was run with 100 V for 45 min in 1X TAE buffer (0.04 M Tris acetate, 0.001 M EDTA) along with EcoR1/Hind III double digest Lamda DNA marker (Banglore Genei, Pvt., Ltd., Bangalore, India).

Amplification of 16S rDNA by Polymerase Chain Reaction (PCR)

In this study, we used (Tamura et al., 2007) universal primer for amplification of 16S rDNA gene in all bacterial species. This primer was custom synthesized by Bangalore Genei Pvt. Ltd., Bangalore, India. The 50 μl of reaction mixture consisted of 50 ng of genomic DNA, 2.5 units of Taq polymerase, 5 μl of 10 X buffer (100 mM Tris-HCl, 500 mM KCl pH-8.3), 200 μM dNTP, 1.5 mM MgCl2 and 10 pmoles of each primer. The forward primer 27F (5′-AGAGTTTGATCCTGGCTCAG-3′) and reverse primer 1492R (5′-TACGGTTAC CTTGTT ACGACTT-3′) (Miller et al., 2013) were used. Amplification was performed under the following thermal (PCR System 2720, Applied Biosystems, Singapore) conditions: initial denaturation at 94°C for 5 min, followed by 34 cycles of denaturation at 94°C for 1 min, annealing at 52°C for 1.5 min, extension at 72°C for 2 min and a final extension at 72°C for 7 min. Amplified PCR products (5 μl) were resolved on a 1.5% (w/v) agarose gel at 100 V for 45 min in 1X TAE buffer containing ethidium bromide (EtBr) along with 500 bp DNA ladder (Bangalore Genei Pvt., Ltd. Bangalore, India). We obtained the expected size of PCR product (1500 bp). It was purified using PCR purification kit (Invitrogen, PureLink^{TM} PCR purification kit, United States) for the sequencing of 16S rDNA.

In order to establish the genetic relationship, we used 16S rDNA gene sequence of isolated species along with reference strains retrieved from ribosomal database project. Multiple sequence alignment was done by Clustal W and MEGA 4.0 software for construction of phylogenetic tree with 500 bootstrap replication. The evolutionary distances were computed using the Maximum Composite Likelihood method (Nei et al., 1985).

Assessment of Genetic Diversity Using ISSR

Genetic diversity of the bacterial species was assessed by ISSR markers. A total 7 ISSR marker selected as it's frequently being used in the laboratory, producing reproducible and polymorphic bands (UBC-807, UBC-808, UBC-809, UBC-811, UBC-823, UBC-824, and UBC-836) primers were used for genetic diversity assessment (**Table 6**). For ISSR PCR was performed in a volume of 15 μl reaction mixture containing 1 μl template DNA (80 ng), 1.5 μl 10 × PCR buffer, 0.35 μl of dNTPs (25 mM), 0.3 μl $MgCl_2$ (1.5 mM), 1.2 μl random primer (10 pM), 0.25 μl Taq polymerase (3 units) and 10.4 μl, sterile distilled water. The reaction was carried out in a DNA Engine Dyad ALD1234 (Biorad, United States). The PCR programmed for an initial denaturation for 4 min at 94°C, then 38 cycles of 1 min denaturation at 94°C, 1 min annealing at 55°C, and 1 min extension at 72°C with a final extension at 72°C for 10 min. The PCR amplification with each ISSR primer was repeated twice to verify the reproducibility of the results. The amplified ISSR amplicons were analyzed by electrophoresis in 2.5% agarose gel using Tris–Acetic acid–Ethylene diamine tetraacetic acid buffer. The number of amplified amplicons was recorded using a gel documentation system (AlphaImager 3400).

ISSR Data Analysis and Scoring

The amplified bands of ISSR on gels were scored as one (1) for present and zero (0) for absent. Evaluation of fragment patterns was carried out by the similarity index (SI). The similarity matrix data was calculated by Jaccard index using NTSYSpc Version 2.11X software. The polymorphism information content (PIC) were calculated by the using formula (Roldàn-Ruiz et al., 2000); PICi = 2fi (1 – fi), for each locus. Where PICi is the polymorphic information content of the locus i, fi is the frequency of the amplified fragments and 1 - fi is the frequency of non-amplified fragments. The effective multiplex ratio (EMR) is calculated by multiplying the proportion of polymorphic loci per their total number with total number of polymorphic loci (per primer) with the following formula as described by Powell et al. (1996) and Nagaraju et al. (2001) EMR = np(np/n) Where np is the number of polymorphic loci, and n is total number of loci. Marker index (MI) is a statistical parameter utilized for estimation of the total utility of the maker system. MI is the product of the PIC value (or expected heterozygosity, HE) and EMR (Powell et al., 1996; Nagaraju et al., 2001) MI = PIC * EMR. The similarity matrix data was subjected to unweighed pair group method for arithmetic average (UPGMA)

TABLE 6 | Detail of ISSR primers and their amplification profile used in genetic diversity assessment.

S. No.	ISSR	Nucleotide sequence (5′. . . . . .3′)	TA (°C)	A B	S R(bp)	PB	MB	PE%	PIC	EMR	MI
1	UBC-807	AGAGAGAGAGAGAGAGT	55.0	8	300–2100	6	2	75.00	0.28	4.50	1.25
2	UBC-808	AGAGAGAGAGAGAGAGC	56.8	10	250–2250	9	1	90.00	0.40	8.10	3.24
3	UBC-809	AGAGAGAGAGAGAGAGG	58.0	7	500–1500	7	0	100.00	0.44	7.00	3.11
4	UBC-811	GAGAGAGAGAGAGAGAC	52.0	8	650–1900	7	1	87.50	0.39	6.13	2.38
5	UBC-823	TCTCTCTCTCTCTCTCC	55.0	13	300–800	12	1	92.30	0.41	11.08	4.54
6	UBC-824	TCTCTCTCTCTCTCTCG	55.0	10	250–1700	9	1	90.00	0.40	8.10	3.24
7	UBC-836	AGAGAGAGAGAGAGACYA	55.0	11	500–2000	11	0	100.00	0.44	11.00	4.89
Performance of primer (Average)			**–**	**9.57**	**–**	**8.71**	**0.86**	**90.69**	**0.40**	**7.99**	**3.24**

TA, Annealing temperature; AB, Amplified bands; SR, Size range base pair; PB, Polymorphic bands; MB, Monomorphic bands; PE, Polymorphism efficiency; PIC, Polymorphism information content; EMR, Effective multiplex ratio; MI, Marker index.

cluster analysis to generate a dendrogram using average linkage procedure.

Effect of Different Treatment Combinations of *Bacillus* Strains on Plant Growth Attributes of *Vigna radiata* Under Plant Growth Chamber

Healthy seeds of *Vigna radiata* was selected and sterilized with 0.02% Mercuric chlorite. The sterilized seed was put on wetted cotton with sterilizing distilled water containing Petri dish for 24 h. The germination percentage of seeds was observed 98% after 24 h. We have filled 150 g sterilized garden soil in sterilized thermocol glass. The bacterial strains were grown in nutrient broth media. After seed sterilization, 10 seeds were inoculated [10^8 colony farming unit (CFU) per seed] as per treatment combinations (**Table 7**). Ten inoculated seeds were showed in respective treatment combination of cups. The experiment was designed with 8 treatments and 3 replications. The experiment setup was put in plant growth chambers. After complete germination in each treatment, only five plants were left for further analysis. After 10 days, plants were uprooted and took length (cm/plant) and fresh weight (g/plant) of shoot and root of *Vigna radiata*.

Statistical Analysis

The experimental setup was prepared with nineteen treatments and three replications. The results were expressed as the mean ± SE of different independent replicates. Analysis of variance (ANOVA) followed by Duncan *post hoc* multiple comparison tests was done using SPSS software (version 16.0). The values of $P \leq 0.05$ were considered as statistically significant.

RESULT

Morphological and Biochemical Characteristics of Thermophilic *Bacillus* Species

Three bacterial strains such as BHUJP-H1, BHUJP-H2, and BHUJP-H3 were isolated, analyzing the cultivable aerobic bacteria from hot spring and checked their morphological, biochemical properties and growth pattern on different temperature range from 30 to 60°C. The cell morphologies of *B. subtilis* species BHUJP-H1 show gram positive character, rod and circular shape, *Bacillus* sp. BHUJP-H2 reflect gram positive characteristic having circular rod and spindle form and *B. licheniformis* BHUJP-H3 show gram positive character having rod and filamentous form were observed (Verma et al., 2010). In the present study, the colonies morphology of strains BHUJP-H1 (flat elevation, curled margin, and creamy white color), BHUJP-H2 (flat elevation, undulate margin and whitish color) and BHUJP-H3 (flat elevation, curled margin and whitish color) were observed (**Table 1**). The amylase production was recorded in isolated bacterial strains BHUJP-H1 and BHUJP-H3 but not in BHUJP-H2. The biochemical characteristic such as catalase and cellulase production was observed in all three isolated *Bacillus* species. Out of which, BHUJP-H1 and BHUJP-H2 showed more cellulase production as compared to BHUJP-H3. These *Bacillus* strains were found good growth on nutrient agar media at 30, 40, 50, and 60°C. *B. subtilis* strain BHUJP-H1, *Bacillus* sp. species BHUJP-H2, and *B. licheniformis* strain BHUJP-H3 have the ability to grow in the broader temperature range from 30 to 60°C. The BHUJP-H1 was found to more growth at an extreme high temperature of 60°C as compared to BHUJP-H2 and BHUJP-H3 (**Table 2**). The *B. subtilis* BHUJP-H1 was showed faster growth rate at 60°C as compared to 30, 40, and 50°C. The variation in growth vs. temperature expressed that this bacterium can survive in adverse environmental condition.

TABLE 7 | Treatment combination of bacterial strains.

S. No.	Treatments
1	Control (Un-inoculated)
2	*Bacillus subtilis* BHUJP- H1
3	*Bacillus* sp. BHUJP-H2
4	*Bacillus licheniformis* BHUJP-H3
5	*Bacillus subtilis* BHUJP- H1+ *Bacillus* sp. BHUJP-H2
6	*Bacillus subtilis* BHUJP- H1+ *Bacillus licheniformis* BHUJP-H3
7	*Bacillus* sp. BHUJP-H2+ *Bacillus licheniformis* BHUJP-H3
8	*Bacillus subtilis* BHUJP- H1+ *Bacillus* sp. BHUJP-H2+ *Bacillus licheniformis* BHUJP-H3

Plant Growth Promoting Properties of Thermophilic *Bacillus* Species

In order to estimate, indole-3-acetic acid (IAA) production was found in three thermophilic *Bacillus* strains at 150 and 300 μg/ml concentration of tryptophan, where the tryptophan is used as a precursor of IAA synthesis. The bacterial strains *Bacillus* sp. BHUJP-H2 and *B. licheniformis* BHUJP-H3 showed a significant IAA production as compared to *B. subtilis* BHUJP-H1 at concentrations (150 and 300 μg/ml) of tryptophan in broth medium after 24, 48, and 72 h incubation. IAA syntheses by bacterial strains have recorded a range from 14.65 to 34.76 μg/ml in broth medium. At the tryptophan concentration of 150 μg/ml, the IAA production was estimated significant increase in *Bacillus* sp. (25.41, 26.36, and 24.06 μg/ml) and *B. licheniformis* (20.93, 24.34, and 31.79 μg/ml) as compared to *B. subtilis* (14.65, 17.12, and 18.35 μg/ml) during incubation period of 24, 48, and 72 h, respectively. Similarly, it was also observed at 300 μg/ml concentration of tryptophan, bacterial strains *B. licheniformis* (24.55, 32.52, and 34.76 μg/ml) *Bacillus* sp. (25.74, 22.94, and 22.27 μg/ml) showed significant IAA production as compared to *B. subtilis* (21.36, 19.08, and 24.85 μg/ml) after incubation period of 24, 48, and 72 h, respectively (**Table 3**). The main entity has observed that the *Bacillus* sp. and *B. licheniformis* were found more significant IAA synthesis than *B. subtilis*.

The thermophilic *B. licheniformis* BHUJP-H3 was showed the more significant increase (33.01 μg/ml) phosphate solubilisation as compared to *Bacillus sp*. BHUJP-H2 (12.76 μg/ml) and *B. subtilis* BHUJP-H1 (12.09 μg/ml) at 3 days' incubation in

Pikovaskaya broth media at 45°C. Whereas, *Bacillus* sp. BHUJP-H2 (63.14 μg/ml) was obtained higher phosphate solubilisation followed by *B. licheniformis* BHUJP-H3 (42.14 μg/ml) as compared to *B. subtilis* BHUJP-H1 (22.48 μg/ml) at 6 days' incubation period. *Bacillus* sp. and *B. licheniformis* BHUJP-H3 were found to be more phosphate solubilising strain than BHUJP-H1 at 3 and 6 days' incubation, respectively (**Table 4**). Ammonia, hydrogen cyanide (HCN) and siderophore production were observed in isolated thermophilic species of *B. subtilis* and *Bacillus* sp. while *B. licheniformis* was unable to produce HCN and siderophore. *B. subtilis* was showed more HCN production as compared to others (**Table 4**).

Insecticide Tolerance of Monocrotophos and Chlorpyrifos Ability of *Bacillus* Strains

The monocrotophos and chlorpyrifos insecticide is the commercial available group of organophosphate insecticide which is widely used in agriculture. We have tested the effect of this insecticide on microbial growth on nutrient agar media by the methods of disk diffusion. The growth inhibition test was performed by using four concentrations of insecticide 1x, 2x, 3x, and 10x. The bacterial strains BHUJP-H1, BHUJP-H2 and BHUJP-H3 were showed no inhibition zone against monocrotophos that means these strains were more tolerant (**Table 5**). While chlorpyrifos, the bacterial strains BHUJP-H1 and BHUJP-H2 showed the zone of inhibition at 1x, 2x, and 3x concentration that means these strains are susceptible. The strain BHUJP-H3 was found tolerance at all concentration of insecticides (**Table 5**), that means strains BHUJP-H3 is tolerant.

The Molecular Characterization of Thermophilic *Bacillus*

The Genomic DNA of all species was resolved on 0.8% agarose gel. The 16S rDNA was amplified by PCR using 16S universal primers 27F forward and 1492R reverse primer. Amplified 16S rDNA gene was resolved on the agaroge gel and their size was found to be 1500 bp. It was sequenced by automated sequencer from Centre of Human Genetic Disorder, Institute of Science, BHU, Varanasi, India. The 16S rDNA analysis followed by BLAST search exhibited close to 16S rDNA database similarity. A comparison with the 16S rDNA sequences available in the GenBank database indicated that the strain BHUJP-H1, showed 98% similarity with *B. subtilis* strain M65 (Accession No. KC315772). The strain BHUJP-H2 showed 93% similarity with *Bacillus* sp. COA-2P (Accession no. KM575935) and also strain BHUJP-H2 showed 99% similarity with *B. licheniformis* strain RA32UN (Accession No. KJ867517). According to similarity index of the partial gene sequence, the microbial strains BHUJP-H1, BHUJP-H2 and BHUJP-H3 were confirmed microbial strains as *Bacillus* genus. The sequences of the strains BHUJP-H1, BHUJP-H2 and BHUJP-H3 were deposited in NCBI GenBank database with different accession number KU312403, KU312404, and KU312405, respectively. The 16S rDNA has confirmed the identification of hot spring bacteria such as *B. subtilis* strain BHUJP-H1 (Accession No. KU312403), *Bacillus* sp. strain BHUJP-H2 (Accession No. KU312404) and *B. licheniformis* strain BHUJP-H3 (Accession No. KU312405). The phylogenetic tree between 9 taxa were generated using the UPGMA method (**Figure 1**). The thermophilic bacterial strains *Bacillus* sp. BHUJP-H2 and *B. subtilis* BHUJP-H1 is more closely related taxa as compared *B. licheniformis* BHUJP-H3. BHUJP-H3 showed similarity with known taxa of HT-W34-B1 and RA32UN.

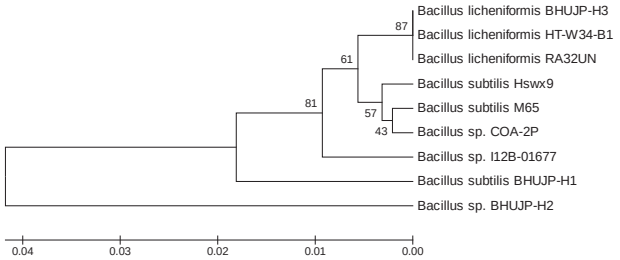


FIGURE 1 | The phylogenetic tree between 9 taxa were generated using the UPGMA method. The thermophilic bacterial strains *Bacillus* sp. BHUJP-H2 and *B. subtilis* BHUJP-H1 is more closely related taxa as compared *B. licheniformis* BHUJP-H3. BHUJP-H3 showed similarity with known taxa of HT-W34-B1 and RA32UN.

Genetic Diversity Assessment of Thermophilic *Bacillus* by ISSR

In order to assess the genetic diversity, the ISSR have been performed among these species. The genomic DNA of thermophilic *B. subtilis* BHUJP-H1, *Bacillus* sp. BHUJP-H2 and *B. licheniformis* BHUJP-H3 has PCR amplified gene in respect of seven primers. Out of all screened primers, UBC-809, UBC-836, shows 100% polymorphism efficiency while UBC-823 shows 92.30%, UBC-808 and UBC-824 shows 90.00%, UBC-811 and UBC-807 showed 87.50 and 75.00% polymorphism respectively and overall polymorphism efficiency was 90.69% (**Table 6**). The average number of polymorphic band amplified by ISSR was 8.71 per primer and the number of amplicons varies from 7 to 13 with a size range of 250-2250 base pair. The highest polymorphism content (PIC) showed by the UBC-809 and UBC-836 while the lowest, 0.28 by UBC-807, the overall average PIC 0.40 obtained (**Table 6**). The highest, 11.08 and lowest, 4.50 EMR obtained for primer UBC-823 and UBC-807, the average EMR of the used primers, 7.99 obtained (**Table 6**). The highest, 4.89 and lowest, 1.25 MI obtained with primer UBC-836 and UBC-807 respectively and an average of 3.24 MI obtained with all used primer. The amplified bands showed similar banding pattern with respect to seven random primers between thermophilic *B. subtilis* BHUJP-H1 and *Bacillus* sp. BHUJP-H2 Whereas, *B. licheniformis* BHUJP-H3 showed different banding patterns that indicate this strain is genetically different from BHUJP-H1 and BHUJP-H2 (**Figure 2**). In dendrogram, *B. subtilis* BHUJP-H1 and *Bacillus* sp. BHUJP-H2 grouped under the same cluster whereas *B. licheniformis* BHUJP-H3 in separate cluster. The ISSR analysis showed high polymorphism among the three isolates.

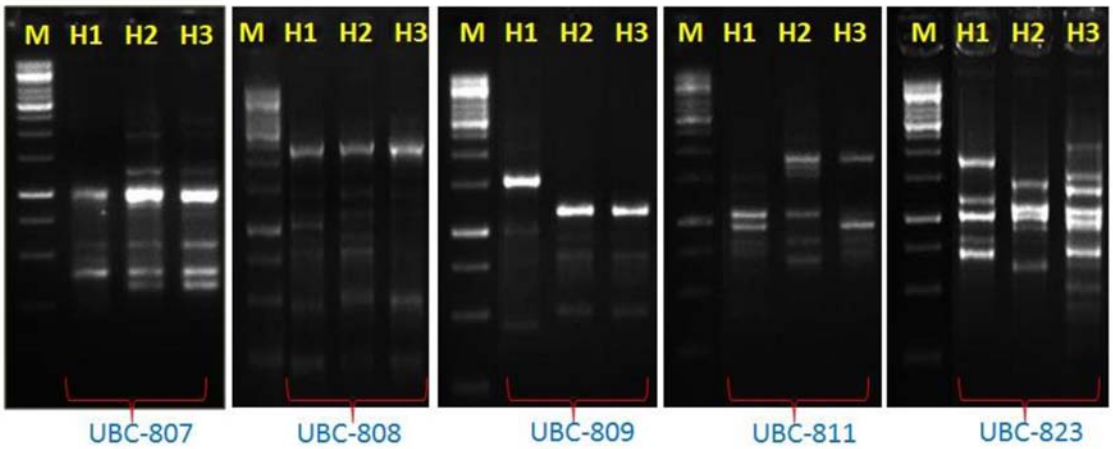


FIGURE 2 | Inter simple sequence repeat (ISSR) analysis of three *Bacillus* strains by using 7 sets of primers: UBC-807, UBC-808, UBC-809, UBC-811, UBC-823, UBC-824, and UBC-836, here gel picture of primers UBC-807, UBC-808, UBC-809, UBC-811, and UBC-823 showed different band pattern on 2.5% gel agarose gel M: Marker size 1 kb plus (Banglore genei), amplification between three strain H1: *B. subtilis* BHUJP-H1; H2: *Bacillus* sp. BHUJP-H2 and H3: *B. licheniformis* BHUJP-H3.

The genetic diversity between isolated thermophilic *B. subtilis* BHUJP-H1, *Bacillus* sp. BHUJP-H2 and *B. licheniformis* BHUJP-H3 was done by ISSR profile, which classified them into two groups based on their capacity for producing various biochemical and plant growth promoting activities (**Figure 3**).

Effect of Different Treatment Combinations of *Bacillus* Strains on Plant Growth Attributes of *Vigna radiata*

The treatment combination of *B. subtilis* BHUJP- H1, *B. subtilis* BHUJP- H1+ *B. licheniformis* BHUJP-H3, and *B. subtilis* BHUJP- H1+ *Bacillus* sp. BHUJP-H2+ *B. licheniformis* BHUJP-H3 were recorded significant growth of shoot length (cm/plant) as compared to control (Un-inoculated) and others after 10 days seedling growth of mungbean plants (**Table 7**). The *B. subtilis* BHUJP- H1+ *B. licheniformis* BHUJP-H3 and *B. subtilis* BHUJP- H1 were found the more significant increase in shoot length as compared to others

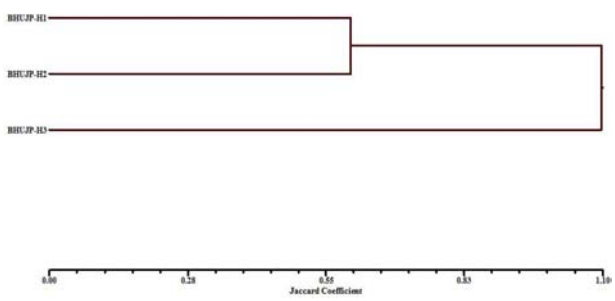


FIGURE 3 | UPGMA dendrogram based on the Nei's genetic similarity index illustrating the genetic relationship among 3 *Bacillus* species.

(**Figure 4A**). In root length, treatment *B. subtilis* BHUJP-H1+ *Bacillus* sp. BHUJP-H2+ *B. licheniformis* BHUJP-H3 was found more significant enhancement followed by *B. subtilis* BHUJP-H1+*Bacillus* sp. BHUJP-H2, *B. subtilis* BHUJP-H1+ *B. licheniformis* BHUJP-H3 and *Bacillus* sp. BHUJP-H2+ *B. licheniformis* BHUJP-H3 than control, *B. subtilis* BHUJP-H1, *Bacillus* sp. BHUJP-H2 and *B. licheniformis* BHUJP-H3 (**Figure 4B**).

The fresh shoot weight of mungbean was recorded after 10 days uprooted plants of different treatment combination. Fresh shoot weight was significantly observed more in all treatment combinations as per controls. Treatment *B. subtilis* BHUJP-H1+ *Bacillus sp.* BHUJP-H2 and *B. subtilis* BHUJP-H1+ *B. licheniformis* BHUJP-H3 were found the more significant fresh weight of shoot followed by *Bacillus* sp. + *B. licheniformis*, *Bacillus* sp. BHUJP-H2, *B. subtilis* + *Bacillus* sp. + *B. licheniformis*, *B. licheniformis* and *Bacillus* sp. as compared to control (**Figure 4C**). But in the case of root weight (g/plant) was found only significant in treatment *B. licheniformis* BHUJP-H3 as compared to all others while the enhancement of root weight was observed in treatment combination of strains BHUJP-H1+ BHUJP-H2, BHUJP- H1+ BHUJP-H3, BHUJP-H2+BHUJP-H3 and BHUJP-H1 as compared to others (**Figure 4D**). The leaf fresh weight (g/plant) was recorded more significant in treatment combination of *B. subtilis* BHUJP- H1+ *B. licheniformis* BHUJP-H3 and *B. subtilis* BHUJP- H1+ *Bacillus* sp. BHUJP-H2 followed by *Bacillus* sp. BHUJP-H2+ *B. licheniformis* BHUJP-H3, *B. licheniformis* BHUJP-H3 and *B. subtilis* BHUJP- H1+ *Bacillus* sp. BHUJP-H2+ *B. licheniformis* BHUJP-H3 as compared to control after 10 days seedling growth (**Figure 4E**). Overall, an impact of different treatment combination of *Bacillus* strains has enhanced the shoot length and fresh shoot weight as compared to control (**Figure 5**).

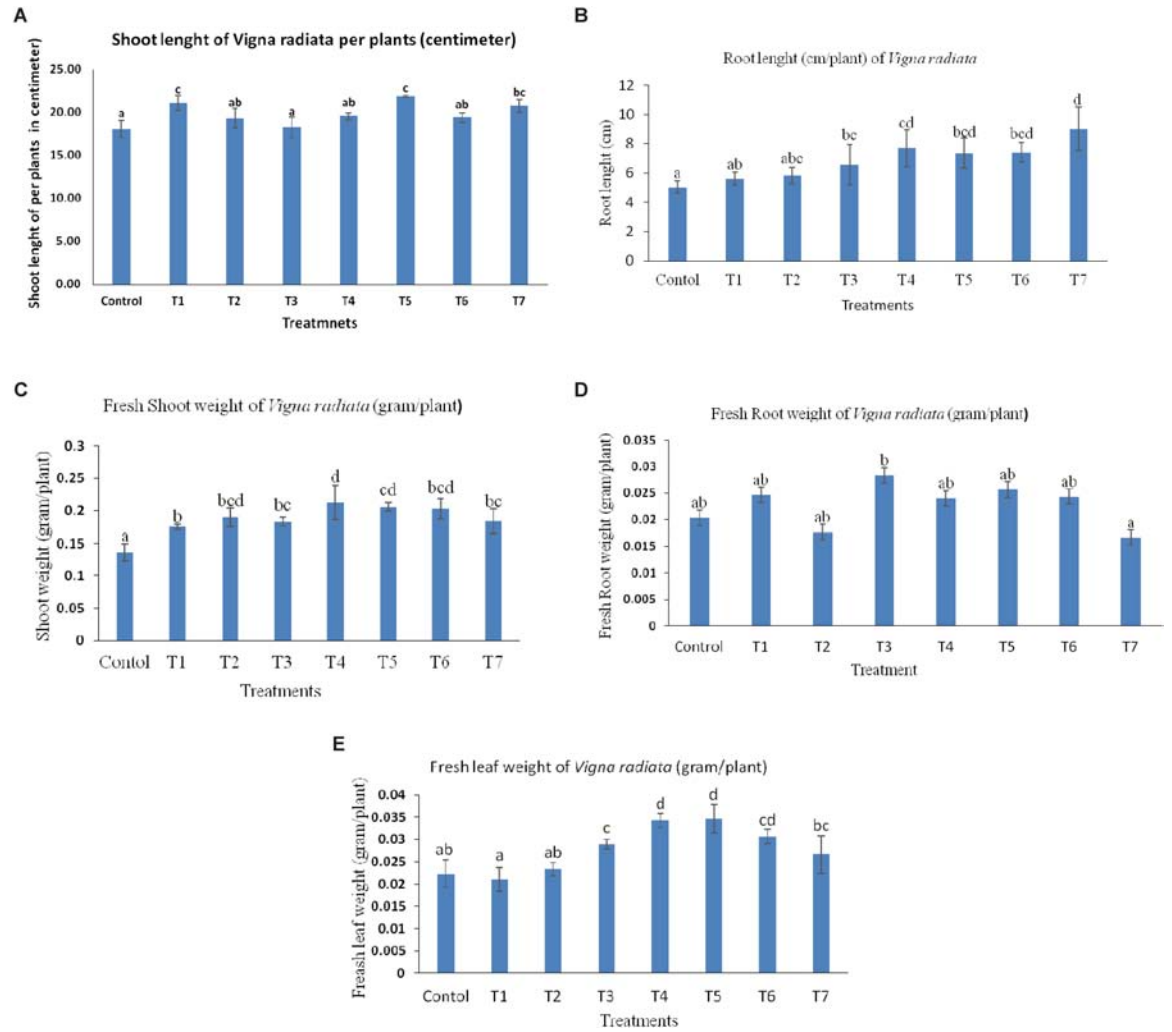


FIGURE 4 | Effect of microbial culture on plant growth attributes after 10 days inoculation, **(A)** shoot length (cm) of *Vigna radiate* per plant; **(B)** Root length (cm) of *Vigna radiata* per plant; **(C)** fresh shoot weight (g) of *Vigna radiata* per plant; **(D)** Fresh root weight (g) of *Vigna radiata* per plant; **(E)** Fresh leaf weight (g) of *Vigna radiata* per plant.

DISCUSSION

Bacterial Screening and Biochemical Properties

There is increasing the global warming and climate changes, a major issue that affects the biodiversity. In the present study, we isolated bacteria from extreme hot spring condition so they have very good properties for producing thermo-tolerant enzymes and novel biomolecules (plant growth promoter compound) that can play a key role for industrial, agricultural and biotechnological applications. The selected species were identified as *Bacillus* by comparing with the reference strains of *B. subtilis* BHUPSB13, *Paenibacillus polymyxa* BHUPSB16 (Verma et al., 2016) and *B. megaterium* BHUPSB14 (Verma and Yadav, 2018). As per morphological, biochemical and molecular characterization, the isolated bacterial strains were identified as *B. subtilis* BHUJP-H1, *Bacillus* sp. BHUJP-H2 and *B. licheniformis* BHUJP-H3. The current study revealed that *Bacillus* strains were found in the sediment of hot spring of Chumathang area, Ladakh region. Previously, Yadav et al. (2015) have studied about a microbial diversity of various hot spring water and sediments that they found *B. megaterium, B. subtilis, B. firmus,* and *B. pumilus*.

The amylase, catalase, and cellulase production were observed in BHUJP-H1, BHUJP-H2 and BHUJP-H3, while straining BHUJP-H2 unable to produce amylase. These enzymes are thermostable and capable of producing various types of enzymes for industrial use and these strains can be used as the future alternative of enzymes production. Also, identifying enzymes producing capability these extremophilic stains are required to access for utility in various fields of medicine, agriculture and microbiology. The characteristic, especially, cellulase enzyme plays a key role in the degradation of cellulosic material for bioethanol production and also degradation of organic residues

FIGURE 5 | Effect of microbial inoculation of enhancing plant height as per different treatment combination of *Vigna radiata* after 10 days seed inoculation.

for enhancing the soil fertility and health. The cellulase enzyme play an important role in composting of agro-cellulosic material in soil to enhance soil fertility and health by providing adequate carbon source for survival of rhizosphere microbes and their proliferation. The catalase is also used for the manufacture of baked goods, beverages preparation, the textile industry and cosmetic industry (Zhang et al., 2010). Amylase also plays a significant role in starch degradation (Abd-Elhalem et al., 2015). *B. subtilis* BHUJP-H1, *Bacillus* sp. strain BHUJP-H2, and *B. licheniformis* strain BHUJP-H3 has the ability to grow in a wide range of temperatures (30–60°C). The variation in growth temperature (30, 40, 50, and 60°C) expressed that this can survive in adverse environmental condition. A number of studies show that is possible because of the bacterial genome have been modified in a stress condition for better survival and fitness in changing environmental conditions. It may be possible to bacterial genes could be regulated and adjusted according to adverse conditions (Panda et al., 2015). Thermophilic microorganisms have recently gained more scientific and industrial importance because of their thermal stability and activity (Coolbear et al., 1992). Similarly, Yadav et al. (2015) have reported that the extreme conditions may be resources for the new genus of *Bacillus* that can be flourished well under the extremes pH, temperature, salinity, and moisture. *Bacillus* strains have the ability to produce endospore that can protect them from the diverse environmental condition.

Plant Growth Promoting Properties of *Bacillus* Strains

Plant growth promoting properties of *B. subtilis* BHUJP-H1, *Bacillus* sp. BHUJP-H2 and *B. licheniformis* BHUJP-H3 were showed production of IAA, HCN, ammonia and siderophore and the phosphate solubilisation except for the BHUJP-H3 that unable to produce HCN and siderophore. In the previous study, *B. subtilis*, IAA production was found more significantly increased in *Bacillus* sp. and *B. licheniformis* according to increase the concentration of tryptophan. Similarly, Ahmad et al. (2005) have also observed that *Azotobacter* and *Pseudomonas* show the ability for enhancing the IAA production according to increase in the concentration of tryptophan, where tryptophan uses a precursor for IAA synthesis under the broth culture. IAA concentration may vary between the different strains and it is also affected by growth, media and nutrient availability (Sridevi and Veera Mallaiah, 2007). IAA plays a vital role in the plant growth hormones for enhancing the cell division, plant growth, and yield (Verma et al., 2010). *Bacillus* sp. was observed more significant IAA production followed by *B. licheniformis* than the *B. subtilis*. Swain et al. (2007) have previously reported IAA producing by *B. subtilis* spp. That showed significantly higher than *Burkholderia* sp., *Bacillus* sp., *Pseudomonas* sp. BHUPSB04, *Pseudomonas* sp. BHUPSB06 and *Paenibacillus* sp. as compared to *Trichoderma* and *Azospirillum* under the broth culture in presence of tryptophan (Verma et al., 2010, 2014). *Bacillus* sp. BHUJP-H2 and *B. licheniformis* BHUJP-H3 has recorded more significant enhancement in the phosphate solubilisation than the Bacillus sp. BHUJP-H1. Phosphate solubilisation occurs by *Bacillus* species because of production of the acids for lowering the pH of broth media that support the conversion of an insoluble form of phosphate into the soluble form. These strains can be used as effective and efficient phosphate solubilising strains for enchanting the agricultural productivity. In previous reports by Rudresh et al. (2005), Valverde et al. (2006), Ahmad et al. (2008), and Verma et al. (2010, 2014) have shown that *Pseudomonas, Bacillus*, *Azotobacter*, and *Mesorhizobium* are potential phosphate solubilizers. In the present study, ammonia production was shown by all isolated strains whereas, the HCN and siderophore estimated by BHUJP-H1 and BHUJP-H2. Ammonia is significantly effective for plant growth and also the enhancing soil fertility. Whereas, the HCN and siderophore were estimated by BHUJP-H1 and BHUJP-H2. Siderophore provides the iron for plant growth. HCN production indirectly helps in plant growth by suppressing the growth of soil borne-phytopathogens and it blocked the electron chain in pathogens for decreasing the population. *Bacillus* sp. BHUJP-H1, *Bacillus* sp. BHUJP-H2 and *B. licheniformis* BHUJP-H3 show the strong ability of plant growth promoting properties. It can provide nutrients to crops by the direct mechanism including these plant growth properties like IAA, ammonia for nitrogen, siderophore for the iron and solubilisation of phosphate for phosphorus whereas, the indirect mechanism such as HCN production to suppress the plant pathogens.

Effect of Monocrotophos and Chlorpyrifos Insecticide on Growth of *Bacillus* Strains

The monocrotophos and chlorpyrifos insecticide are a commercially available group of organophosphate insecticide which are the broad range of insecticide used in agricultural production. The bacterial strains BHUJP-H1, BHUJP-H2 and BHUJP-H3 were showed no inhibition zone against monocrotophos that means these strains are more tolerant. The strains BHUJP-H1 and BHUJP-H2 showed a zone of inhibition at 1x, 2x, and 3x concentration chlorpyrifos and these strains are more susceptible. While strain BHUJP-H3 was found tolerance at all concentration of insecticides. The tolerant strain can have

the ability to degrade or resistance against monocrotophos and chlorpyrifos while the others strains but some strains have shown growth inhibition zone at a different concentration, these types stains may not have the ability to degrade the insecticides. Those strains may be degraded the insecticide and used as a sole source of carbon for their growth and development (Verma et al., 2014; Hamada et al., 2015; Jadhav and David, 2016).

Molecular Identification by 16S rDNA and Diversity Assessment of Thermophilic *Bacillus* by ISSR

The partial 16S rDNA gene sequencing, thermophilic bacterial strains BHUJP-H1, BHUJP-H2 and BHUJP-H3 were identified as Bacillus sp. BHUJP-H1 (KU312403), *B. subtilis* BHUJP-H2 (KU312404) and *B. licheniformis* BHUJP-H3 (KU312405). Yadav et al., 2015 have previously reported that 13 genera, 9 (*Bacillus, Halobacillus, Lysinibacillus, Oceanobacillus, Paenibacillus, Salinibacillus, Sediminibacillus, Thalassobacillus,* and *Virgibacillus*) belong to Bacillaceae and 4 (*Ammoniphilus, Aneurinibacillus, Brevibacillus,* and *Paenibacillus*) that belongs to Paenibacillaceae were isolated from thermal plants. Similarly, Acharya and Chaudhary (2012) isolated from Barkeshwar hot spring in West Bengal and identified as *B. licheniformis* WBS1 and *Bacillus* sp. WBS3 by 16S rRNA. The 16S rDNA is easy tools for identification of microorganisms using BLAST. Similar sequences of known bacterial strains were identified for construction of the phylogenetic tree. The phylogenetic tree between 9 taxa was constructed by using UPGMA. The thermophilic BHUJP-H3 showed similarity with the known taxa of strains HT-W34-B1 and RA32UN.

Inter simple sequence repeat markers are one of the cheapest and easiest marker systems with high efficiency in generating polymorphism among populations and being a PCR based fingerprint, very helpful and informative tool in genetic diversity studies as well as it is also a fast genotyping technique which is widely used in characterization of genetic diversity among populations (Baysal et al., 2011; Akramipour et al., 2017). In the present study the genetic diversity assessment performed by ISSR molecular markers which is generally used in microbial diversity assessment (Baysal et al., 2011; Baysal, 2015; Rayar et al., 2015; Akramipour et al., 2017). Total 7 ISSR primers used for assessment produced 7-13 bands and 67 total bands out of which 61 polymorphic bands with an average 8.71 bands per primer and 90.68 overall polymorphism efficiency. The PIC, the ability of a marker to establish polymorphism in the population depending on the number of alleles detected and on their distribution frequency (Botstein et al., 1980). Thus, PIC identifies the discriminatory ability of the marker. In present study the highest and the lowest PIC obtained by the UBC-809 and UBC-836 while the lowest, 0.28 the overall PIC value 0.40 obtained by the used marker system (**Table 6**) suggest high polymorphism among the species. For the dominant markers, the maximum PIC value is 0.5. The markers having similar distribution in population higher the PIC values. The PIC value also depends on the distribution frequency of the alleles (Chesnokov and Artemyeva, 2015). The total number of polymorphic loci (per primer) is the measure of EMR, the higher EMR value, higher the effectiveness of the primer marker system. In the present study the highest, 11.08 and lowest, 4.50 EMR obtained for primer UBC-823 and UBC-807, the average EMR value for the used primers, 7.99 obtained (**Table 6**). The high EMR suggest that the used ISSR markers are potential for the study of genetic diversity within bacterial population, our result is also supported by recent study where the ISSR markers potentially used for assessment of genetic diversity (Baysal et al., 2011; Rayar et al., 2015; Akramipour et al., 2017). The MI is a statistical factor which estimate whole effectiveness of the used maker system. The higher MI value indicate better is the method (Nagaraju et al., 2001; Chesnokov and Artemyeva, 2015). In present study the highest, 4.89 and lowest, 1.25 MI obtained with primer UBC-836 and UBC-807 respectively and with an average of 3.24 MI obtained with all used primer. The high MI value proved the suitability of ISSR marker for genetic assessment in bacterial population, the result is supported by the recent (Baysal et al., 2011; Rayar et al., 2015; Akramipour et al., 2017) findings. The ISSR assessment showed that the thermophilic *B. subtilis* BHUJP-H1 and *Bacillus* sp. BHUJP-H2 showed similar band pattern. While, *B. licheniformis* BHUJP-H3 showed different banding patterns which means this strain is genetically different from strains BHUJP-H1 and BHUJP-H2. The dendrogram was constructed by UPGMA method based to find out that the *B. subtilis* BHUJP-H1 and *Bacillus* sp. BHUJP-H2 showed the close relationship between them as compared to *B. licheniformis* BHUJP-H3. Our result also showed that all seven ISSR markers worked efficiently in genetic diversity assessment. The genetic diversity of *B. subtilis* BHUJP-H1, *Bacillus* sp. BHUJP-H2 and *B. licheniformis* BHUJP-H3 were done by ISSR profile, which classified them in two groups according to their capacity for producing various biochemical and plant growth promoting properties.

In the present study, the cluster I showed only single *B. licheniformis* BHUJP-H3 and cluster II showed two species *B. subtilis* BHUJP-H1 and *Bacillus* sp. BHUJP-H2. In both clusters, some biochemical and plant growth promoting activities were found to significant difference including IAA production, phosphate solubilisation, and ammonia while HCN and siderophore production only produced in the cluster I which belongs to group of *Bacillus* strains BHUJP-H1 and BHUJP-H2. The genetic polymorphism of *Bacillus* strains from hot spring sources by RAPD and phenotypic characteristic has been studied by Hazem and Manar (2003) and resulted in 5 major clusters with the 60% similarity. The thermophilic strains have the ability to play a key role for industrial applications. Furthermore, *Bacillus* sp. BHUJP-H1, *Bacillus* sp. BHUJP-H2 and *B. licheniformis* BHUJP-H3 can be used as drought resistance plant growth promoting strains for sustainable agricultural production. Also, these strains can be used for harnessing some industrially important enzymes because of improving the growth under the extreme environmental conditions. It can be useful for maintaining the soil health and early warning indicators of environmental changes, it became essential to study its genetic diversity.

Effect of Bio-inoculant of *Bacillus* Strains on Plant Growth Attributes of *Vigna radiata* Under Plant Growth Chamber

Plant growth of any plants was affected by soil nutrient content which is totally governed by the different types of microbes and their physiological and biological process to help in enhancing soil fertility and health under rhizosphere. The healthy rhizosphere plays an important role in enhancing the plant growth by the direct indirect mechanism of soil microbes Verma et al. (2010). We have attempted to take different treatment combination of plant growth promoting bacillus strains for enhancing plant growth attributed under plant growth chamber. The significant enhancement of shoot length (cm/plant) in treatment combination of *B. subtilis* BHUJP-H1, *Bacillus* sp. BHUJP-H2, *B. subtilis* BHUJP-H1+ *Bacillus* sp. BHUJP-H2, *B. subtilis* BHUJP-H1+ *B. licheniformis* BHUJP-H3, *Bacillus* sp. BHUJP-H2+ *B. licheniformis* BHUJP-H3 and *B. subtilis* BHUJP-H1+ *Bacillus* sp. BHUJP-H2+ *B. licheniformis* BHUJP-H3 were recorded as compared to control (Un-inoculated) and *Bacillus licheniformis* BHUJP-H3 after 10 days seed inoculation. The combination *B. subtilis* BHUJP-H1+ *B. licheniformis* BHUJP-H3, *B. subtilis* BHUJP-H1 and *B. subtilis* BHUJP-H1+ *Bacillus sp.* BHUJP-H2+ *B. licheniformis* BHUJP-H3 were found a more significant increase in shoot length followed by others, because the *B. subtilis* BHUJP-H1 and *B. licheniformis* BHUJP-H3 produce more IAA which help as plant growth hormones to promote shoot growth as compared to *Bacillus* sp. BHUJP-H3. While the strain *B. subtilis* BHUJP-H1 was not enhanced significant growth of root length but root length recorded higher than control. The possibility of non-significant root length growth by *Bacillus subtilis* BHUJP-H1 can be very short experiment in small cup or may be production of HCN can be inhibit the growth of root length. Some studies by Bakker and Schippers (1987) and Bakker et al. (1989) have been reported that the *Pseudomonas* spp. suspected to inhibit potato root development by their production of hydrogen cyanide. Cyanide producing *Pseudomonas* spp. also causes growth inhibition in lettuce and bean (Alstrom and Burns, 1989; Schippers et al., 1990).

Others, strains BHUJP-H3 and BHUJP-H3 showed maximum phosphate solubilisation and ammonia production while *Bacillus subtilis* BHUJP-H1 showed average phosphate solubilisation, Ammonia, HCN, and siderophore. These parameters may be support for enhancing shoot length of mungbean plant in soils under plant growth chambers. The combination of BHUJP-H1+ BHUJP-H2+ BHUJP-H3, BHUJP-H1+ BHUJP-H2, BHUJP-H1+ BHUJP-H3, and BHUJP-H2+ BHUJP-H3 were recorded more significant increase in root length as compared to control. Fresh shoot weight was significantly observed more in all treatment combinations as per control. The treatment *Bacillus licheniformis* BHUJP-H3 gave only significant enhancement of root weight (g/plant) than others while the enhancement of root weight was observed in others treatment combination. The leaf fresh weight (g/plant) was recorded more significant in treatment combination of BHUJP-H1+ BHUJP-H3 and BHUJP-H1+ BHUJP-H2, BHUJP-H1+ BHUJP-H2+ BHUJP-H3 as compared to control after 10 days seedling growth. Overall, the impact of different treatment combination of *Bacillus* strains has enhanced the shoot length and fresh shoot weight as compared to control. Similarly, the enhancement of plant growth was found due to plant growth promoting properties of different strains. Ait Kaki et al. (2017) reported that *B. amyloliquefaciens* (4RH) strain showed very significant property of biocontrol and biofertilization characteristics under *in vitro* so he recommended a potential agent for future bioinsecticide for integrate pest management and organic agricultural productions. Figueiredo et al. (2008) have been found that CIAT 899 rhizobia strains co-inoculated with *Paenibacillus polymyxa* strain DSM 36 which enhance higher shoot and root dry weight than single inoculation with CIAT 899 strain in common bean. Similarly, Elkoca et al. (2010) reported an increased shoot dry weight as a result of co-inoculation of common bean with *B. megaterium* (M-3) strain and *Rhizobium* strain. The treatment combination of *B. subtilis* BHUJP-H1, *B. subtilis* BHUJP-H1+ *B. licheniformis* BHUJP-H3 and *B. subtilis* BHUJP-H1+ *Bacillus* sp. BHUJP-H2+ *Bacillus licheniformis* BHUJP-H3 were recorded more better combination for enhancing plant growth attributes of *Vigna radiata* followed by *B. subtilis* BHUJP-H1+ *Bacillus* sp. BHUJP-H2 and *Bacillus* sp. BHUJP-H2+ *B. licheniformis* BHUJP-H3 as compared to control and others.

CONCLUSION

The optimal growth of strain BHUJP-H1 was at the extreme temperature of 60°C as compared to BHUJP-H2 and BHUJP-H3. BHUJP-H1 was found efficient growth at temperature 60°C than 30, 40, and 50°C. *Bacillus* sp. BHUJP-H1, *Bacillus* sp. BHUJP-H2 and *B. licheniformis* BHUJP-H3 were produced catalase, cellulase, ammonia, HCN, siderophore, IAA and also solubilised the phosphate, while *B. licheniformis* BHUJP-H3 could not be produced HCN and siderophore. Strain BHUJP-H1, *Bacillus* sp. BHUJP-H2 and *B. licheniformis* BHUJP-H3 gave good cellulase activities and tolerant against monocrotophos insecticide while straining BHUJP-H1 and BHUJP-H2 showed susceptible against chlorpyrifos at 1x, 2x, and 3x concentration. The treatment combination of *B. subtilis* BHUJP-H1, *B. subtilis* BHUJP-H1+ *B. licheniformis* BHUJP-H3 and *B. subtilis* BHUJP-H1+ *Bacillus* sp. BHUJP-H2+ *B. licheniformis* BHUJP-H3 were recorded better combination for enhancing plant growth attributes of *Vigna radiata* followed by as compared to control and others. The stains *B. subtilis* BHUJP-H1, *B. subtilis* BHUJP-H1+ *B. licheniformis* BHUJP-H3, and *B. subtilis* BHUJP-H1+ *Bacillus* sp. BHUJP-H2+ *B. licheniformis* BHUJP-H3 can be further used as effective microbial inoculant for enhancing production of mungbean under field conditions. Others strain *Bacillus* sp. BHUJP-H1 and *Bacillus* sp. BHUJP-H2 can be used as drought tolerant plant growth promoting bacteria for enhancing the sustainable agriculture production. In future, these strains can be used as a consortium for drought tolerant bio-inoculants for agricultural farming.

AUTHOR CONTRIBUTIONS

JV wrote and edited the manuscript as well as made design experiment. DJ did all the experiment from microbial isolation to their properties and plant growth analysis. SP provided the soil samples for microbial isolation, helped with editing, and provided suggestions for the experiment. JY provided the lab facility for microbial isolation, helped with editing and the experiment designing. VS helped with manuscript editing and provided suggestions for molecular diversity. RK helped with molecular diversity analysis of bacterial strains by ISSR primers and wrote this art in manuscript.

ACKNOWLEDGMENTS

The authors thank CSIR, New Delhi for providing the fund for research and development on cellulose degrading bacteria and also thanks to DST and DBT for developing plant growth promoting consortium as biofertilizers.

REFERENCES

Abd-Elhalem, B. T., El-Sawy, M., Gamal, R. F., and Abou-Taleb, K. A. (2015). Production of amylases from *Bacillus amyloliquefaciens* under submerged fermentation using some agro-industrial by-products. *Ann. Agric. Sci.* 60, 193–202. doi: 10.1016/j.aoas.2015.06.001

Acharya, S., and Chaudhary, A. (2012). Alkaline cellulase produced by a newly isolated thermophilic *Aneurinibacillus thermoaerophilus* WBS2 from hot spring, India. *Afr. J. Microbiol. Res.* 6, 5453–5458.

Ahmad, F., Ahmad, I., and Khan, M. S. (2005). Indole acetic acid production by the indigenous isolates of *Azotobacter* and fluorescent *Pseudomonas* in the presence and absence of tryptophan. *Turkish J. Biol.* 29, 29–34.

Ahmad, F., Ahmad, I., and Khan, M. S. (2008). Screening of free-living rhizospheric bacteria for their multiple plant growth promoting activities. *Microbiol. Res.* 163, 173–181. doi: 10.1016/j.micres.2006.04.001

Ait Kaki, A., Kara Ali, M., Milet, A., Moula, N., Thonart, P., and Kacem Chaouche, N. (2017). In vitro biocontrol and biofertilization features study of a *Bacillus amyloliquefaciens* (4RH) strain isolated from a hot spring soil in Algeria. *Afr. J. Microbiol. Res.* 11, 1564–1572. doi: 10.5897/AJMR2017.8745

Akramipour, N., Abdollahi, M., Taghavi, S. M., and Rezaei, R. (2017). Genetic diversity of soft rot strains of *Pectobacterium*, isolated from different hosts, using ISSR marker. *Arch. Phytopathol. Plant Prot.* 50, 789–801. doi: 10.1080/03235408.2017.1384195

Alstrom, S., and Burns, R. G. (1989). Cyanide production by rhizobacteria as a possible mechanism of plant growth inhibition. *Biol. Fertil. Soils* 7, 232–238. doi: 10.1007/BF00709654

Aneja, K. R. (2003). *Experiments in Microbiology, Plant Pathology and Biotechnology*, 4th Edn. Daryaganj: New Age International Publishers.

Bakker, A. W., Bakker, P. A. H. M., and Schippers, B. (1989). "Deleterious cyanide-producing rhizosphere pseudomonads as a factor limiting potato root growth and tuber yield in high frequency potato-cropping soil," in *Effects of Crop Rotation on Potato Production in the Temperate Zones*, eds J. Vos, C. D. van Loon, and G. J. Bollen (Dordrecht: Kluwer Academic Publishers), 153–162.

Bakker, A. W., and Schippers, B. (1987). Microbial cyanide production in the rhizosphere in relation to potato yield reduction and *Pseudomonas* spp.-mediated plant growth stimulation. *Soil Biol. Biochem.* 19, 249–256. doi: 10.1016/0038-0717(87)90037-X

Bauer, A. W., Kirby, W. M. M., Sherris, J. C., and Turck, M. (1966). Antibiotic susceptibility testing by a standardized single disk method. *Am. J. Clin. Pathol.* 45, 493–496. doi: 10.1093/ajcp/45.4_ts.493

Baysal, Ö. (2015). "Host resistance: SAR and ISR to plant pathogenic bacteria," in *Sustainable Approaches to Controlling Plant Pathogenic Bacteria*, eds V. Kannan and K. Bastas (Boca Raton, FL: CRC Press), 205–222. doi: 10.1201/b18892-11

Baysal, Ö., Mercati, F., İkten, H., Yıldız, R. Ç, Carimi, F., Aysan, Y., et al. (2011). *Clavibacter michiganensis* subsp. *michiganesis*: tracking strains using their genetic differentiations by ISSR markers in Southern Turkey. *Physiol. Mol. Plant Pathol.* 75, 113–119. doi: 10.1016/j.pmpp.2010.10.002

Belkova, N. L., Tazaki, K., Zakharova, J. R., and Parfenova, V. V. (2007). The activity of bacteria in water of hot springs from Southern and Central Kamchatskaya geothermal provinces, Kamchatka Peninsula, Russia. *Microbiol. Res.* 162, 99–107. doi: 10.1016/j.micres.2006.01.006

Botstein, D., White, R. L., Skolnick, M., and Davis, R. W. (1980). Construction of a genetic linkage map in man using restriction fragment length polymorphisms. *Am. J. Hum. Genet.* 32, 314–331.

Bric, J. M., Bostock, R. M., and Silverstone, S. E. (1991). Rapid in situ assay for indole acetic acid production by bacteria immobilized on nitrocellulose membrane. *Appl. Environ. Microbiol.* 57, 535–538.

Cappuccino, J. G., and Sherman, N. (1992). *Microbiology, a Laboratory Manual*, 3rd Edn. New York, NY: Benjamin/Cummings Publication Co, 125–179.

Chesnokov, Y. V., and Artemyeva, A. M. (2015). Evaluation of the measure of polymorphism information of genetic diversity. *Agric. Biol.* 50, 571–578.

Cihan, A. C., Tekin, N., Ozcan, B., and Cokmus, C. (2012). The genetic diversity of genus *Bacillus* and the related genera revealed by 16S rRNA gene sequences and ardra analyses isolated from geothermal regions of turkey. *Braz. J. Microbiol.* 43, 309–324. doi: 10.1590/S1517-83822012000 1000037

Claus, D., and Fritz, D. (1989). "Taxonomy of bacillus," in *Bacillus*, ed. C. R. Harwood (New York, NY: Plenum Press), 5–26.

Coolbear, T., Daniel, R., and Morgan, H. W. (1992). The enzymes from extreme thermophiles, bacterial sources, thermos ability and industrial relevance. *Adv. Biochem. Eng. Biotechnol.* 45, 57–98. doi: 10.1007/BFb0008756

Elkoca, E., Turan, M., and Donmez, M. F. (2010). Effects of single, dual and triple inoculations with *Bacillus subtilis*, *Bacillus megaterium* and *Rhizobium leguminosarum* BV. *phaseoli* on nodulation, nutrient uptake, yield and yield parameters of common bean (*Phaseolus vulgaris* L. CV. 'ELKOCA-05'). *J. Plant Nutr.* 33, 2104–2119. doi: 10.1080/01904167.2010.519084

Figueiredo, M. V. B., Martinez, C. R., Burity, H. A., and Chanway, C. P. (2008). Plant growth-promoting rhizobacteria for improving nodulation and nitrogen fixation in the common bean (*Phaseolus vulgaris* L.). *World J. Microbiol. Biotechnol.* 24, 1187–1193. doi: 10.1007/s11274-007-9591-4

Ghati, A., Sarkar, K., and Paul, G. (2013). Isolation, characterization and molecular identification of esterolytic thermophilic bacteria from an Indian hot spring. *Curr. Res. Microbiol. Biotechnol.* 1, 196–202.

Ghosh, D., Bal, B., Kashyap, V. K., and Pal, S. (2003). Molecular phylogenetic exploration of bacterial diversity in a Bakreshwar (India) hot spring and culture of Shewanella related thermophiles. *Appl. Environ. Microbial.* 69, 4332–4336. doi: 10.1128/AEM.69.7.4332-4336.2003

Gilbert, J. J., and Jack, J. D. (1993). Rotifers as predators on small ciliates. *Hydrobiologia* 255, 247–253. doi: 10.1007/BF00025845

Gutiérrez-Manero, F. J., Ramos-Solano, B., Probanza, A., Mehouachi, J., R Tadeo, F., and Talon, M. (2001). The plant-growth-promoting rhizobacteria *Bacillus pumilus* and *Bacillus licheniformis* produce high amounts of physiologically active gibberellins. *Physiol. Plant.* 111, 206–211. doi: 10.1034/j.1399-3054.2001.1110211.x

Hamada, M., Matar, A., and Bashir, A. (2015). Carbaryl degradation by bacterial isolates from a soil ecosystem of the gaza strip. *Braz. J. Microbiol.* 46, 1087–1091. doi: 10.1590/S1517-838246420150177

Hazem, A., and Manar, A. (2003). Genetic polymorphism by RAPD-PCR and phenotypic characteristics of isolated thermotolerant Bacillus strains from hot spring sources. *New Microbiol.* 26, 249–256.

Huber, R., Eder, W., Heldwein, S., Wanner, G., Huber, H., Rachel, R., et al. (1998). *Thermocrinis ruber* gen. nov., sp. nov., a pink filament-forming hyperthermophilic bacterium isolated from Yellowstone National Park. *Appl. Environ. Microbiol.* 164, 3576–3583.

Jadhav, S. S., and David, M. (2016). Biodegradation of flubendiamide by a newly isolated *Chryseobacterium* sp. strain SSJ1. *3 Biotech* 6:31. doi: 10.1007/s13205-015-0347-9

Kayasth, M., Kumar, V., and Gera, R. (2013). Exploring the potential of PGPR strain bacillus licheniformis to be developed as multifunctional biofertilizer. *Cent. Eur. J. Biol.* 2, 12–17.

Kecha, M., Benallaoua, S., Touzel, J. P., Bonaly, R., and Duchiron, F. (2007). Biochemical and phylogenetic characterization of novel terrestrial hyperthermophilic archaeon pertaining to the genus *Pyrococcus* from an Algerian hydrothermal hot spring. *Extremophiles* 11, 65–73. doi: 10.1007/s00792-006-0010-9

Khan, M., and Patel, C. B. (2007). Plant growth promoting the effect of *Bacillus firmus* strain NARS1 isolated from the central Himalayan region of India on *Cicer arietinum* at low temperature. *Egypt Afr. Crop Sci. Soc.* 8, 1179–1181.

Kloepper, J. W., Rodríguez-Kábana, R., Mcinroy, J. A., and Young, R. W. (1992). Rhizosphere bacteria antagonistic to soybean cyst (*Heterodera glycines*) and root-knot (*Meloidogyne incognita*) nematodes: identification by fatty acid analysis and frequency of biological control activity. *Plant Soil* 139, 75–84. doi: 10.1007/BF00012844

Kuddus, M., and Ramtekke, P. W. (2012). Recent developments in production and biotechnological applications of cold-active microbial proteases. *Crit. Rev. Microbiol.* 38, 380–388. doi: 10.3109/1040841X.2012.678477

Kumar, P., Patel, S. K., Lee, J. K., and Kalia, V. C. (2013). Extending the limits of Bacillus for novel biotechnological applications. *Biotechnol. Adv.* 31, 1543–1561. doi: 10.1016/j.biotechadv.2013.08.007

Lele, O. H., and Deshmukh, P. V. (2016). Isolation and characterization of thermophilic *Bacillus* sp. with extracellular enzymatic activities from hot spring of Ganeshpuri, Maharashtra, India. *Int. J. Appl. Res.* 2, 427–430.

Lorck, H. (1948). Production of hydrocyanic acid by bacteria. *Physiol. Plant.* 1, 142–146. doi: 10.1111/j.1399-3054.1948.tb07118.x

Mallik, M. A. B., and Tesfai, K. (1983). Compatibility of *Rhizobium japonicum* with commercial pesticides *in vitro*. *Bull. Environ. Contam. Toxicol.* 31, 432–437. doi: 10.1007/BF01622274

Martensson, A. M. (1992). Effects of agrochemicals and heavy metals on fast-growing rhizobia and their symbiosis with small-seeded legumes. *Soil Biol. Biochem.* 24, 435–445. doi: 10.1016/0038-0717(92)90206-D

Miller, C. S., Handley, K. M., Wrighton, K. C., Frischkorn, K. R., Thomas, B. C., and Banfield, J. F. (2013). Short-read assembly of full-length 16S amplicons reveals bacterial diversity in subsurface sediments. *PLoS One* 8:56018. doi: 10.1371/journal.pone.0056018

Mohammad, B. T., Al Daghistani, H. I., Jaouani, A., Abdel-Latif, S., and Kennes, C. (2017). Isolation and characterization of thermophilic bacteria from Jordanian hot springs: *Bacillus licheniformis* and *Thermomonas hydrothermalis* isolates as potential producers of thermostable enzymes. *Int. J. Microbiol.* 2017:6943952. doi: 10.1155/2017/6943952

Nagaraju, J., Reddy, K. D., Nagaraja, G. M., and Sethuraman, B. N. (2001). Comparison of multilocus RFLPs and PCR-based marker systems for genetic analysis of the silkworm, *Bombyx mori*. *Heredity* 86, 588–597. doi: 10.1046/j.1365-2540.2001.00861.x

Nazina, T. N., Lebedeva, E. V., Poltaraus, A. B., Tourova, T. P., Grigoryan, A. A., and Sokolova, D. S. H. (2004). *Geobacillus gargensis* sp. Nov., a novel thermophile from a hot spring, and the reclassification of *Bacillus vulcanic* as *Geobacillus vulcani* comb. nov. *Int. J. Syst. Evol. Microbiol.* 54, 2019–2024. doi: 10.1099/ijs.0.02932-0

Nei, M., Stephens, J. C., and Saitou, N. (1985). Methods for computing the standard errors of branching points in an evolutionary tree and their application to molecular data from humans and apes. *Mol. Biol. Evol.* 2, 66–85.

Panda, A. K., Bisht, S. S., Kumar, N. S., and Mandal, S. D. (2015). Investigations on the microbial diversity of Jakrem hot spring, Meghalaya, India using cultivation-independent approach. *Genomics* 4, 156–157. doi: 10.1016/j.gdata.2015.04.016

Perez, C., Munoz-Garay, C., Portugal, L. C., Sanchez, J., Gill, S. S., Soberon, M., et al. (2007). *Bacillus thuringiensis* ssp. Israelis Cyt1Aa enhances an activity of Cry11Aa toxin by facilitating the formation of a pre-pore oligomeric structure. *Cell Microbiol.* 9, 2931–2937. doi: 10.1111/j.1462-5822.2007.01007.x

Pikovskaya, R. I. (1948). Mobilization of phosphorus in soil in connection with the vital activity of some microbial species. *Microbiology* 17, 362–370.

Powell, W., Morgante, M., Andre, C., Hanafey, M., Vogel, J., Tingey, S., et al. (1996). The comparison of RFLP, RAPD, AFLP and SSR (microsatellite) markers for germplasm analysis. *Mol. Breed.* 2, 225–238. doi: 10.1007/BF00564200

Rayar, J. K., Arif, M., and Singh, U. S. (2015). Relative efficiency of RAPD and ISSR markers in assessment of DNA polymorphism and genetic diversity among *Pseudomonas* strains. *Afr. J. Biotechnol.* 14, 1097–1106. doi: 10.5897/AJB10.1951

Reysenbach, A. L., Ehringer, M., and Hershberger, K. (2000). Microbial diversity at 83°C in the calcite springs, Yellowstone national park: another environment where the *Aquificales* and "Korarchaeota" coexist. *Extremophiles* 4, 61–67.

Reysenbach, A. L., Wickham, G. S., and Pace, N. R. (1994). Phylogenetic analysis of the hyperthermophilic pink filament community in Octopus spring, Yellowstone national park. *Appl. Environ. Microbiol.* 60, 2113–2119.

Roldàn-Ruiz, I., Dendauw, J., Van Bockstaele, E., Depicker, A., and De Loose, M. (2000). AFLP markers reveal high polymorphic rates in ryegrasses (*Lolium* spp.). *Mol. Breed.* 6, 125–134. doi: 10.1023/A:1009680614564

Rudresh, D. L., Shivaprakash, M. K., and Prasad, R. D. (2005). Effect of combined application of Rhizobium, phosphate solubilizing bacterium and *Trichoderma* spp. on growth, nutrient uptake and yield of chickpea (*Cicer aritenium* L.). *Appl. Soil Ecol.* 28, 139–146. doi: 10.1016/j.apsoil.2004.07.005

Sadfi, N., Cherif, M., Fliss, I., Boudabbous, A., and Antoun, H. (2001). Evaluation of bacterial isolates from salty soils and bacillus thuringiensis strains for the biocontrol of fusarium dry rot of potato tubers. *J. Plant Pathol.* 101–117. doi: 10.4454/jpp.v83i2.1118

Saharan, B. S., and Verma, S. (2014). Potential plant growth promoting activity of *Bacillus licheniformis* UHI(II)7. *Int. J. Microbial Res. Technol.* 2, 22–27.

Sambrook, J., and Russel, D. W. (2001). *Molecular Cloning: A Laboratory Manual*, 3rd Edn. Cold Spring Harbor, NY: Coldspring-Harbour Laboratory Press.

Satyanarayana, T., Raghukumar, C., and Shivaji, S. (2005). Extremophilic microbes: diversity and perspectives. *Curr. Sci.* 89, 78–90.

Schaffer, C., Franck, W. L., Scheberl, A., Kosma, P., McDermott, T. R., and Messner, P. (2004). Classification of isolates from locations in Austria and Yellowstone National Park as *Geobacillus tepidamans* sp. nov. *Int. J. Syst. Evol. Microbiol.* 54, 2361–2368. doi: 10.1099/ijs.0.63227-0

Schippers, B., Bakker, A. W., Bakker, P. A. H. M., and Van Peer, R. (1990). Beneficial and deleterious effects of HCN-producing pseudomonads on rhizosphere interactions. *Plant Soil* 129, 75–83. doi: 10.1007/BF00011693

Sharma, A., Pandey, A., Shouche, Y. S., Kumar, B., and Kulkarni, G. (2009). Characterization and identification of *Geobacillus* spp. isolated from Soldhar hot spring site of Garhwal Himalaya, India. *J. Basic Microbiol.* 49, 187–194. doi: 10.1002/jobm.200800194

Siangbood, H., and Ramanujam, P. (2011). A report on thermophilic *Cyanophyta* (Cyanobacteria) from Jakremhotspring, Meghalaya. *Int. J. Algae* 13, 178–185. doi: 10.1615/InterJAlgae.v13.i2.70

Soltanpour, P. N., and Workman, S. M. (1981). Soil-testing methods used at Colorado State University Soil-Testing Laboratory for the Evaluation of Fertility, Salinity, Sodicity, and Trace-Element Toxicity. Fort Collins, CO: Colorado Technology Bulletin, 14–22.

Sridevi, M., and Veera Mallaiah, K. (2007). Bioproduction of indole acetic acid by *Rhizobium* strains isolated from root nodules of green manure crop, *Sesbania sesban* (L.) Merr. *Iran. J. Biotechnol.* 5, 178–182.

Srinivas, T. N. R., Rao, S. N., Reddy, P. V. V., Pratibha, M. S., Sailaja, B., Kavya, B., et al. (2009). Bacterial diversity and bio prospecting for cold-active lipases, amylases and proteases, from culturable bacteria of Kongsfjorden and Ny-Ålesund, Svalbard, Arctic. *Curr. Microbiol.* 59, 537–554. doi: 10.1007/s00284-009-9473-0

Swain, M. R., Naskar, S. K., and Ray, R. C. (2007). Indole-3-acetic acid production and effect on sprouting of yam (*Dioscorea rotundata* L.) minisetts by *Bacillus subtilis* isolated from culturable cow dung microflora. *Pol. J. Microbiol.* 56, 103–110.

Tamura, K., Dudley, J., Nei, M., and Kumar, S. (2007). MEGA4: molecular evolutionary genetics analysis (MEGA) software version 4.0. *Mol. Biol. Evol.* 24, 1596–1599. doi: 10.1093/molbev/msm092

Tringe, S. G., and Hugenholtz, P. (2008). A renaissance for the pioneering 16S rRNA gene. *Curr. Opin. Microbiol.* 11, 442–446. doi: 10.1016/j.mib.2008.09.011

Valverde, A., Burgos, A., Fiscella, T., Rivas, R., Velaz-quez, E., Rodriguez-Barrueco, C., et al. (2006). Differential effects of inoculations with *Pseudomonas jessenii* PS06 (a phosphate-solubilizing bacteria) and *Mesorhizobium ciceri* C-2/2 strains on the growth and seed yield of chickpea under greenhouse and field conditions. *Plant Soil* 287, 43–50. doi: 10.1007/s11104-006-9057-8

Van den Burg, B. B. (2003). Extremophiles as a source of novel enzymes. *Curr. Opin. Microbiol.* 6, 213–218. doi: 10.1016/S1369-5274(03)00060-2

Verma, J. P., Tiwari, K. N., Yadav, J., and Mishra, A. K. (2016). Development of microbial consortia for growth attributes and protein content in micropropagated *Bacopa monnieri* (L.). *Proc. Natl. Acad. Sci. India Sec. B Biol. Sci.* 88, 143–151.

Verma, J. P., and Yadav, J. (2018). Implication of microbial consortium on biomass and yield of chickpea under sustainable agriculture. *Environ. Eng. Manage. J.* 17, 513–522.

Verma, J. P., Yadav, J., Tiwari, K. N., and Jaiswal, D. K. (2014). Evaluation of plant growth promoting activities of microbial strains and their effect on growth and yield of chickpea (*Cicer arietinum* L.) in India. *Soil Biol. Biochem.* 70, 33–37. doi: 10.1016/j.soilbio.2013.12.001

Verma, J. P., Yadav, J., Tiwari, K. N., and Kumar, A. (2013). Effect of indigenous *Mesorhizobium* spp. and plant growth promoting rhizobacteria on yields and nutrients uptake of chickpea (*Cicer arietinum* L.) under sustainable agriculture. *Ecol. Eng.* 51, 282–286. doi: 10.1016/j.ecoleng.2012.12.022

Verma, J. P., Yadav, J., Tiwari, K. N., Lavakush, and Singh, V. (2010). The impact of Plant growth promoting rhizobacteria on crop production. *Int. J. Agric. Res.* 954–983. doi: 10.3923/ijar.2010.954.983

Watanabe, F. S., and Olsen, S. R. (1965). Test of an ascorbic acid method for determining phosphorus in water and $NaHCO_3$ extracts from soil. *Soil Sci. Soc. Am. Proc.* 29, 677–678. doi: 10.2136/sssaj1965.03615995002900060025x

Yadav, A. N., Verma, P., Kumar, M., Pal, K. K., Dey, R., Gupta, A., et al. (2015). Diversity and phylogenetic profiling of niche-specific Bacilli from extreme environments of India. *Ann. Microbiol.* 65, 611–629. doi: 10.1007/s13213-014-0897-9

Yadav, S., Kaushik, R., Saxena, A. K., and Arora, D. K. (2011). Diversity and phylogeny of plant growth-promoting bacilli from the moderately acidic soil. *J. Basic Microbiol.* 51, 98–106. doi: 10.1002/jobm.201000098

Zhang, D., Du, G., and Chen, J. (2010). Fermentation production of microbial catalase and its application in textile industry. *Sheng Wu Gong Cheng Xue Bao* 26, 1473–1481.

Conflict of Interest Statement: The authors declare that the research was conducted in the absence of any commercial or financial relationships that could be construed as a potential conflict of interest.

frontiers in Plant Science

ORIGINAL RESEARCH
published: 17 July 2018
doi: 10.3389/fpls.2018.01017

Earthworm Grazed-*Trichoderma harzianum* Biofortified Spent Mushroom Substrates Modulate Accumulation of Natural Antioxidants and Bio-Fortification of Mineral Nutrients in Tomato

*Udai B. Singh[1], Deepti Malviya[1†], Wasiullah Khan[1†], Shailendra Singh[1†], N. Karthikeyan[1], Mohd. Imran[2], Jai P. Rai[3], B. K. Sarma[4], M. C. Manna[5], Rajan Chaurasia[6], Arun K. Sharma[1], Diby Paul[7] and Jae-Wook Oh[8]**

[1] Plant-Microbe Interaction and Rhizosphere Biology Lab, ICAR-National Bureau of Agriculturally Important Microorganisms (ICAR-NBAIM), Maunath Bhanjan, India, [2] Department of Biosciences, Faculty of Science, Integral University, Lucknow, India, [3] Department of Mycology and Plant Pathology (Krishi Vigyan Kendra), Institute of Agricultural Sciences, Banaras Hindu University, Varanasi, India, [4] Department of Mycology and Plant Pathology, Institute of Agricultural Sciences, Banaras Hindu University, Varanasi, India, [5] Division of Soil Biology, ICAR-Indian Institute of Soil Science, Bhopal, India, [6] Department of Botany, Faculty of Science, Banaras Hindu University, Varanasi, India, [7] Pilgram Marpeck School of STEM, Truett McConnel University, Cleveland, GA, United States, [8] Department of Animal Biotechnology, Konkuk University, Seoul, South Korea

OPEN ACCESS

Edited by:
Youssef Rouphael,
Università degli Studi di Napoli Federico II, Italy

Reviewed by:
Alessandra Salvioli,
Università degli Studi di Torino, Italy
László Kredics,
University of Szeged, Hungary

***Correspondence:**
Jae-Wook Oh
ohjw@konkuk.ac.kr

[†] These authors have contributed equally to this work.

Specialty section:
This article was submitted to Plant Microbe Interactions, a section of the journal Frontiers in Plant Science

Received: 28 March 2018
Accepted: 22 June 2018
Published: 17 July 2018

Citation:
Singh UB, Malviya D, Khan W, Singh S, Karthikeyan N, Imran M, Rai JP, Sarma BK, Manna MC, Chaurasia R, Sharma AK, Paul D and Oh J-W (2018) Earthworm Grazed-Trichoderma harzianum Biofortified Spent Mushroom Substrates Modulate Accumulation of Natural Antioxidants and Bio-Fortification of Mineral Nutrients in Tomato. Front. Plant Sci. 9:1017. doi: 10.3389/fpls.2018.01017

The present investigation was aimed at evaluating the impact of earthworm grazed and *Trichoderma harzianum* biofortified spent mushroom substrate (SMS) on natural antioxidant and nutritional properties of tomato. Results of the investigation reveal that earthworm grazing and *T. harzianum* bio-fortification led to significant improvement in the physico-chemical properties of fresh SMS and its application increased the accumulation of natural antioxidants and mineral content in tomato as compared to either *T. harzianum* biofortified SMS or fresh SMS. In particular, the earthworm grazed, *T. harzianum* biofortified SMS (EGTHB-SMS) was found to inhibit lipid peroxidation and protein oxidation with significant increase in total polyphenol and flavonoid content in tomato. Further, it increased Fe^{2+}/Fe^{3+} chelating activity, superoxide anion radical scavenging activity compared to other treatments. The results thus suggest an augmented elicitation of natural antioxidant properties in tomato treated with EGTHB-SMS, resulting in a higher radical scavenging activity, that is highly desirable for human health. In addition, the use of SMS to enhance the nutritional value of tomato fruits becomes an environment friendly approach in sustainable crop production.

Keywords: *Trichoderma harzianum*, solid-state cultivation, spent mushroom substrate, antioxidants, ROS, lipid peroxidation, protein oxidation

INTRODUCTION

People are increasingly becoming more conscious about their health and more attention is being paid to the nutritional value of human diet with an eye on role of mineral nutrients and antioxidants in health. Several epidemiological studies have reported that high intake of green vegetables, fruits and pulses is associated with reduced risk of a number of chronic diseases (Singh et al., 2010)

due to imbalance in the antioxidant and prooxidant homeostasis in human body (Nautiyal et al., 2008). Such conditions dominate either due to increased generation of free radicals (FRs) caused by excessive oxidative stress or due to poor scavenging/quenching of FRs in the body (Bhanja et al., 2009; Ahmad et al., 2016). Free radicals are chemically unstable compounds that results in damage to lipid cells, proteins and DNA (Ahmad et al., 2016). Reactive oxygen species (ROS) are produced intra-cellularly through multiple mechanisms and depending on the cell and tissue types, the major sources being the 'professional' producers of ROS NADPH oxidase (NOX) complexes in cell membranes, mitochondria, peroxisomes, and endoplasmic reticulum (Singh et al., 2009b; Singh et al., 2010). Biological systems have several antioxidant defense mechanisms that prevent the destructive effects of ROS and FRs such as antioxidative compounds and enzymes (Nautiyal et al., 2008; Ahmad et al., 2016). In aerobic organisms, the major target sites for ROS and FRs are the cellular membrane lipids, proteins and DNA and they cause alterations in membrane structure and its function (Ahmad et al., 2016). A wide range of antioxidants, both natural and synthetic have been used as external supplements in the regular diet that could influence and suppress/slow down the processes including lipid oxidation, binding of transitional metal ion catalysts, decomposition of peroxides, etc. Increase in FR scavenging through simple or complex cellular mechanisms both in living organisms as well as in the food industry has been well documented (Prakash et al., 2007b; Singh et al., 2009b, 2010). Due to the apparent toxicity of synthetic antioxidants, currently, the interest of the society in natural antioxidants is increasing day by day (Huang and Wang, 2004).

Tomato (*Solanum lycopersicum*) is one of the most important vegetable crops consumed across the various climatic regions of the world. Tomato and its products serve as rich sources of dietary bioactive molecules such as lycopene, beta-carotene, phytoene, phytofluene, folic acid, flavonoids and vitamin C (George et al., 2004; Hyman et al., 2004). These bioactive compounds are well-studied for their health protective abilities especially against cardiovascular diseases, coronary heart diseases and various types of cancer. Similarly, *Trichoderma harzianum* (avirulent, plant beneficial fungus) is a common soil inhabitant broadly distributed in terrestrial ecosystems (Harman et al., 2004) and plays many important roles in diverse natural environments. Although the fungus absorbs nutrition from dead organic materials as a saprophyte, majority of its population is concentrated in nutrient rich niches like the rhizosphere. In agriculture, *T. harzianum* is primarily known for its biocontrol activities against soilborne phytopathogens but other multifarious activities have also been recorded by various researchers. Therefore, the ability of the fungus to colonize roots of crop plants is of a great advantage when it is used as a nutrient mobilizer, plant growth promoter and biological control agent (Harman et al., 2004; Sarma et al., 2015). The ability to colonize root and endophytic behavior of *Trichoderma* spp. in plant roots are proven and it was found that *Trichoderma* responds chemotropically to roots. The response of root cells to colonization by fungi may have profound implications in the performance of these organisms as plant growth promoting as well as biocontrol agents of soilborne plant pathogens (Harman et al., 2004). There are strains of *T. harzianum* which improve nutrient uptake and translocation in plants, resulting in enhanced nutritional value of the produce there from Glick (1995), Berg (2009).

Spent mushroom substrate (SMS) is a by-product of mushroom industry, rich in organic matter and other essential nutrients for plant growth. Annually, mushroom industries release more than 50 million tons of SMS as waste material (Fox and Chorover, 1999) which is vastly under utilized. A larger portion of SMS is carelessly disposed outside the production unit itself begetting a number of environmental issues. Piling-up of SMS may cause various environmental problems/pollution which further leads to groundwater contamination and production of greenhouse gasses such as carbon dioxide and nitrous oxide causing global warming and loss of essential plant nutrients (Beyer, 1996). In recent years, attention has been made toward more amicable ways of SMS disposal. These surplus residues can be recycled for sustaining soil health and enhancing crop productivity because the SMS is rich source of organic matter and mineral nutrients. Some of the researchers used SMS as organic amendment in various vegetable and high value crops and reported positive effect on crop yield (Beyer, 1996; Ahlawat et al., 2006). Earthworm grazing is a biooxidation process accelerating the stabilization of organic matter involving the joint action with microorganisms which are responsible for the physico-biochemical degradation of organic matter present in the waste. Earthworms are the important drivers of the process, conditioning the substrate and altering physico-biological activities (Aira et al., 2002; Domınguez, 2004). Further, earthworm has indirect effects on the structure and activities of microbial communities present in the waste through stimulation of microbial populations, inoculum dispersal, litter comminution, grazing, gut passage and aggregate formation (Anderson, 1987; Aira et al., 2002). Earthworms are involved in the fragmentation and ingestion of fresh organic matter, providing a greater surface area for the microbial colonization, and thereby drastically altering biological activity (Lores et al., 2006). Earthworms also have a great impact on nutrient mineralization and transformation during vermicomposting through modifications of the environmental conditions in the organic wastes and their interactions with microbes which favor the nitrogen transformation by rapid conversion of ammonia-nitrogen into nitrates (Aira et al., 2008; Domınguez et al., 2010). It increases the macro- and micronutrients, plant growth hormones auxins, gibberellins and cytokinins (Krishnamoorthy and Vajrabhiah, 1986), humic acids (Atiyeh et al., 2002) and enzymes in the decomposed organic manures (Sarma et al., 2010) which not only enhance plant growth but also hold nutrients for longer periods (Ndegwa and Thompson, 2001; Singh et al., 2013a). But so far no attempt seems to be made on value addition of SMS using *T. harzianum* and its synergistic effect with earthworm (*Eisenia fetida*). Moreover, the effect of earthworm grazed and *T. harzianum* bio-fortified SMS on nutritional quality and natural antioxidant properties of tomato is also an unexplored area. Recognizing the importance of nutritional value of tomato in terms of antioxidant properties and other mineral

nutrients and their role in human health, the present study was undertaken with the objectives to study (1) the effect of earthworm grazing and *T. harzianum* bio-fortification on value addition of SMS, and (2) the impact of earthworm grazed and *T. harzianum* bio-fortified SMS on natural antioxidant and other nutritional properties of tomato.

MATERIALS AND METHODS

Source of Reagents and Media

Culture media were procured from HiMedia, India, whereas, chemical reagents including standards were procured from Sigma–Aldrich, India. Bovine serum albumin (BSA), lycopene, β-carotene, organic solvents and other chemicals and analytical grade solvents were purchased from Merck Biosciences, India.

Fungal Strain, Earthworm (*Eisenia fetida*) and Culture Condition

The test fungal strain, *T. harzianum* UBSTH-501 (GenBank Accession No: MG972984) was obtained from Plant-Microbe Interaction and Rhizosphere Biology Lab, ICAR-National Bureau of Agriculturally Important Microorganisms (ICAR-NBAIM), Kushmaur, Maunath Bhanjan, India and maintained on potato dextrose agar (PDA) by sub-culturing at 25 ± 2°C at 15 days interval. Earthworms (*E. fetida*) were obtained from Dr. M. C. Manna, Division of Soil Biology, ICAR-Indian Institute of Soil Science, Bhopal, India.

In Planta Study

Experimental Design

The effects of earthworm grazed, *T. harzianum* biofortified SMS on nutritional quality of tomato were evaluated under nethouse conditions at ICAR-NBAIM, India. The treatments were: T_1- Control (without SMS), T_2- Fresh SMS, T_3-earthworm grazed SMS, T_4- *T. harzianum* biofortified SMS and T_5-earthworm grazed, *T. harzianum* biofortified SMS. Experiments were arranged in a completely randomized block design under nethouse conditions and each treatment consisted of five replications.

Preparation of Earthworm Grazed and *T. harzianum* Bio-Fortified SMS

Fresh SMS of white button mushroom (*Agaricus bisporus*) was collected from Department of Mycology and Plant Pathology, Institute of Agricultural Sciences, Banaras Hindu University, Varanasi, India. It was subsequently air-dried under shade, sieved (2 mm pore size) and filled into Vermi-bed of the dimensions of 5 × 3 × 2 ft. Grain-based bioformulation of *T. harzianum* was prepared following the procedure described by Singh et al. (2012). *T. harzianum* bio-fortified SMS was prepared as per the methods/steps described in **Figure 1**. Physico-chemical properties of fresh SMS, earthworm grazed SMS, *T. harzianum* biofortified SMS and earthworm grazed and *T. harzianum* biofortified SMS were analyzed. At the time of application, the population of *T. harzianum* in earthworm grazed and *T. harzianum* biofortified and only *T. harzianum* biofortified SMS were 3.20×10^6 and 2.25×10^6 cfu g^{-1}, respectively.

Planting Material and Growth Condition

Tomato seedlings (*cv*. Dev, Nunhems Pvt. Ltd., India) were grown in sterile potting mixture adopting standard horticultural practices at ICAR-NBAIM, Maunath Bhanjan, India. Forty-five days old tomato plants were uprooted gently without much disturbance to the roots and transplanted into pots in nethouse. The experiments were conducted during November to February with the range of relative humidity being 80–85% under 11/13 h light/dark photoperiod.

Soil Collection, Preparation and Analysis

Soil was collected from an agricultural farm, Indian Institute of Seed Science (formerly known as Directorate of Seed Research), Kushmaur, Maunath Bhanjan, India. Such soil was further processed for the study by sieving (2 mm pore size) and air drying. After processing, the soil was mixed with vermicompost in a 4:1 ratio (w/w) and chemical fertilizers, *viz.* diammonium phosphate, urea and muriate of potash at 174, 326, and 100 kg per hectare, respectively, followed by its sterilization by autoclaving at 121°C for 60 min twice at 12 h interval and storage in the same position. The physico-chemical properties of experimental soil were analyzed after 5 days of sterilization (**Table 1**).

Nethouse Study

Forty-five days old tomato plants were transplanted into pots each containing 200 g of fresh and bio-fortified SMS. The SMS was thoroughly mixed in the pots containing experimental soil (5 kg) and a single plant was transplanted into each pot. SMS free soil served as the negative control and soil with fresh SMS was taken as positive control. Further, two blocks were randomly arranged to replicate the five treatments and each treatment with five replicates (pots) was randomly arranged within each block.

Estimation of Natural Antioxidants and Nutritional Quality of Ripe Tomato Fruits

Five ripe tomato fruits were randomly picked from each treatment for further analysis. The contents of total carbohydrate, soluble sugar, protein and lutein zeaxanthin were determined according to Sadasivam and Manickam (1996), whereas vitamin A (as retinal) and ascorbic acid were quantified according to Thimmaiah (2012). Lycopene and β-carotene content in ripe tomato fruits were measured according to Hyman et al. (2004). N content in the ripe fruits was quantified by Kjeldahl method, whereas the composition of minerals and micronutrients was determined according to Sahu (2011).

A modified thiobarbituric acid-reactive species (TBARS) assay of Ohkawa et al. (1979) was used to measure lipid peroxidation with slight modifications (Singh et al., 2010). Similarly, protein oxidation was estimated according to the methods of Singh et al. (2009b) with slight modifications (Singh et al., 2010). The contents of total polyphenols and flavonoids were determined according to Singh et al. (2009a). The ferrous and ferric ion chelating properties and superoxide anion radical scavenging ability of ripe tomato extract were determined

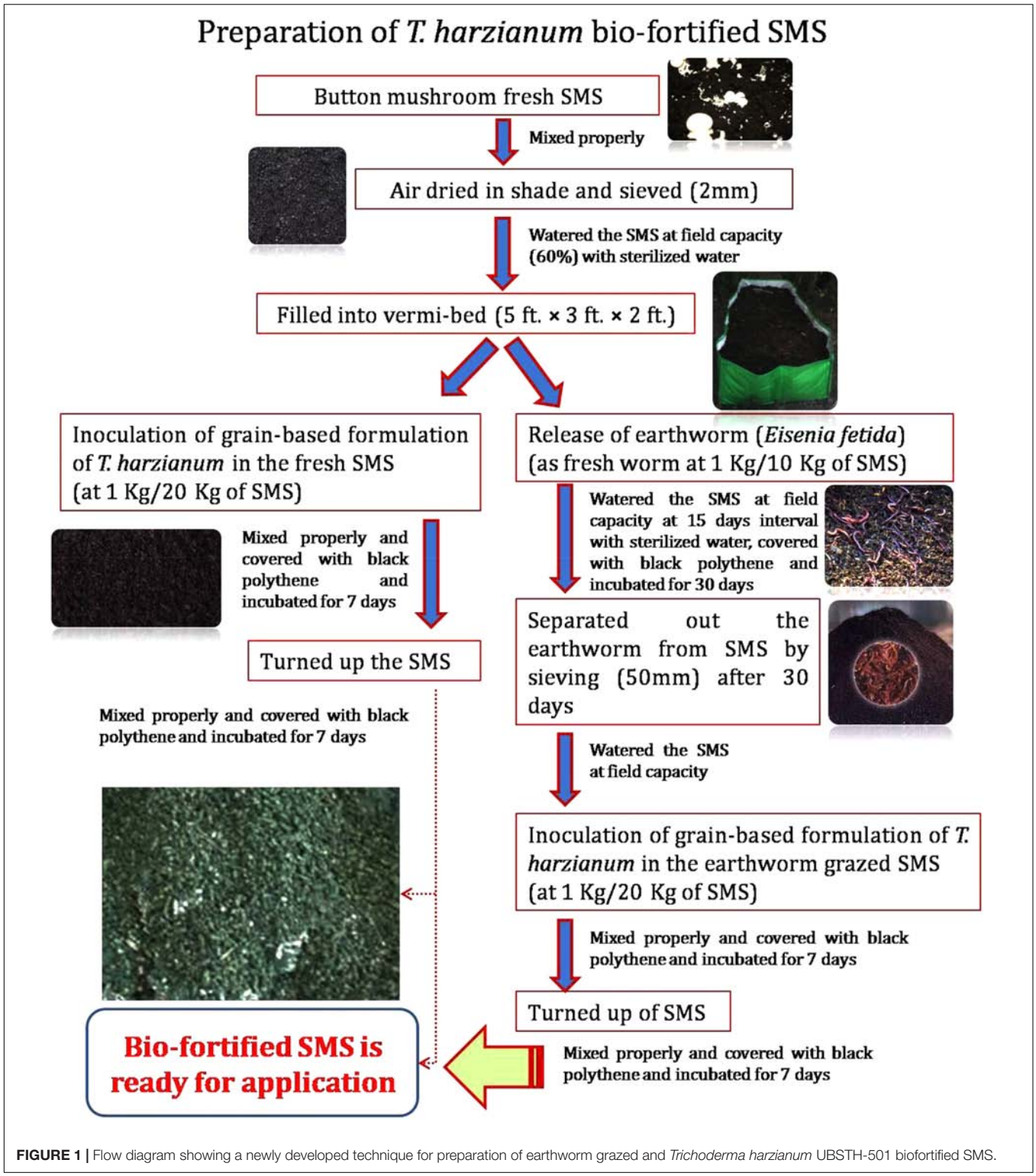


FIGURE 1 | Flow diagram showing a newly developed technique for preparation of earthworm grazed and *Trichoderma harzianum* UBSTH-501 biofortified SMS.

according to Singh et al. (2010). β-carotene bleaching assay was performed by the auto-oxidation of β-carotene and linoleic acid coupled reaction method according to Singh et al. (2010). Peroxidase and catalase activities were measured according to Thimmaiah (2012). A unit of catalase is defined as the quantity of enzyme necessary to decompose 1 μM of H_2O_2 per minute at 25°C, whereas 1 unit of peroxidase is equal to 0.1 of absorbance. Superoxide dismutase activity of tomato extracts was determined following the method of Sadasivam and Manickam (1996). The effect of *T. harzianum* biofortified SMS on number of fruit per

TABLE 1 | Physico-chemical characteristics of initial experimental soil.

S.N.	Soil properties	Values
(1)	Textural class	Silt loam
(2)	pH	7.2
(3)	EC (dS m^{-1})	0.98
(4)	OC (g kg^{-1})	5.4
(5)	OM (g kg^{-1})	9.04
(6)	Bulk density (Mg m^{-3})	1.40
(7)	Particle density (Mg m^{-3})	2.50
(8)	$CaCO_3$ (%)	6.55
(9)	**Available macronutrients (kg ha^{-1})**	
(i)	N	189.41
(ii)	P	29.70
(iii)	K	149.79
(iv)	S	8.65
(10)	**Available micronutrients (mg kg^{-1})**	
(i)	Fe	14.68
(ii)	Mn	2.50
(iii)	Cu	0.97
(iv)	Zn	0.64
(v)	B	0.11
(vi)	Mo	0.06

plants and yield was measured after final harvest of fruits. For this purpose, five plants were randomly selected from each treatments and first picking was done after 75 days of transplanting. A total of five picking was done at 10 days interval and finally average number of fruits per plants and yield (kg per plant) were calculated.

Effect of Earthworm Grazed and *T. harzianum* Bio-Fortified SMS on Physico-Biochemical Properties of Soil

The effects of earthworm grazed and *T. harzianum* bio-fortified SMS on physico-biochemical properties of soil were analyzed at the end of the experiment. Soil samples (200 g) were collected from each treatment and stored at 4°C till analysis. Soil pH and EC were measured in 1:2.5 soil-water mixture. Soil organic carbon was measured by using modified Walkley-Black method. However, bulk density, particle density $CaCO_3$, available micronutrients (N, P, K, and S) and available micronutrients (Fe, Mn, Cu, Zn, B, and Mo) in the soil samples were analyzed by following the standard procedures described by Sahu (2011).

Statistical Analyses

In vitro laboratory experiments were performed in a completely randomized design and the nethouse experiments were conducted in a completely randomized block design. Laboratory experiments were repeated three times, whereas nethouse experiments were repeated twice in five replications each. Data were subjected to analysis of variance (ANOVA) and Duncan's Multiple Range Test using SPSS software Version 16.0 program. Data were compared with DMRT at $p \leq 0.05$. Graphs and figures were drawn using the statistical package Origin Version 8.0.

RESULTS

Effect of Earthworm Grazing and *T. harzianum* Bio-Fortification on Nutritional Value of SMS

Results showed that earthworm grazing and *T. harzianum* bio-fortification alone or in combination significantly increased the biochemical and nutritional properties of fresh SMS (**Table 2**). The physico-chemical properties in terms of pH, EC, bulk and particle density showed significant improvement in EGTHB-SMS and only *T. harzianum* biofortified SMS (THB-SMS) over the earthworm grazed SMS (EG-SMS) and fresh SMS. However, marginal increase was recorded with respect to nitrogen content, whereas phosphorus and potassium content were significantly higher in EGTHB-SMS (1.79 and 285.50, respectively) over only THB-SMS (1.65 and 256.25, respectively), EG-SMS (1.25 and 241.50, respectively) and fresh SMS (1.18 and 231.0, respectively). Similar trend was recorded in case of calcium content (**Table 2**). Further, results of the investigation reveal the fact that *T. harzianum* biofortification and earthworm grazing significantly decrease the sodium, chloride and nitrate content, and simultaneously increased porosity and moisture content in the biofortified SMS as compared to other treatments (**Table 2**).

Effect on Accumulation of Total Carbohydrate, Soluble Sugar, Proteins and Lutein Zeaxanthin Content

Plants treated with *T. harzianum* bio-fortified SMS showed higher accumulation of total carbohydrate, soluble sugar, proteins and lutein zeaxanthin in the ripe tomato fruits. The accumulation of total carbohydrate was significantly higher in the tomato fruits obtained from the plants treated with EGTHB-SMS (53.90 $\mu g\ g^{-1}$ fresh wt.) when compared to only THB-SMS (46.92 $\mu g\ g^{-1}$ fresh wt.), EG-SMS (42.35 $\mu g\ g^{-1}$ fresh wt.), fresh SMS treated (41.04 $\mu g\ g^{-1}$ fresh wt.) and control plants (35.67 $\mu g\ g^{-1}$ fresh wt.) (**Figure 2A**). Similarly, significant increase in total soluble sugar content in ripe fruits was recorded in plants treated with EGTHB-SMS (48.67 $\mu g\ g^{-1}$ fresh wt.) followed by only THB-SMS (39.56 $\mu g\ g^{-1}$ fresh wt.), EG-SMS (32.45 $\mu g\ g^{-1}$ fresh wt.), fresh SMS (30.92 $\mu g\ g^{-1}$ fresh wt.) compared to the untreated control plants (26.33 $\mu g\ g^{-1}$ fresh wt.) under nethouse conditions (**Figure 2B**). Similarly, the total protein content (**Figure 2C**) and lutein zeaxanthin content (**Figure 2D**) in ripe fresh tomato fruits were also significantly higher when treated with EGTHB-SMS compared to the other treatments under investigation.

Effect on Accumulation of Vitamin A, β-Carotene, Lycopene and Ascorbic Acid

In the present study, EGTHB-SMS recorded considerable increase in synthesis and accumulation of vitamin A, β-carotene, lycopene and ascorbic acid in ripe tomato fruits under nethouse experiments. Quantitative estimation showed that vitamin A in tomato fruits was significantly higher in the plants inoculated with EGTHB-SMS (8.33 $\mu g\ g^{-1}$ fresh wt.) compared to other treatments (**Figure 3A**). Similarly, the highest β-carotene content

TABLE 2 | Effect of solid-state cultivation of *Trichoderma harzianum* UBSTH-501 and earthworm (*Eisenia fetida*) grazing on physico-chemical properties of spent button mushroom substrate.

S.N.	Parameters tested	Fresh SMS	*E. fetida* grazed SMS	*T. harzianum* UBSTH-501 fortified SMS	*T. harzianum UBSTH-501* fortified and *E. fetida* grazed SMS
(1)	pH	8.4[a]	8.1[b]	7.8[c]	7.2[d]
(2)	EC	5.50[a]	4.15[b]	4.02[b]	3.21[c]
(3)	Organic carbon (%)	4.90[b]	4.95[b]	4.50[c]	5.20[a]
(4)	Nitrogen (%)	2.71[c]	2.75[c]	2.85[b]	2.96[a]
(5)	Phosphorus (%)	1.18[d]	1.25[c]	1.65[b]	1.79[a]
(6)	Potassium (ppm)	231.0[d]	241.50[c]	256.25[b]	285.50[a]
(7)	Calcium (ppm)	543.33[d]	552.35[c]	600.05[b]	660.50[a]
(8)	Sodium (ppm)	260.25[a]	260.12[a]	210.45[b]	180.00[c]
(9)	Chloride (ppm)	146.10[a]	120.25[b]	75.04[c]	60.66[d]
(10)	Nitrate (%)	12.80[a]	10.33[b]	8.96[c]	8.02[c]
(11)	Total dissolved solid (ppm)	1910.00[a]	1450.50[b]	1469.60[b]	1262.55[c]
(12)	Bulk density (g cm^3)	0.57[a]	0.50[a]	0.40[b]	0.44[b]
(13)	Particle density (g cm^3)	2.20[a]	2.10[a]	1.84[b]	1.75[b]
(14)	Porosity (%)	20.00[d]	24.25[c]	30.00[b]	39.60[a]
(15)	Moisture (%)	59.00[c]	62.33[b]	65.50[a]	66.90[a]

EC represents Electrical Conductivity, ppm- part per million, cm-centimeter, data are means (n = 3), data with different letters show significant difference in row data in Complete Randomized Design at $p < 0.05$ under Duncan's multiple range test.

was also recorded in tomato treated with EGTHB-SMS (7.01 μg g^{-1} fresh wt.) compared to only THB-SMS treated (6.50 μg g^{-1} fresh wt.), EG-SMS (5.75 μg g^{-1} fresh wt.), fresh SMS (5.54 μg g^{-1} fresh wt.) and untreated control plants (4.49 μg g^{-1} fresh wt.) (**Figure 3B**). Further, more or less similar trend was observed with lycopene (**Figure 3C**) and ascorbic acid content in ripe tomato fruits (**Figure 3D**).

Effect on Accumulation of Mineral Content in Ripe Tomato Fruits

The present study reveals a significant increase in mineral contents of ripe tomato fruits when inoculated with EGTHB-SMS compared to the only THB-SMS, EG-SMS, fresh SMS and control plants (**Figure 4**). Results of the investigation also establish that in ripe fruits from tomato plants inoculated with EGTHB-SMS induced 1.5–2 times more uptake and accumulation of nitrogen, phosphorus, potassium and calcium compared to those from plants treated with only THB-SMS, EG-SMS and fresh SMS (**Figures 4A–D**, respectively). Also, a similar trend was recorded for the magnesium and iron content of the ripe tomato fruits (**Figures 4E,F**). Again, maximum zinc, manganese and sodium content was recorded in tomato fruits from plants treated with EGTHB-SMS followed by only THB-SMS, fresh SMS and EG-SMS treated plants (**Figures 4G–I**, respectively), whereas minimum mineral content was recorded in control plants (**Figure 4**).

Effect on Accumulation of Natural Antioxidants and Enzymatic Activities

In the present study, antioxidant activity was measured by different methods, namely presence of lipid peroxidation and protein oxidation assay, total polyphenols and flavonoids, ferrous and ferric ion chelating activity, superoxide anion radical scavenging activity, β-carotene bleaching assay and antioxidant enzymatic activity.

Effect on Lipid Peroxidation and Protein Oxidation

The activity of extracts to scavenge $HO^{\cdot}$ was also measured by protein oxidation method; extract obtained from plants treated with EGTHB-SMS inhibited the degree of lipid peroxidation and protein oxidation significantly ($p < 0.01$). Maximum inhibition in lipid peroxidation (34.34%) and protein oxidation (33.66%) was recorded in the fresh extract obtained from EGTHB-SMS treated plants followed by those treated with only THB-SMS, EG-SMS and fresh SMS treated plants at 10 mg ml^{-1} (**Figures 5A,B**). However, minimum inhibition of the mentioned processes was recorded in control plants. Further, the reference compound quercetin exhibited better inhibitory effects on lipid peroxidation and protein oxidation than other treatments (**Figure 5**).

Total Polyphenol and Flavonoid Content

It was observed that total polyphenol and flavonoid content of ripe tomato fruits increased significantly in plants treated with EGTHB-SMS (125.19 mg of gallic acid equivalents g^{-1} of extract and 62.97 mg of quercetin equivalents g^{-1} of extract) as compared to the plants treated with only THB-SMS (95.23 mg of gallic acid equivalents g^{-1} of extract and 59.82 mg of quercetin equivalents g^{-1} of extract), EG-SMS (82.50 mg of gallic acid equivalents g^{-1} of extract and 49.52 mg of quercetin equivalents g^{-1} of extract) and fresh SMS (81.55 mg of gallic acid equivalents g^{-1} of extract and 46.33 mg of quercetin equivalents g^{-1} of extract). Minimum polyphenol and flavonoid content was recorded in ripe fruits obtained from control plants (65.66 mg

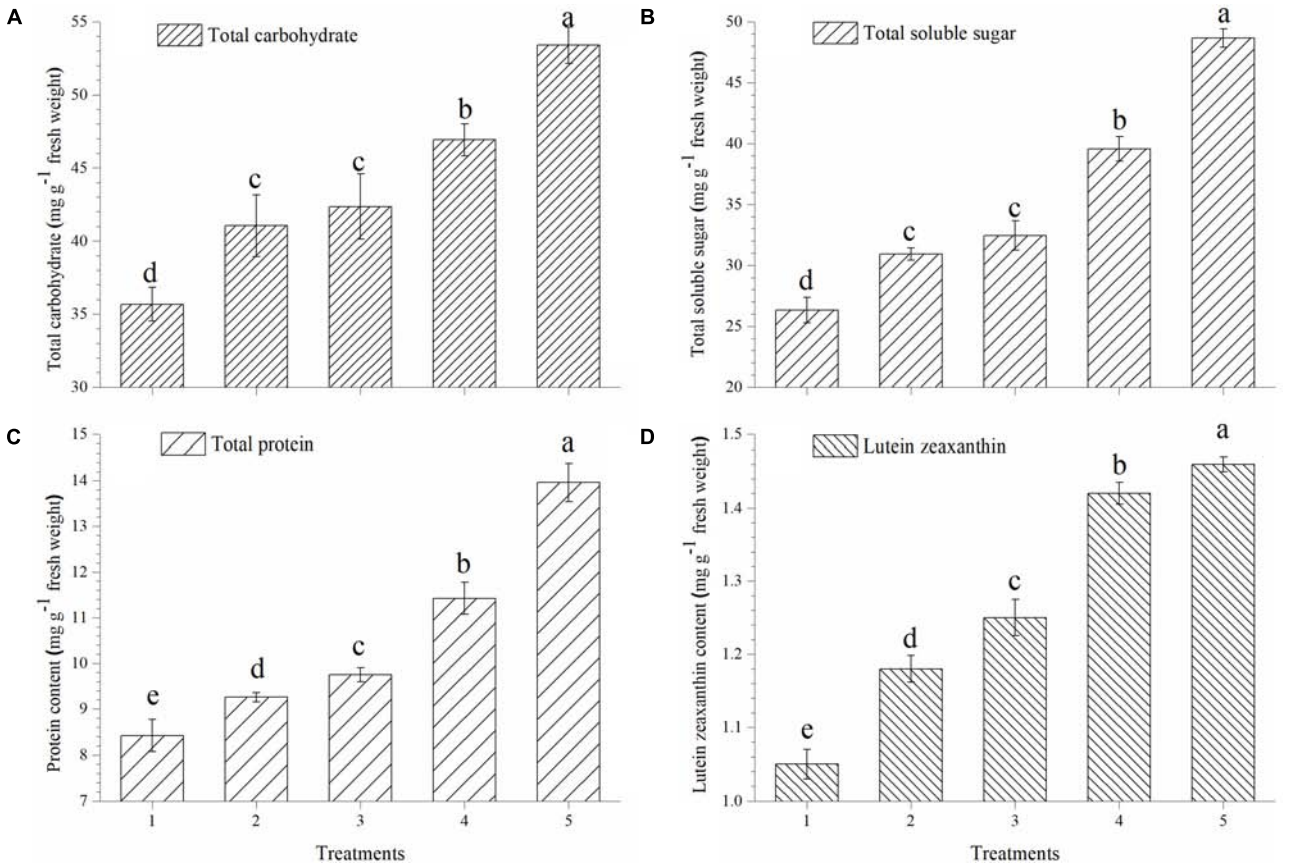


FIGURE 2 | Effect of solid state cultivated *T. harzianum* UBSTH-501 on nutritional quality of tomato at harvest, **(A)** total carbohydrate, **(B)** total soluble sugar, **(C)** protein content, and **(D)** lutein zeaxanthin content. Treatments: 1- Control (untreated), 2- Fresh SMS, 3- Earthworm grazed SMS, 4- *T. harzianum* UBSTH-501 biofortified SMS, and 5- Earthworm grazed and *T. harzianum* UBSTH-501 biofortified SMS. Data are means ($n = 5$) and vertical bars represent standard error of means. Different letters above columns show significant difference in randomized block design test at $p < 0.05$ under Duncan's multiple range test.

of gallic acid equivalents g^{-1} of extract and 32.59 mg of quercetin equivalents g^{-1} of extract) (**Figures 6A,B**, respectively).

Ferrous and Ferric Ion Chelating Activity

In the current study, further investigations were done on the role of the ripe tomato fruit extract on Fe^{2+} and Fe^{3+}chelation, because these are the most effective pro-oxidants that are present in the food system (Halliwell et al., 1987). Maximum chelating effects on Fe^{2+} and Fe^{3+} ions (27.65 and 40.70%, respectively) were observed in the extracts of ripe fruits from plants treated with EGTHB-SMS followed by THB-SMS, EG-SMS and fresh SMS. However, minimum Fe^{2+} and Fe^{3+} ions chelation (12.40 and 19.65%, respectively) was recorded in the extracts of ripe fruits obtained from control plants at 10 mg ml^{-1} under nethouse conditions (**Figures 6C,D**).

Effects on Superoxide Anion Radical Scavenging Activity and β-Carotene Bleaching Assay

The extract of fruits from plants treated with EGTHB-SMS was found to be the most potent superoxide scavenger (41.23%) compared to that of fruits from only THB-SMS (36.59%), EG-SMS (30.25%), fresh SMS (28.92%) and control (21.67%) plants (**Figure 6E**).

Auto-oxidation of β-carotene (β-carotene chelating assay) was used to evaluate the antioxidant activity of the ripe tomato fruit extracts. Results showed that extract taken from plants treated with EGTHB-SMS exhibited higher antioxidant activities (57.89%) compared to that from plants treated with THB-SMS (57.32%), EG-SMS (50.22%) and fresh SMS (49.67%), whereas extract of fruits from control plants was found to be least antioxidative (42.91%) on the selected parameter (**Figure 6F**).

Antioxidant Enzymatic Activity

Earthworm grazed, *T. harzianum* biofortified SMS treated tomato exhibited higher antioxidant activities. The results exhibited more or less similar pattern for antioxidant enzymes activity in ripe tomato fruit extract as recorded for mineral content (**Figures 6G–I**). **Figure 6G** reflects higher peroxidase activity in ripe fruits from plants treated with EGTHB-SMS (130.49 unit mg^{-1} min^{-1} fresh wt.) compared to other treatments. As revealed in **Figure 6H**, the activity of

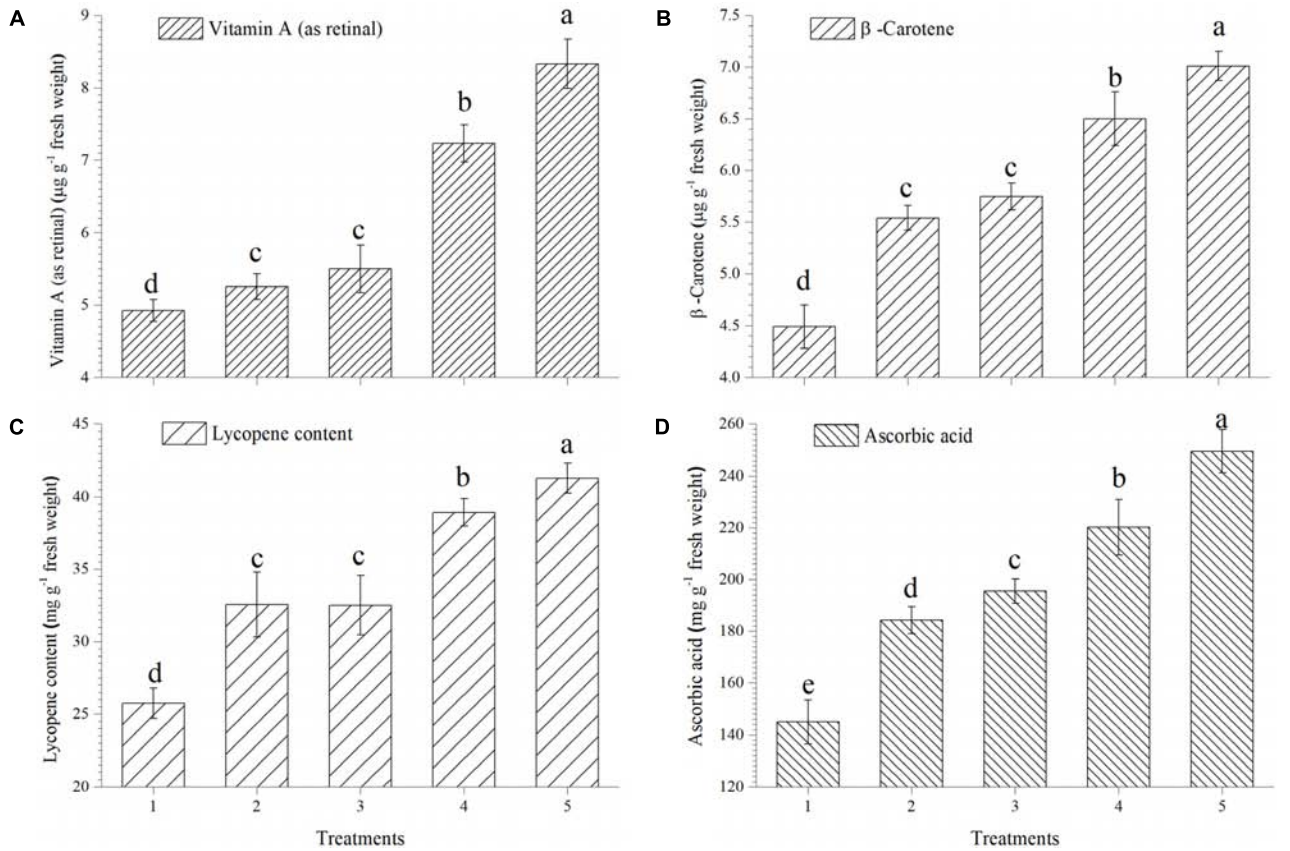


FIGURE 3 | Effect of solid state cultivated *T. harzianum* UBSTH-501 on antioxidant compound in ripe tomato fruits at harvest. **(A)** Vitamin A (as retinal), **(B)** β-carotene content, **(C)** lycopene content, and **(D)** ascorbic acid content. Treatments: 1- Control (untreated), 2- Fresh SMS, 3- Earthworm grazed SMS, 4- *T. harzianum* UBSTH-501 biofortified SMS, and 5- Earthworm grazed and *T. harzianum* UBSTH-501 biofortified SMS. Data are means (n = 5) and vertical bars represent standard error of means. Different letters above columns show significant difference in randomized block design test at $p < 0.05$ under Duncan's multiple range test.

catalase showed similar behavior, responding significantly to the different treatments. The superoxide dismutase (SOD) activity was highest in ripe fruits taken from plants treated with EGTHB-SMS (4.21 unit mg^{-1} min^{-1} fresh wt.) compared to plants treated with only THB-SMS (3.89 unit mg^{-1} min^{-1} fresh wt.), EG-SMS (3.00 unit mg^{-1} min^{-1} fresh wt.) and fresh SMS (3.25 unit mg^{-1} min^{-1} fresh wt.). However, least SOD activity was recorded in the extract of fruits taken from control plants grown under nethouse conditions (**Figure 6I**).

Effect on Fruit Yield

Results showed that plants treated with EGTHB-SMS produced significantly higher number of fruits per plants and yield (25.75 and 1.69 kg/plant, respectively) as compared to other treatments including plants treated with only THB-SMS (21.67 and 1.55 kg/plant, respectively), EG-SMS (18.15 and 1.45 kg/plant, respectively), fresh SMS (18.35 and 1.45 kg/plant, respectively) and control (14.52 and 1.25 kg/plant, respectively) under nethouse conditions after final harvest. However, lowest fruit yield was recorded in untreated control plants (**Figure 7**).

Effect of Bio-Fortified SMS on Physico-Biochemical Properties of Soil

Results showed that amendment of fresh and biofortified SMS significantly affect the biochemical properties of the soil (**Table 3**). A significant increase in the soil organic carbon, organic matter and available macronutrients (N, P, K, and S) was recorded in the soil amended with earthworm grazed-*T. harzianum* biofortified SMS as compared to other treatments and initial value as presented in **Table 1**. Further, maximum available Fe, Mn, Zn, and Mo was recorded in the soil treated with earthworm grazed-*T. harzianum* biofortified SMS other treatments (**Table 3**). However, minimum $CaCO_3$ was found in the soil amended with earthworm grazed-*T. harzianum* biofortified SMS.

DISCUSSION

Natural antioxidants and their role of in human health maintenance is a complex process and understanding these complex processes is the key to recognize cell responses against damage by free radicals. Free radicals (syn. reactive oxygen

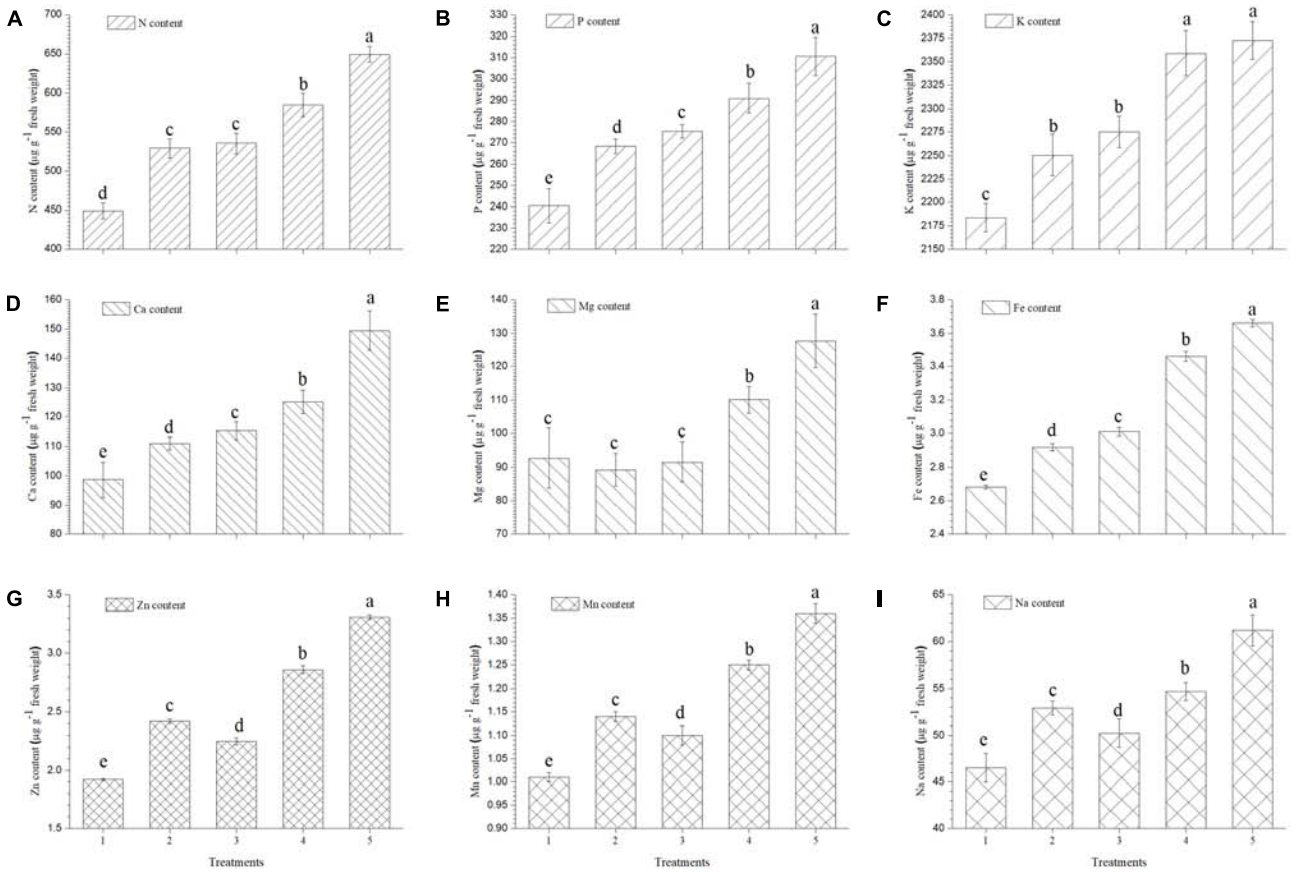


FIGURE 4 | Effect of solid state cultivated *T. harzianum* UBSTH-501 on mineral content in ripe tomato fruits at harvest. **(A)** N content, **(B)** P content, **(C)** K content, **(D)** Ca content, **(E)** Mg content, **(F)** Fe content, **(G)** Zn content, **(H)** Mn content, and **(I)** Na content. Treatments: 1- Control (untreated), 2- Fresh SMS, 3- Earthworm grazed SMS, 4- *T. harzianum* UBSTH-501 biofortified SMS, and 5- Earthworm grazed and *T. harzianum* UBSTH-501 biofortified SMS. Data are means ($n = 5$) and vertical bars represent standard error of means. Different letters above columns show significant difference in randomized block design test at $p < 0.05$ under Duncan's multiple range test.

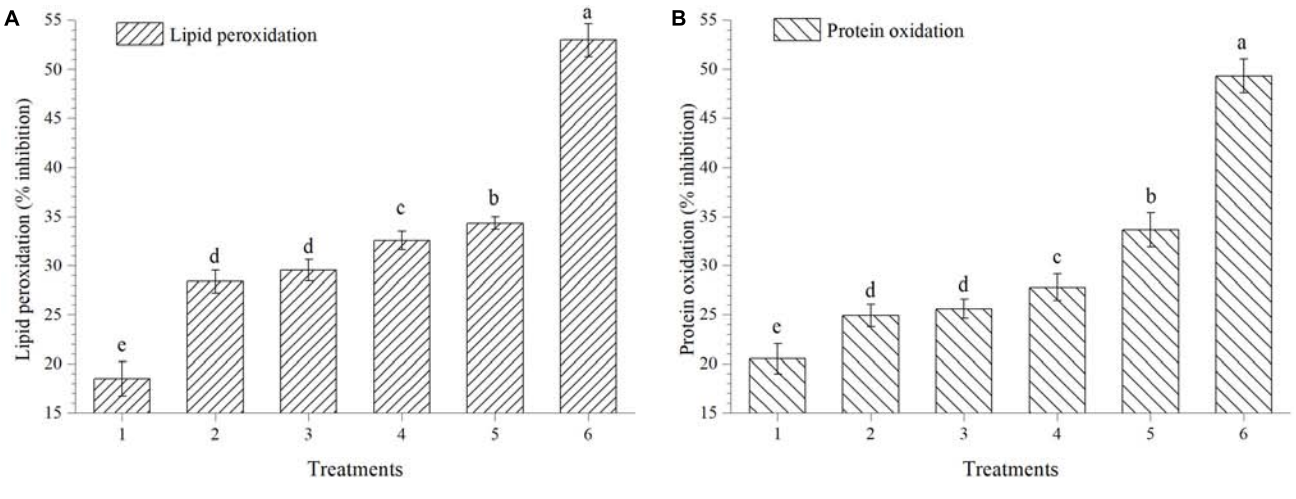


FIGURE 5 | Effect of solid state cultivated *T. harzianum* UBSTH-501 on **(A)** lipid peroxidation and **(B)** protein oxidation in ripe tomato fruits at harvest. Treatments: 1- Control (untreated), 2- Fresh SMS, 3- Earthworm grazed SMS, 4- *T. harzianum* UBSTH-501 biofortified SMS, 5- Earthworm grazed and *T. harzianum* UBSTH-501 biofortified SMS and 6- Reference compound quercetin. Data are means ($n = 5$) and vertical bars represent standard error of means. Different letters above columns show significant difference in randomized block design test at $p < 0.05$ under Duncan's multiple range test.

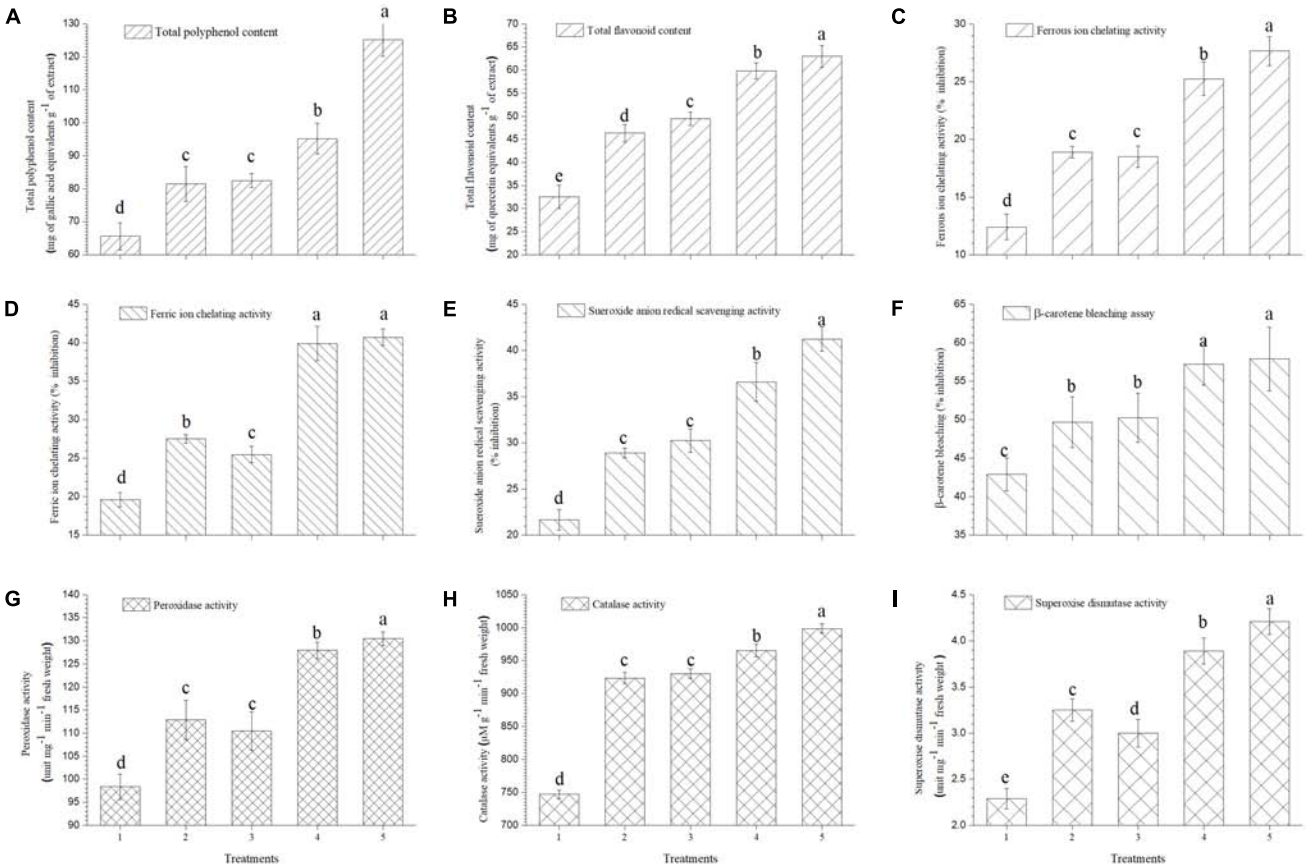


FIGURE 6 | Effect of solid state cultivated *T. harzianum* UBSTH-501 on antioxidant enzymatic activities in ripe tomato fruits at harvest **(A)** Total polyphenol content, **(B)** total flavonoid content, **(C)** ferrous ion chelating activity, **(D)** ferric ion chelating activity, **(E)** superoxide anion radical scavenging activity, **(F)** β-carotene bleaching assay, **(G)** peroxidase activity, **(H)** catalase activity, and **(I)** superoxide dismutase activity. Treatments: 1- Control (untreated), 2- Fresh SMS, 3- Earthworm grazed SMS, 4- *T. harzianum* UBSTH-501 biofortified SMS, and 5- Earthworm grazed and *T. harzianum* UBSTH-501 biofortified SMS. Data are means ($n = 5$) and vertical bars represent standard error of means. Different letters above columns show significant difference in randomized block design test at $p < 0.05$ under Duncan's multiple range test.

species-ROS) are produced in the cells as a by-product of normal metabolism and/or as a result of biotic and abiotic stresses (Sardesai, 1995). A prominent role of antioxidants in the reduction of ROS in the stressed tissues has already been established and reported by several workers (Halliwell, 1996; Devasagayam et al., 2004; Singh et al., 2010). ROS/FRs are normally neutralized by efficient systems in the body itself that include the antioxidant enzymes (peroxidase, catalase, SOD and glutathione peroxidase) and the nutrient-derived antioxidant small molecules such as lycopene, vitamin A, vitamin E, vitamin C, β-carotenes, polyphenolics, flavonoids, glutathione, etc. (Sardesai, 1995; Halliwell, 1996, 1997; Devasagayam et al., 2004). Since ancient era, fresh fruits, vegetables and herbal medicines have been the rich source of antioxidants in traditional human diet that protect tissues from the damage caused by free radicals (Sardesai, 1995; Sen and Chakraborty, 2011). In the present study, it has been demonstrated that application of *T. harzianum* biofortified SMS modulates accumulation of natural antioxidants and bio-fortification of mineral nutrients in tomato (*S. lycopersicum*) fruits rendering them more valuable as a food. Tomato constitutes an important source of antioxidants in Indian diet (George et al., 2004). The antioxidant activity of ripe tomato fruits has been tested using a wide variety of methods. Antioxidant activity is the capacity to prevent auto-oxidation of FR-mediated oxidation of the substrates when present at low concentration (Halliwell, 1992; Deepa et al., 2006; Bhanja et al., 2009). All these assays have been frequently used to assess antioxidant activity in the substrates (Deepa et al., 2006; Bhanja et al., 2009; Singh et al., 2010). Results from the present study involving earthworm grazing and *T. harzianum* biofortification of fresh SMS increases its physico-chemical and nutritional properties to significant extents. The nutritional quality of fresh SMS was even higher when the fresh SMS was co-inoculated with *T. harzianum* and earthworm in an additive manner as compared to only *T. harzianum* biofortified and earthworm grazed SMS. Results showed that optimized composting system with appropriate microorganisms and earthworm, *E. fetida* proved highly efficient in transforming SMS into odorless, porous and homogenized biofortified SMS. The improvement in nutritional quality of SMS may be attributed to accelerated

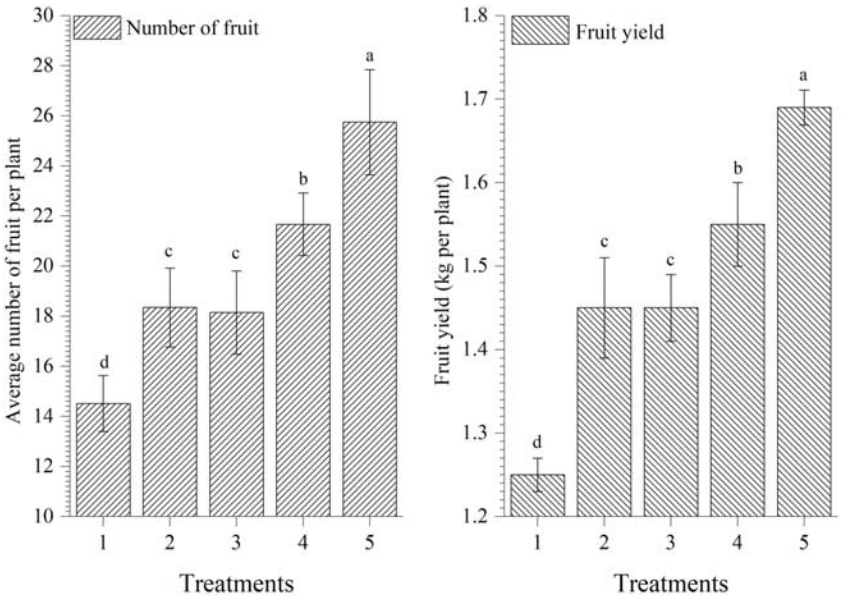


FIGURE 7 | Effect of solid state cultivated *T. harzianum* UBSTH-501 on number of fruits per plant and yield of tomato at harvest. Treatments: 1- Control (untreated), 2- Fresh SMS, 3- Earthworm grazed SMS, 4- *T. harzianum* UBSTH-501 biofortified SMS, and 5- Earthworm grazed and *T. harzianum* UBSTH-501 biofortified SMS. Data are means ($n = 5$) and vertical bars represent standard error of means. Different letters above columns show significant difference in randomized block design test at $p < 0.05$ under Duncan's multiple range test.

TABLE 3 | Effect of earthworm grazed and *T. harzianum* bio-fortified spent mushroom substrate on the physico-biochemical properties of post-harvested soil.

S.N.	Soil properties	Fresh SMS	*E. fetida* grazed SMS	*T. harzianum* UBSTH-501 fortified SMS	*E. fetida* grazed and *T. harzianum* UBSTH-501 fortified SMS
(1)	pH	7.2^{b}	7.3^{a}	7.3^{a}	7.2^{b}
(2)	EC (dSm^{-1})	0.89^{b}	0.90^{b}	0.95^{a}	0.96^{a}
(3)	Organic carbon ($g\ kg^{-1}$)	5.66^{d}	5.75^{c}	5.82^{b}	5.96^{a}
(4)	Organic matter ($g\ kg^{-1}$)	9.56^{a}	10.05^{a}	9.85^{a}	10.33^{a}
(5)	Bulk density ($g\ cm^{-3}$)	1.38^{a}	1.39^{a}	1.37^{a}	1.38^{a}
(6)	Particle density ($g\ cm^{-3}$)	2.48^{b}	2.53^{a}	2.50^{b}	2.55^{a}
(7)	$CaCO_3$ (%)	6.75^{a}	6.84^{a}	6.72^{a}	6.58^{b}
(8)	**Available Macronutrients ($kg\ ha^{-1}$)**				
(i)	N	195.60^{c}	196.72^{c}	198.35^{b}	201.15^{a}
(ii)	P	29.70^{d}	30.95^{c}	32.55^{b}	35.19^{a}
(iii)	K	152.33^{c}	155.75^{b}	153.82^{c}	158.69^{a}
(iv)	S	7.90^{d}	8.25^{c}	8.50^{b}	8.96^{a}
(9)	**Available micronutrient ($mg\ kg^{-1}$)**				
(i)	Fe	18.90^{c}	20.65^{b}	20.25^{b}	22.33^{a}
(ii)	Mn	2.10^{b}	2.66^{a}	2.45^{a}	2.50^{a}
(iii)	Cu	1.10^{b}	0.98^{c}	1.25^{a}	1.15^{b}
(iv)	Zn	0.69^{c}	0.69^{c}	0.71^{b}	0.76^{a}
(v)	B	0.08^{c}	0.10^{b}	0.12^{a}	0.09^{b}
(vi)	Mo	0.05^{c}	0.05^{c}	0.06^{b}	0.07^{a}

EC represents Electrical Conductivity, data are means (n = 3), data with different letters show significant difference in row data in Complete Randomized Design at $p < 0.05$ under Duncan's multiple range test.

mineralisation of the organic matter by *T. harzianum* and further enrichment by the earthworm feeding of biofortified SMS (Jordan et al., 2008). Further, several reports showed that earthworm grazing enhances physical property of the organic waste (Lalander et al., 2015). Recycling of these organics in agriculture after bio-fortification and earthworm grazing has several benefits such as improving soil carbon content and sustaining soil health that eventually lead to better crop produce

and enhanced productivity (Lau et al., 2003; Jordan et al., 2008; Jonathan et al., 2011). In the current study, it was observed that application of EGTHB-SMS significantly increased the total carbohydrate, soluble sugar, proteins and lutein zeaxanthin in ripe tomato extract. Carbohydrate, sugars and proteins are the primary building blocks for any living cell (Singh et al., 2009a). The biofortified SMS carries substantial amount of essential plant nutrients, organics and enzymes which, on one hand, are useful for the better crop growth leading to improvement in the yield and returns thereby and on the other, help improve the nutritional quality of the produce (Lau et al., 2003; Jordan et al., 2008; Jonathan et al., 2011; Lalander et al., 2015).

Further application of biofortified SMS increases the antioxidant compounds namely lycopene and β-carotene in the ripe tomato fruits. There is an increase in concentration of β-carotene as the lycopene content increases, but the degree of increase is not similar. In ripe fresh tomato fruits, significant positive correlation has been found between lycopene and β-carotene content. Tomato lycopene and β-carotene have more bioactivity and bioavailability and also β-carotene is the precursor of vitamin A. Significant variation in concentrations of these compounds in different treatments might be due to the effect of solid-state cultivated *T. harzianum* and bioactive compounds present in the biofortified SMS. Organically cultivated fruits and vegetables generally tend to have higher concentrations of various essential nutrients like iron, phosphorous, potassium, vitamin C and several antioxidants compared to their conventionally grown counterparts (Barron, 2010; Montalba et al., 2010). The present investigation also validates the positive effects of *T. harzianum* enriched SMS on yield and quality of tomato fruits. While investigating the impact of nitrogen and bioinoculant *Bacillus licheniformis*, Ochoa-Velasco et al. (2016) have reported a positive effect of application of *B. licheniformis* on vitamin C and lycopene content in tomato. Similarly, enhancement in quality parameters of tomato due to the application of bio-augmented compost with effective microorganisms was reported by Verma et al. (2015). Currently, increased attention is being paid to evaluate the role of antioxidants in human diet and health (Singh et al., 2013c). Increase in lycopene, β-carotene, ascorbic acid and other antioxidant compounds in tomato under the influence of *T. harzianum* could help in improving dietary antioxidants and lowering cholesterol and oxidative burst effects, thereby aiding the prevention of several chronic diseases (Singh et al., 2013c) by better scavenging/quenching activities in the body (Bhanja et al., 2009; Singh et al., 2009a).

Moreover, lipid peroxidation by ROS/FRs may lead to the formation of toxic by-products/compounds such as malondialdehyde and 4-hydroxynonenal which can attack on membrane, DNA, inducing mutagenicity and carcinogenicity (Nautiyal et al., 2008; Singh et al., 2009a; Ademoyegun et al., 2011). The ability of any external agent to inhibit the oxidation process is often used to evaluate its antioxidant activity. In the present investigation, we used rat liver homogenate assay to investigate, whether the fresh extract of ripe tomato was able to protect the lipids from oxidation provoked by $FeSO_4$-based $HO^{\cdot}$. In the light of the above findings, the most probable reason for fresh tomato extracts as FRs and/or ROS-scavengers might be the enhanced concentration of antioxidant compounds in the extract that have been reported to inhibit lipid peroxidation and protein oxidation by quenching FRs (Yang et al., 2000; Lee et al., 2008; Singh et al., 2009b, 2010). On the other hand, results also register the fact that plants treated with EGTHB-SMS accumulate more isoflavonoids and phenolics in the ripe tomato fruits. These are also powerful protecting agents against the lethal effects of oxidative damage and protect macromolecules by chelating redox-active transition metal ions (Bhanja et al., 2009; Singh et al., 2010). The present investigation indicates synergistic action of phenolic compounds in scavenging ROS, repairing protein and lipid peroxidation and metal chelation (Prakash et al., 2007a; Lee et al., 2008; Moktan et al., 2008; Singh et al., 2010).

The antioxidant efficiency of phenolic compounds has been related to the number of $OH^{\cdot}$ groups in their structures and also to their hydrogen radical donating abilities (Lemanska et al., 2001; Altunkaya et al., 2009). This observation is in agreement with the findings of other investigators (Lin et al., 2006; Lee et al., 2008; Singh et al., 2010). Higher polyphenolics in the extract might be due to *T. harzianum*-mediated formation or mobilization of free phenolic and flavonoid molecules in the tomato fruits from site of synthesis (Singh et al., 2010; Ahmad et al., 2016; Altunkaya et al., 2016). We further investigated the role of the ripe tomato fruit extract on Fe^{2+} and Fe^{3+}chelation, because these are the most effective pro-oxidants that are present in the food system (Halliwell et al., 1987). The results also reveal that the tomato extracts showed a marked capacity for iron binding which might be helping in the lipid peroxidation and protein protection under stressed condition. The reducing capacity of a compound Fe^{3+} complex to the ferrous form may serve as a significant indicator of its antioxidant capacity (Roy and Rice-Evans, 1994). The existence of reductions is the key of the reducing power, which exhibit their antioxidant activities by breaking the free radical chain by donating a hydrogen atom (Schally and Nagy, 1999). The reduction of the Fe^{3+}/ferric cyanide complex to the ferrous form occurs due to the presence of resultants in the solution. Chelating agents have been reported to be effective as secondary antioxidants as they reduce the redox potential, thereby stabilizing the oxidized form of the metal ions (Halliwell et al., 1987; Prakash et al., 2007b; Singh et al., 2009a, 2010). Superoxide ions directly and/or indirectly initiate oxidation of biomolecules such as nucleic acids, proteins, lipids and carbohydrates as a result of superoxide and hydrogen peroxide serving as precursors of singlet oxygen and $HO^{\cdot}$ (Singh et al., 2010). The present study also revealed that high flavonoid content in the extract leads to scavenging of superoxide radicals. This result is in agreement with the earlier findings of Singh et al. (2009a). Our results also indicate better $O_2^{\cdot-}$ scavenging abilities in the extracts of plants treated with EGTHB-SMS than the control, and it might be due to formation of a glycones from glycosides of total phenolic and flavonoid and activation of superoxide dismutase (SOD) activity in the tomato fruits from *T. harzianum* treated plants. Furthermore, tomato is reported to contain sufficient amount of polyphenols (Singh et al., 2010, 2013b; Altunkaya et al., 2016), which are naturally occurring

antioxidants, that possess an $O_2^{\cdot-}$ scavenging effect similar to flavonoids (Singh et al., 2010; Ahmad et al., 2016).

The antioxidant activity assayed was the ability to inhibit the peroxidation of lipids. The higher antioxidant activity of extracts might be due to the inhibition of chain reaction, decomposing peroxides, preventing continued hydrogen abstraction and also attributed to the presence of antioxidant enzymes and phytochemicals, such as phenolics and isoflavones, ascorbic acid etc. (Singh et al., 2010). Our findings demonstrate a direct correlation between antioxidant activities and levels of enzymatic activity and are in agreement with the findings of some previous studies (Yang et al., 2008; Gao et al., 2010; Singh et al., 2013b,c). Further, the increase in the tomato yield is directly related to the higher nutritional content, enzymes and other plant growth promoting substrates present in the EGTHB-SMS. Furthermore, soil physico-biochemical properties were significantly affected by the application of biofortified SMS. Previous studies showed that application of organic matter significantly increases the soil biological properties (Sahu, 2011; Singh et al., 2013a; Lim et al., 2015). However, the available macro- and micronutrients in vermicompost are generally higher than in traditional compost produced from the same raw material (Lim et al., 2015). The biofortification further increases the quality of the compost and ultimately, influences the plant growth and quality of end products (Pramanik et al., 2007; Singh et al., 2008, 2013a). Additional indirect mechanisms include enhancement in the population of other beneficial microbes in the rhizosphere, enhanced nutrient use efficiency, and modulation of host physio-biological pathways leading to better plant growth and productivity (Ahlawat et al., 2006).

CONCLUSION

The demand for organically grown fruits and vegetables has been growing steadily over the past few decades due to the awareness developed among people for a healthy lifestyle. Addition of organic manure and bioinoculants enhances the nutrient mobilization by the plants and helps them defend against various stresses by enhanced production of antioxidant enzymes and phenolics. The study describes the multifarious effects of EGTHB-SMS in tomato plants that significantly increase various attributes in the tomato fruits. Further, it was noted that EGTHB-SMS was found to be more potential in enhancing natural antioxidants, mineral nutrients and enzymes in the fruits. Encouraging results were also obtained with increase in nutritional quality of ripe tomato fruits (mineral nutrients and natural antioxidant properties) modulated by the application of EGTHB-SMS. Results of the investigation suggest that application of EGTHB-SMS in tomato not only helps in increasing yield and enhancing the nutritional value of ripe fruits but also reduces the risk of environmental pollution caused by piling and rather unsafe disposal of SMS.

AUTHOR CONTRIBUTIONS

UBS, DM, JPR, BKS, AKS, RC, MCM, and DP were involved in conceiving the idea and designing the work. UBS, DM, WK, SS, NK, MI, MCM, and RC conducted the actual research work. UBS, DM, WK, SS, NK, JPR, BKS, AKS, RC, MCM, and DP wrote the paper. However, J-WO contributed to the general designing and also in revising the MS during the review process. All authors were contributed to interpretation of results and discussion and approved the final version of the manuscript.

ACKNOWLEDGMENTS

We would like to express our special thanks to Dr. Pratap Singh, Department of Plant Physiology and Dr. Alok Jha, Centre of Food Science and Technology, Institute of Agricultural Sciences, BHU, Varanasi for providing experimental support to carry out a part of this research work. We wish to thank Dr. A. K. Saxena, ICAR-NBAIM, Kushmaur, Maunath Bhanjan for technical assistance in writing and preparation of the manuscript. We also thank Dr. H. B. Singh, Department of Mycology and Plant Pathology, Institute of Agricultural Sciences, BHU, Varanasi for technical assistance in planning the experiments.

REFERENCES

Ademoyegun, O. T., Fariyike, T. A., and Aminu-Taiwo, R. B. (2011). Effects of poultry dropping on the biologically active compounds in *Capsicum annuum* L. (var. Nsukka yellow). *Agric. Biol. J. N. Am.* 2, 665–672. doi: 10.5251/abjna.2011.2.4.665.672

Ahlawat, O. P., Dev, R., Sagar, M. P., Gupta, P., and Vijay, B. (2006). Effect of recomposted spent mushroom substrate on yield and quality of cauliflower (*Brassica oleracea* L. var. botrytis). *Mushroom Res.* 15, 149–152.

Ahmad, N., Zuo, Y., Lu, X., Anwar, F., and Hameed, S. (2016). Characterization of free and conjugated phenolic compounds in fruits of selected wild plants. *Food Chem.* 190, 80–89. doi: 10.1016/j.foodchem.2015.05.077

Aira, M., Monroy, F., Domınguez, J., and Mato, S. (2002). How earthworm density affects microbial biomass and activity in pig manure. *Eur. J. Soil Biol.* 38, 7–10. doi: 10.1016/S1164-5563(01)01116-5

Aira, M., Sampedro, L., Monroy, F., and Domınguez, J. (2008). Detritivorous earthworms directly modify the structure, thus altering the functioning of a microdecomposer food web. *Soil Biol. Biochem.* 40, 2511–2516. doi: 10.1016/j.soilbio.2008.06.010

Altunkaya, A., Becker, E. M., Gokmen, V., and Skibsted, L. H. (2009). Effect of various inhibitors on enzymatic browning, antioxidant activity and total phenol content of fresh lettuce. *Food Chem.* 115, 163–168. doi: 10.1016/j.foodchem.2008.11.082

Altunkaya, A., Gokmen, V., and Skibsted, L. H. (2016). pH dependent antioxidant activities of lettuce (*L. sativa*) and synergism with added phenolic antioxidants. *Food Chem.* 190, 25–32. doi: 10.1016/j.foodchem.2015.05.069

Anderson, J. M. (1987). "Interactions between invertebrates and microorganisms: noise or necessity for soil processes," in *Ecology of Microbial Communities*, eds J. M. Anderson, A. D. M. Rayner, and D. W. H. Walton (Cambridge: Cambridge University Press), 125–145.

Atiyeh, R. M., Lee, S., Edwards, C. A., Arancon, N. Q., and Metzger, J. D. (2002). The influence of humic acids derived from organic wastes on plant growth. *Bioresour. Technol.* 84, 7–14. doi: 10.1016/S0960-8524(02)00017-2

Barron, J. (2010). Organic vs. *conventional. Nat. Health Newsl.* 9, 1–11.

Berg, G. (2009). Plant–microbe interactions promoting plant growth and health: perspectives for controlled use of microorganisms in agriculture. *Appl. Microbiol. Biotechnol.* 84, 11–18. doi: 10.1007/s00253-009-2092-7

Beyer, M. (1996). The impact of the mushroom industry on the environment. *Mushroom News* 44, 6–13.

Bhanja, T., Kumari, A., and Banerjee, R. (2009). Enrichment of phenolics and free radical scavenging property of wheat koji prepared with two filamentous fungi. *Bioresour. Technol.* 100, 2861–2866. doi: 10.1016/j.biortech.2008.12.055

Deepa, N., Kaur, C., Singh, B., and Kapoor, H. C. (2006). Antioxidant activity in some red sweet pepper cultivars. *J. Food Compost. Anal.* 19, 572–578. doi: 10.1016/j.jfca.2005.03.005

Domınguez, J. (2004). "State of the art and new perspectives on vermicomposting research," in *Earthworm Ecology*, 2nd Edn, ed. C. A. Edwards (Boca Raton: CRC), 401–424.

Domınguez, J., Aira, M., and Gomez-Brandon, M. (2010). "Vermicomposting: earthworms enhance the work of microbes," in *Microbes at Work*, ed. H. Insam (Berlin: Springer-Verlag), 93–114.

Devasagayam, T. P., Tilak, J. C., Boloor, K. K., Sane, K. S., Ghaskadbi, S. S., and Lele, R. D. (2004). Free radicals and antioxidants in human health: current status and future prospects. *J. Assoc. Physicians India* 52, 794–804.

Fox, R., and Chorover, J. (1999). *Seeking a Cash Crop in Spent Substrate*. Available at: http://aginfo.psu.edu/psa/sgg/mushrooms.html

Gao, D., Du, L., Yang, J., Wu, W. M., and Hong Ling, H. (2010). A critical review of the application of white rot fungus to environmental pollution control. *Crit. Rev. Biotechnol.* 30, 70–77. doi: 10.3109/07388550903427272

George, B., Kaur, C., Khurdiya, D. S., and Kapoor, H. C. (2004). Antioxidants in tomato (*Lycopersium esculentum*) as a function of genotype. *Food Chem.* 84, 45–51. doi: 10.1016/S0308-8146(03)00165-1

Glick, B. R. (1995). The enhancement of plant growth by free-living bacteria. *Can. J. Microbiol.* 41, 109–117. doi: 10.1139/m95-015

Halliwell, B. (1992). How to characterize biological antioxidants? *Free Radic. Res. Commun.* 9, 1–32. doi: 10.3109/10715769009148569

Halliwell, B. (1996). Antioxidants in human health and disease. *Annu. Rev. Nutr.* 16, 33–50. doi: 10.1146/annurev.nu.16.070196.000341

Halliwell, B. (1997). Antioxidants and human disease: a general introduction. *Nutr. Rev.* 55, S44–S49. doi: 10.1111/j.1753-4887.1997.tb06100.x

Halliwell, B., Gutteridge, J. M. C., and Aruoma, O. I. (1987). The deoxyribose method: a simple test tube assay for the determination of rate constants for the reaction of hydroxyl radicals. *Anal. Biochem.* 165, 215–219. doi: 10.1016/0003-2697(87)90222-3

Harman, G. E., Howell, C. R., Viterbo, A., Chet, I., and Lorito, M. (2004). Trichoderma species- opportunistic, avirulent plant symbionts. *Nat. Rev. Microbiol.* 2, 43–56. doi: 10.1038/nrmicro797

Huang, H. L., and Wang, B. G. (2004). Antioxidant capacity and lipophilic content of sea weeds collected from the Qingdao coastline. *J. Agric. Food Chem.* 52, 4993–4997. doi: 10.1021/jf049575w

Hyman, J. R., Gaus, J., and Foolad, M. R. (2004). A rapid and accurate method for estimating tomato lycopene content by measuring chromaticity values of fruit puree. *J. Am. Soc. Hortic. Sci.* 129, 717–723.

Jonathan, S. G., Lawal, M. M., and Oyetunji, O. J. (2011). Effect of spent mushroom compost of *Pleurotus pulmonarius* on growth performance of four Nigerian vegetables. *Mycobiology* 39, 164–169. doi: 10.5941/MYCO.2011.39.3.164

Jordan, S. N., Mullen, G. J., and Murphy, M. C. (2008). Composition variability of spent mushroom compost in Ireland. *Bioresour. Technol.* 99, 411–418. doi: 10.1016/j.biortech.2006.12.012

Krishnamoorthy, R. V., and Vajrabhiah, S. N. (1986). Biological activity of earthworm casts: an assessment of plant growth promoter levels in casts. *Proc. Natl. Acad. Sci. U.S.A.* 95, 341–351. doi: 10.1007/BF03179368

Lalander, C. H., Komakech, A. J., and Vinnerås, B. (2015). Vermicomposting as manure management strategy for urbansmall-holder animal farms – Kampala case study. *Waste Manag.* 39, 96–103. doi: 10.1016/j.wasman.2015.02.009

Lau, K. L., Tsang, Y. Y., and Chiu, S. W. (2003). Use of spent mushroom compost to bioremediate PAH-contaminated samples. *Chemosphere* 52, 1539–1546. doi: 10.1016/S0045-6535(03)00493-4

Lee, Y. L., Yang, J. H., and Mau, J. L. (2008). Antioxidant properties of water extracts from Monascus fermented soybeans. *Food Chem.* 106, 1128–1137. doi: 10.1016/j.foodchem.2007.07.047

Lemanska, K., Szymusiak, H., Tyrakowska, B., Zielinski, R., Soffers, A. E. M. F., and Rietjens, I. M. C. M. (2001). The influence of pH on antioxidant properties and the mechanism of antioxidant action of hydroxyflavones. *Free Radic. Biol. Med.* 31, 869–881. doi: 10.1016/S0891-5849(01)00638-4

Lim, S. L., Wu, T. Y., Lim, P. N., and Shak, K. P. Y. (2015). The use of vermicompost in organic farming: overview, effects on soil and economics. *J. Sci. Food Agric.* 95, 1143–1156. doi: 10.1002/jsfa.6849

Lin, C. H., Wei, Y. T., and Chou, C. C. (2006). Enhanced antioxidative activity of soybean koji prepared with various filamentous fungi. *Food Microbiol.* 23, 628–633. doi: 10.1016/j.fm.2005.12.004

Lores, M., Gomez-Brandon, M., Perez-Dıaz, D., and Domınguez, J. (2006). Using FAME profiles for the characterization of animal wastes and vermicomposts. *Soil Biol. Biochem.* 38, 2993–2996. doi: 10.1016/j.soilbio.2006.05.001

Moktan, B., Saha, J., and Sarkar, P. K. (2008). Antioxidant activities of soybean as affected by Bacillus-fermentation to kinema. *Food Res. Int.* 41, 586–593. doi: 10.1016/j.foodres.2008.04.003

Montalba, R., Arriagada, C., Alvear, M., and Zuniga, G. E. (2010). Effects of conventional and organic nitrogen fertilizers on soil microbial activity, mycorrhizal colonization, leaf antioxidant content, and *Fusarium wilt* in high bush blue berry (*Vaccinium corymbosum* L.). *Sci. Hortic.* 125, 775–778. doi: 10.1016/j.scienta.2010.04.046

Nautiyal, C. S., Govindarajan, R., Lavania, M., and Pushpangadan, P. (2008). Novel mechanism of modulating natural antioxidants in functional foods: involvement of plant growth promoting rhizobacteria NRRL B-30488. *J. Agric. Food Chem.* 56, 4474–4481. doi: 10.1021/jf073258i

Ndegwa, P. M., and Thompson, S. A. (2001). Integrating composting and vermicomposting in the treatment and bioconversion of biosolids. *Bioresour. Technol.* 76, 107–112. doi: 10.1016/S0960-8524(00)00104-8

Ochoa-Velasco, C. E., Valadez-Blanco, R., Salas-Coronado, R., Sustaita-Rivera, F., Hernández-Carlos, B., García-Ortega, S., et al. (2016). Effect of nitrogen fertilization and *Bacillus licheniformis* biofertilizer addition on the antioxidants compounds and antioxidant activity of greenhouse cultivated tomato fruits (*Solanum lycopersicum* L. var. Sheva). *Sci. Hortic.* 201, 338–345. doi: 10.1016/j.scienta.2016.02.015

Ohkawa, H., Ohishi, N., and Yagi, K. (1979). Assay for lipid peroxides in animal tissues by thiobarbituric acid reaction. *Anal. Biochem.* 95, 351–358. doi: 10.1016/0003-2697(79)90738-3

Prakash, D., Singh, B. N., and Upadhyay, G. (2007a). Antioxidant and free radical scavenging activities of phenols from onion (*Allium cepa*). *Food Chem.* 102, 1389–1393. doi: 10.1016/j.foodchem.2006.06.063

Prakash, D., Upadhyay, G., Singh, B. N., and Singh, H. B. (2007b). Antioxidant and free radical scavenging activities of seeds and agri-wastes of some varieties of soybean (*Glycine max*). *Food Chem.* 104, 783–790. doi: 10.1016/j.foodchem.2006.12.029

Pramanik, P., Ghosh, G. K., Ghosal, P. K., and Banik, P. (2007). Changes in organic-C, N, P and K and enzyme activities in vermicompost of biodegradable organic wastes under liming and microbial inoculants. *Bioresour. Technol.* 98, 2485–2494. doi: 10.1016/j.biortech.2006.09.017

Roy, B. H., and Rice-Evans, C. A. (1994). *Free Radical Damage and its Control*. Amsterdam: Elsevier Science, 125.

Sadasivam, S., and Manickam, A. (1996). *Biochemical Methods*. New Delhi: New Age International (P) Ltd., 256.

Sahu, A. (2011). *Soil Properties in Relation to Cadmium Toxicity and Phytoremediation Potential of African Marigold (Tagetes erecta L.)*. Varanasi: BHU.

Sardesai, V. M. (1995). Role of antioxidants in health maintenance. *Nutr. Clin. Pract.* 10, 19–25. doi: 10.1177/011542659501000119

Sarma, B. K., Singh, P., Pandey, S. K., and Singh, H. B. (2010). "Vermicompost as modulator of plant growth and disease suppression," in *Vermitechnology II. Dynamic Soil, Dynamic Plant*, Vol. 4, ed. N. Karmegam (Varanasi: Banaras Hindu University), 58–66.

Sarma, B. K., Yadav, S. K., Singh, S., and Singh, H. B. (2015). Microbial consortium-mediated plant defense against phytopathogens: readdressing for enhancing efficacy. *Soil Biol. Biochem.* 87, 25–33. doi: 10.1016/j.soilbio.2015.04.001

Schally, A. V., and Nagy, A. (1999). Cancer chemotherapy based on targeting of cytotoxic peptide conjugates to their receptor on tumors. *Eur. J. Endocrinol.* 141, 1–14. doi: 10.1530/eje.0.1410001

Sen, S., and Chakraborty, R. (2011). "The role of antioxidants in human health," in *Oxidative Stress: Diagnostics, Prevention, and Therapy. ACS Symposium Series*, Vol. 1083, (Washington, DC: American Chemical Society), 1–37. doi: 10.1021/bk-2011-1083.ch001

Singh, A., Jain, A., Sarma, B. K., Abhilash, P. C., and Singh, H. B. (2013a). Solid waste management of temple floral offerings by vermicomposting using *Eisenia fetida*. *Waste Manag.* 33, 1113–1118. doi: 10.1016/j.wasman.2013.01.022

Singh, A., Sarma, B. K., Upadhyay, R. S., and Singh, H. B. (2013b). Compatible rhizosphere microbes mediated alleviation of biotic stress in chickpea through enhanced antioxidant and phenylpropanoid activities. *Microbiol. Res.* 168, 33–40. doi: 10.1016/j.micres.2012.07.001

Singh, B. N., Singh, B. R., Singh, R. L., Prakash, D., Sarma, B. K., and Singh, H. B. (2009a). Antioxidant and anti-quorum sensing activities of green pod of *Acacia nilotica* L. *Food Chem. Toxicol.* 47, 778–786. doi: 10.1016/j.fct.2009.01.009

Singh, B. N., Singh, B. R., Singh, R. L., Prakash, D., Singh, D. P., Sarma, B. K., et al. (2009b). Polyphenolics from various extracts/fractions of red onion (*Allium cepa*) peel with potential antioxidants and antimutagenic activities. *Food Chem. Toxicol.* 47, 1161–1167. doi: 10.1016/j.fct.2009.02.004

Singh, H. B., Singh, B. N., Singh, S. P., and Nautiyal, C. S. (2010). Solid-state cultivation of *Trichoderma harzianum* NBRI-1055 for modulating natural antioxidants in soybean seed matrix. *Bioresour. Technol.* 101, 6444–6453. doi: 10.1016/j.biortech.2010.03.057

Singh, R., Sharma, R. R., Kumar, S., Gupta, R. K., and Patil, R. T. (2008). Vermicompost substitution influences growth, physiological disorders, fruit yield and quality of strawberry (*Fragaria ananassa* Duch.). *Bioresour. Technol.* 99, 8507–8511. doi: 10.1016/j.biortech.2008.03.034

Singh, U. B., Sahu, A., Sahu, N., Singh, B. P., Singh, R. K., Renu, S. D. P., et al. (2013c). Can endophytic *Arthrobotrys oligospora* modulate accumulation of defence related biomolecules and induced systemic resistance in tomato (*Lycopersicon esculentum* Mill.) against root knot disease caused by *Meloidogyne incognita*. *Appl. Soil Ecol.* 63, 45–56. doi: 10.1016/j.apsoil.2012.08.007

Singh, U. B., Sahu, A., Singh, R. K., Singh Dhananjaya, P., Meena, et al. (2012). Evaluation of biocontrol potential of *Arthrobotrys oligospora* against *Meloidogyne graminicola* and *Rhizoctonia solani* in rice (*Oryza sativa* L.). *Biol. Control* 60, 262–270. doi: 10.1016/j.biocontrol.2011.10.006

Thimmaiah, S. R. (2012). *Standard Methods of Biochemical Analysis*. New Delhi: Kalyani Publishers, 278–284.

Verma, S., Sharma, A., Kumar, R., Kaur, C., Arora, A., Shah, R., et al. (2015). Improvement of antioxidant and defense properties of tomato (var. *Pusa Rohini)* by application of bioaugmented compost. *Saudi J. Biol. Sci.* 22, 256–264. doi: 10.1016/j.sjbs.2014.11.003

Yang, J., Kloepper, J. W., and Ryu, C. M. (2008). Rhizosphere bacteria help plants tolerate abiotic stress. *Trends Plant Sci.* 14, 1–4. doi: 10.1016/j.tplants.2008.10.004

Yang, J. H., Mau, J. L., Ko, P. T., and Huang, L. C. (2000). Antioxidant properties of fermented soybean broth. *Food Chem.* 71, 249–254. doi: 10.1111/j.1750-3841.2011.02443.x

Conflict of Interest Statement: The authors declare that the research was conducted in the absence of any commercial or financial relationships that could be construed as a potential conflict of interest.

frontiers in Plant Science

ORIGINAL RESEARCH
published: 20 July 2018
doi: 10.3389/fpls.2018.01028

Biostimulant Potential of Humic Acids Extracted From an Amendment Obtained via Combination of Olive Mill Wastewaters (OMW) and a Pre-treated Organic Material Derived From Municipal Solid Waste (MSW)

Giuseppe Palumbo[1], Michela Schiavon[2], Serenella Nardi[2], Andrea Ertani[2], Giuseppe Celano[3] and Claudio M. Colombo[1]*

[1] Dipartimento di Agricoltura, Ambiente e Alimenti, Università degli Studi del Molise, Campobasso, Italy, [2] Dipartimento di Agronomia, Animali, Alimenti, Risorse Naturali e Ambiente, Università di Padova, Legnaro, Italy, [3] Dipartimento di Farmacia, Università degli Studi di Salerno, Fisciano, Italy

OPEN ACCESS

Edited by:
Youssef Rouphael, Università degli Studi di Napoli Federico II, Italy

Reviewed by:
Francesco Di Gioia, University of Florida, United States
Michele Di Foggia, Università degli Studi di Bologna, Italy
Giacomo Cocetta, Università degli Studi di Milano, Italy

***Correspondence:**
Andrea Ertani
andrea.ertani@unipd.it

Specialty section:
This article was submitted to Crop and Product Physiology, a section of the journal Frontiers in Plant Science

Received: *05 April 2018*
Accepted: *25 June 2018*
Published: *20 July 2018*

Citation:
Palumbo G, Schiavon M, Nardi S, Ertani A, Celano G and Colombo CM (2018) Biostimulant Potential of Humic Acids Extracted From an Amendment Obtained via Combination of Olive Mill Wastewaters (OMW) and a Pre-treated Organic Material Derived From Municipal Solid Waste (MSW). Front. Plant Sci. 9:1028. doi: 10.3389/fpls.2018.01028

Olive mill wastewaters (OMW) contain significant levels of phenolic compounds with antimicrobial/phytotoxic activity and high amounts of undecomposed organic matter that may exert negative effects on soil biology. Among OMW detoxification techniques, those focusing on oxidative degradation of phenolic compounds are relevant. The composting (bio-oxidation) process in particular, exploits exothermic oxidation reactions by microorganisms to transform the organic matrix of OMW into an amendment biologically stable and feasible to use in agriculture. This process consists of an active phase during which organic compounds are rapidly decomposed, and a curing phase characterized by a slow breakdown of the remaining materials with the formation of humic substances (HS) as by-products. In this study, bio-oxidation of OMW was performed using a pre-treated organic material derived from municipal solid waste (MSW). The obtained amendment (OMWF) was stable and in accordance with the legislative parameters of mixed organic amendments. HS were then extracted from OMWF and MSW (control amendment, Amd-C), and differences in structural properties of their humic acid (HA) fraction were highlighted via spectroscopy (Fourier Transform Infrared) and Dynamic Light Scattering. To assay a potential use of HA as biostimulants for crops, 12-day old *Zea Mays* L. plants were supplied with HA at 0.5 mg and 1 mg C L^{-1} for 2 days. HA from both amendments increased plant growth, but HA from OMWF was more effective at both dosages (plus 35–37%). Also, HA from OMWF enhanced both nitrogen assimilation and glycolysis by increasing the activity of nitrate reductase (∼1.8–1.9 fold), phosphoglucose isomerase (PGI) (∼1.8–2 fold) and pyruvate kinase (PK) (∼1.5–1.8 fold), while HA from Amd-C targeted glycolysis preferentially. HA from OMWF, however, significantly stimulated plant nutrition only at lower dosage, perhaps because certain undetermined compounds from detoxified OMW and incorporated in HA altered the root membrane permeability, thus preventing the increase of nutrient uptake. Conversely, HA from Amd-C increased nutrient accumulation in maize at both

dosages. In conclusion, our results indicate that the amendment obtained via OMW composting using MSW had a reduced pollution load in terms of phenolic compounds, and HA extracted from OMWF could be used as valuable biostimulants during maize cultivation.

Keywords: *Zea Mays* L., bio-oxidation, amendments, humic acids, biostimulants, FT-IR, nitrogen assimilation, glycolysis

INTRODUCTION

Olive mill wastewaters (OMW), also named olive vegetable waters, are endowed with properties that depend on the fruit variety and maturity, climate, soil type and extraction procedure (Borja et al., 2006). The disposal of OMW poses a concern to the olive oil industry, which is widely developed in the Mediterranean countries, such as Spain, Italy, Greece, and Tunisia (Niaounakis and Halvadakis, 2006; International Olive Council, 2014; Koutsos et al., 2018). These countries produce on average 2.74 million tons of olive oil per year, which account for 98% of the world production (Ahmadi-Esfahani, 2006). Italy is the second largest olive oil producer in the European Union (EU), with an estimated olive oil production of 500–600 thousand tons per year (Incelli et al., 2016).

The oil extraction process requires water in large amounts and generates an annual world production of wastewaters to be treated as high as 30 million tons (Roig et al., 2006; Mechri et al., 2007; Sellami et al., 2008). OMW are characterized by a high pollutant load because of their significant content in phenolic compounds with antimicrobial-phytotoxic action and limited biodegradability (Komilis et al., 2005; Justino et al., 2012). They also contain significant amounts of undecomposed organic substance, which may exert negative effects on soil biology and properties (Di Serio et al., 2008; Ntougias et al., 2013).

Unfortunately, a common European legislation framework concerning the management and the recycling of OMW in agriculture does not still exist. Therefore, EU countries discretely set the threshold limit values for the safe OMW disposal and re-use (Koutsos et al., 2018). In Italy, the disposal of OMW as amendments in agriculture is currently regulated by Law 574/96, which allows the shedding of OMW on soil within the range of 50–80 m^3 ha^{-1}. Spreading practices of OMW on soil exceeding this threshold might be harmful to the ecosystem and cause failure of crop cultivation (Ouzounidou et al., 2010; Incelli et al., 2016). However, whether properly detoxified, OMW could be used as organic fertilizers to improve soil fertility under conditions of nutrient and organic matter shortage (Mekki et al., 2006; Nasini et al., 2013). Poor and unstructured soils for instance, can be enriched in nitrogen (N), phosphorus (P), and potassium (K) through spreading of detoxified OMW (Isidori et al., 2005).

Olive mill wastewaters detoxification techniques include treatments focused on the degradation of bioactive molecules, primarily phenolic compounds (Casa et al., 2003; Silva et al., 2007). Among these techniques, the most widely employed are those based on flocculation/coagulation in water (Khoufi et al., 2008; Flores et al., 2018), ozonization (Andreozzi et al., 1998), centrifugation and ultrafiltration (Arvanitoyannis et al., 2007), sonication (Oztekin and Sponza, 2013), anaerobic digestion, dilution (Hamdi et al., 1991; Andreozzi et al., 2008; El-Gohary et al., 2009), oxidative degradation via addition of manganese and iron oxides (Colarieti et al., 2006), and composting or bio-oxidation (Paredes et al., 2002; Sanchez Monedero et al., 2002; Sampedro et al., 2007). This last approach in particular, exploits exothermic oxidation reactions by microorganisms to transform the organic matrix of OMW into a biologically stable and odorless amendment, feasible to use in agriculture (Ghanbari et al., 2012; Gigliotti et al., 2012). The quality of the amendment is evaluated based on the absence of pathogens and heavy metals.

Specifically, the composting process consists of two stages in sequence: an active, thermophilic phase, during which organic components undergo intense and rapid degradation activity and the break down of phytotoxic compounds occurs and proceed until the biological stability of the process is achieved; a curing phase, characterized by the degradation and further transformation of recalcitrant organic components, with the formation of humic substances (HS) (Adani et al., 1997). In this process, it is possible that some of the phenolic compounds contained in OMW detoxified in this way become part of the HS instead of being degraded.

Humic substances comprise humic and fulvic acids, and consist of small molecules of amphiphilic nature able to generate molecular aggregates or supramolecular assemblies in solution and on mineral surfaces (Wershaw, 1999; Piccolo, 2001; Schaumann, 2006). HS influence plant physiology by triggering complex transcriptional networks through an intricate mechanism of action involving auxin- dependent and independent signaling pathways (Muscolo et al., 2013; Nardi et al., 2016, 2017). They are also widely recognized as biostimulants, i.e., products containing substances and/or microorganisms whose function in trivial amounts is to promote plant growth-related processes, enhance plant nutrient uptake and use efficiency, resistance and tolerance to abiotic stress, and improve the quality of crop-derived products (European Biostimulants Industry Council [EBIC], 2013). The effects of HS in plants depends on their concentration, molecular weight and physical-chemical properties. Strong evidence exists that HS effects in plants are in part due to their content in substances displaying hormone-like activity (Nardi et al., 1994; Mora et al., 2010). HS can stimulate plant nitrogen (N) uptake and assimilation (Varanini and Pinton, 2001; Vaccaro et al., 2009), and induce changes in root architecture, especially in the early phases of plant

development (Canellas et al., 2002; Nardi et al., 2002; Zandonadi et al., 2007). Additionally, HS enhance the root H^+-ATPase activity (Nardi et al., 1991; Zandonadi et al., 2010) and control nutrient availability in maize (Eyheraguibel et al., 2008).

Combining the importance of HS in plant productivity and the idea of recycling OMW for agricultural purposes, the aim of this study consisted in: (i) detoxifying OMW via a bio-oxidation process using a pre-treated organic material derived from municipal solid waste (MSW); (ii) extracting HA from the resulting amendment and compare their properties with those of HA obtained from a control amendment (MSW without OMW); (iii) testing whether the obtained HA displayed beneficial properties on maize (*Zea Mays* L.) plant metabolism and could be used as valuable biostimulants.

MATERIALS AND METHODS

Chemical and Physical Analyses of OMW

Olive mill wastewaters were furnished by an olive mill farm located in Isernia (Molise region, Italy) and produced via olive oil centrifugation using a three-phase process. Three samples (100 g each) of OMW were collected and analyzed. With respect to nitrogen (N) forms, the content of ammonium (NH_4^+) was determined using the Nessler method reported by Trombetta et al. (1998), while NO_3^- and NO_2 were quantified via steam distillation procedures described by Bremner and Keeney (1965).

The remaining chemical analyses were performed according to the analytical procedures reported in the official methods of Italian soil chemical analysis (Violante, 2000).

For total phenol determination, 5 mL of OMW were centrifuged for 5 min at 5,000 g; 0.25 mL of supernatant were then added with 0.5 mL ethyl acetate, and the obtained extract was stirred and centrifuged for 5 min at 5,000 *g*. The extraction procedure was repeated three times with further additions of ethyl acetate (0.5 mL). Finally, the supernatant was dried at room temperature for about 48 h. The solid extract was solubilized using 0.25 mL of a mixture containing methanol and water in the ratio 4:1 (v:v), and then vortexed for 2 min. The extract was placed in 10 mL tubes, added with 1 mL of distilled H_2O, 0.9 mL of 0.5 M $NaHCO_3$ (pH 8.5), and 1 mL of diluted acetate 1/10 (v/v). The extract was then stirred for 2 min and after 2 h the content of total phenols was determined via spectrophotometer at λ = 765 nm according to the Folin–Ciocalteu method described by Zullo et al. (2014). The amount of total polyphenols was expressed in mg dm^{-3} of gallic acid. Quantification of individual phenols was performed as described below for MSW.

Analyses were all conducted on 100 g of homogenized of the same OMW.

Chemical and Microbiological Analyses of MSW

Before being processed with OMW, the organic material derived from MSW was analyzed for the presence of microorganisms in order to exclude the existence of human pathogens, mainly enteric bacteria, viruses, protozoa, and helminthes (Bonadonna et al., 2002). Total and fecal coliforms were absent, thus attesting the good hygienic conditions of the matrix. The determination of total organic carbon, Kjeldahl nitrogen, phosphorus Olsen, electrical conductivity and pH were performed according to the methods of Violante (2000). Specifically, total organic carbon was analyzed by the method Springer-Klee, nitrogen was determined using the Kjeldahl method, phosphorus (P) Olsen was measured spectrophotometrically at λ = 720 nm, electrical conductivity and pH were measured in a sample suspension added with distilled water in the ratio 1:1 (v:v). Elemental analysis was conducted via ICP-OES (Inductive Coupled Plasma Optical Emission), after the sample was digested with a solution of 65% HNO_3/37% HCl (ratio HNO_3/HCl 1:3 v/v), warmed until boiling for 30 min under agitation, according to the manufacturer's instructions, and filtered at 0.45 μm filter (Millipore). Analyses were all performed on three samples (100 g each) of MSW.

Bio-Oxidative Process for OMW Composting

Detoxification of OMW was performed through the addition of OMW to a pre-treated organic material derived from MSW, under conditions (T = 65°C) that prevented the growth of pathogenic organisms, while favored the development of bacteria required for OMW composting. The content in polyphenols was measured after 6 months since the beginning of the bio-oxidative process.

The OMW (20 L) was gradually added to MSW to favor the adsorption process and get a ratio of 1:1 on the organic matrix. Urea (2%, v/v) was also added. The resulting amendment was named OMWF (olive mill water filter plus MSW pre-treated organic material). The MSW pre-treated organic material added with 2% (v/v) urea was used as control (Amd-C). The organic material lodged in the bins was turned over periodically, every 20 days. The composting process was monitored for 6 months and total carbon and total nitrogen were determined in the amendments using the Springer–Klee and Kjeldahl methods, respectively (Violante, 2000) in three replicates. The efficacy of the bio-oxidative process in reducing OMW toxicity was evaluated by measuring the variation in content and profile of phenolic compounds. Extraction of total polyphenols from the amendments was performed using ethyl acetate as described previously, and their content was determined via HPLC using a UV-VIS detector DAD at 280 nm and a column Synchronis C18, 15 cm in length and 4.6 cm in diameter, with a particle size diameter of 5 μm. The separation of the different fractions was performed according to the following conditions: 0 to 15 min by using a gradient mixture of 95% acetic acid 0.5% (A) and 5% acetonitrile (B) up to 80% A and 20% B, with isocratic separation up to 30 min. The identification of phenolic compounds was carried out by comparing their retention times (RT) and online UV spectra with those of reference standards corresponding to phenolic compounds commonly present in most OMW (hydroxybenzoic acid, syringic acid, verbascoside and ferulic acid). Standards were provided by Sigma-Aldrich Ltd.

Chemical Extraction of Humic Acids (HA) From the Amendments

Humic substances extraction was carried out according to Nardi et al. (1994). Chemical fractioning in humic acids (HA), fulvic acids (FA), and humin (HU) was based on the differential solubility of the organic fractions of the amendment depending on the pH. Control (Amd-C) and OMWF amendments were placed in individual 500 mL Erlenmeyer flasks and added with 0.5 M NaOH (40 mL) and distilled water (80 mL). Nitrogen (N) in each flask was insufflated for a few seconds. The suspensions were shaken for 6 h and left to rest for further 12 h. Each suspension was then centrifuged at 6,000 × *g* in order to obtain two fractions, one containing the total extractable carbon (TEC). The TEC-containing solution was placed in a cylinder and acidified with HCl (ratio 1:1, v/v) to achieve a pH lower than 2, which allowed the separation of the HA fraction from the supernatant (FA fraction). Both HA and FA were purified and dialyzed. HA were initially freeze-dried and their content in C was measured using an elemental analyzer (Vario MACRO CNS, Hanau, Germany). Then, an amount of them corresponding to 10 mg C was re-suspended in deionized water in the presence of few drops of pure NH_3 to obtain a HA stock solution with 1 mg ml^{-1} final concentration. NH_3 was removed using rotavapor in the presence of acidic trap.

FTIR Spectroscopy of Humic Acids

Before performing spectroscopic analyses, samples of HSs obtained from Amd-C and OMWF amendments were reduced to an impalpable powder in agate mortar using potassium bromide (KBr), and kept in a desiccator for 24 h with silica gel.

FTIR spectra of Amd-C and OMWF were obtained via a VERTEX70/70v high-resolution spectrophotometer (Bruker, Italy). The absorbance spectra were collected at a spectral resolution of 4 cm^{-1} with 256 scans, between 400 and 4,000 cm^{-1} and converted to absorbance using the software OPUS 6.5 (Bruker Optics).

Spectroscopic analyses were performed on three samples of HA. We only show one representative FTIR spectrum.

Dynamic Light Scattering (DLS) of Humic Acids: Particle Size (PS) and Electrophoretic Mobility (EM)

Both PS and EM measurements were performed at 25 ± 0.1°C with a Zetasizer Nano-ZS (Malvern, Instruments), consisting of an Avalanche photodiode (APD) detector and a 4 mW He-Ne laser (λ = 633 nm). This instrument was widely used for a large variety of colloidal dispersions. ζ potential data were calculated from EM by the Henry equation (1):

$$EM = \frac{2\varepsilon\zeta}{3\eta} f(\kappa R) \quad (1)$$

Where ε is the dielectric constant, η the viscosity, *R* the particle hydrodynamic radius and κR the ratio of R to Debye length. To convert EM into ζ the Smoluchowski factor $f(\kappa R) = 1.5$ was used (valid for $\kappa R > 1$). Effective voltage gradient was in the range 40–140 mV mm^{-1}.

Particle size distributions and Poly Dispersity Index (PDI) were obtained from the intensity autocorrelation function by the cumulate and CONTIN methods, respectively, using the Malvern software (DTS Version 6.01). The apparent hydrodynamic diameter *D* was calculated from the *Z*-average translation diffusion coefficient through the Stokes–Einstein equation (2) assuming spherical particles:

$$D = \frac{k_B T}{3\pi\eta D} \quad (2)$$

Where k_B is the Boltzmann constant, *T* is temperature, η the viscosity and *D* is the apparent hydrodynamic diameter.

Particle size and ξ data of aqueous dispersions of humic acids extracted from amendment and humic acids extracted from amendment adsorbed with vegetation waters were monitored in the pH range 2–10. Data represented the mean of three replicates.

Plant Growth Experimental Design

Zea mays L. seeds (var. DKc 5783, DeKalb, Lodi, IT) were soaked in distilled water for one night and then surface-sterilized in 5% (v/v) sodium hypochlorite for 10 min while shaking (Ertani et al., 2009). The seeds were left to germinate for 60 h in the dark at 25°C on a filter paper wetted with 1 mM $CaSO_4$. Germinated seedlings were transplanted into 3 L pots (density of plants = 10 per pot) equipped with net holds into the top, which provided a hydroponic floating system for plant growth. Roots floated in a modified Hoagland nutrient solution (Hoagland and Arnon, 1950) that was maintained aerated via air insufflation. The nutrient solution was renewed every 48 h and had the following composition: (μM): KH_2PO_4 (40), $Ca(NO_3)_2$ (200), KNO_3 (200), $MgSO_4$ (200), FeNaEDTA (10), H_3BO_3 (4.6), $CuCl_2{\cdot}2H_2O$ (0.036), $MnCl_2{\cdot}4H_2O$ (0.9), $ZnCl_2$ (0.09), $NaMoO{\cdot}2H_2O$ (0.01). Plants were cultivated in a climate chamber under 14 h light/10 h dark cycle, with an air temperature of 27/21°C, relative humidity of 70/85% and at a photon flux density of 280 mol m^{-2} s^{-1}. On the 12th day, part of the plants was treated for 48 h with humic acids (HA) extracted from Amd-C and OMWF amendments. Based on their C content (28 and 26% w/w for Amd-C and OMWF, respectively), different volumes of HA from stock solutions (1 mg ml^{-1}) were added to the nutrient solution in order to supply plants with two different C concentrations: 0.5 mg and 1 mg carbon per liter (C L^{-1}). The HA concentrations were chosen based on previous studies where the most pronounced effects of HS in plants were observed at 0.5 mg and 1 mg C L^{-1} in a period as short as 48 h.

The remaining part of the untreated plants was used as a control (C). For each experimental condition, three pots were prepared. The experiment was repeated three times and was performed according to a randomized block design.

At the end of the 48 h, the plantlets were collected and divided in leaves and roots. Roots were carefully washed with distilled water first, and then with 10 mM EDTA for 15 min to remove any metal remained in root apoplast. Plant material was blot-dried and analyzed for dry weight and activities of marker enzymes.

For dry weight measurement, 30 plants were used (10 per treatment from each pot). Plants were divided into roots and leaves, and weighed separately. The samples were placed in a drying oven for 2 days at 70°C and allowed to cool down for 2 h inside a closed bell jar.

Mineral-Nutrient Determination

The determination of mineral nutrients in leaves and roots of maize plants was performed after an acid-digestion procedure (HNO_3/HCl 1:3, v/v). All digestion reactions were carried out in closed Teflon vessels of 120mL volume using 500 mg plant material and 10 mL of 30% (v/v) HCl as a solvent. Digested samples were then diluted in 10 mL ultrapure water and assayed via Inductively Coupled Plasma–Optical Emission Spectroscopy (ICP-OES, Optima 2000 DV, Perkin Elmer Instruments Germany).

Enzyme Extraction and Assay Conditions, Protein Quantification

For enzyme activity assays, leaf material from five individual plants (biological replicates) per treatment was used.

For the extraction of phosphoglucose isomerase (PGI) and pyruvate kinase (PK) enzymes, leaves (1 g) from maize plants subjected to different treatments were ground in a mortar using liquid nitrogen and homogenized for 5 min in the presence of 100 mM HEPES-NaOH pH 7.7, 10 mM $MgCl_2 \cdot 6H_2O$, 0.4 mM Na_2EDTA, 100 mM Na ascorbate, 1% (w/v) polyvinylpyrrolidone (PVP), 1% (w/v) BSA (bovine serum albumin), and 5 mM reduced glutathione (GSH). The homogenate was then filtered and centrifuged at 20,000 × *g* for 20 min at 4°C.

Phosphoglucose isomerase (PGI, EC 5.3.1.9) assay: 530 μL 0.2 M Tris adjusted with 0.1 M HCl to pH 9.0 were added with 75 μL 20 mM β-NADH-Na_2-salt in distilled water, 75 μL 80 mM fructose-6-phosphate-Na_2 in 0.2 M Tris pH 9.0, and 20 μL glucose 6-phosphate dehydrogenase (from yeast) diluted to 30 U mL^{-1} with 0.2 M Tris pH 9.0. The reaction was started by adding 50 μL extract after a lag time of 20 min at 30°C (Nowotny et al., 1998). Measurements were performed spectrophotometrically for 60 s at λ = 340 nm.

Pyruvate kinase (PK, EC 2.7.1. 40) assay: 450 μL 0.1 M triethanolamine (TEA) adjusted with NaOH to pH 7.75 were added with 50 μL 3 mM β-NADH-Na_2-salt in 0.1 M TEA pH 7.75, 50 μL 0.15 M $MgSO_4 \cdot 6H_2O$ and 0.15 M KCl in 0.1 M TEA (pH 7.75), 50 μL L-lactic dehydrogenase diluted to 225 U mL^{-1} with TEA (pH 7.75), and 50 μL extract. The reaction was started after a lag time of 10 min at 30°C by adding 50 μL 0.225 M 2-phosphoenolpyruvate-Na-H_2O in 0.1 M TEA (pH 7.75) (Nowotny et al., 1998). Measurements were performed spectrophotometrically at λ = 340 nm.

For the extraction of nitrate reductase (NR, E.C.1.7.1.1), leaf tissues (1 g) were ground in a mortar and added with 100 mM HEPES-NaOH pH 7.5, 5 mM MgCl2, and 1 mM dithiothreitol (DTT). The ratio of plant material to mixture solution was 1:3 (v/v). The extract was filtered through two layers of muslin and clarified by centrifugation at 20,000 *g* × 15 min. The supernatant was then used for enzymatic analysis. All steps were carefully performed at 4°C (Schiavon et al., 2008). The activity of nitrate reductase (NR) was assayed in a solution containing 100 mM KH_2PO_4, 100 mM KNO_3, and 400 mL of enzyme extract. The activity was measured spectrophotometrically at λ = 540 nm, and the calibration curve was plotted with known concentrations of $NaNO_2$ (Lewis et al., 1982).

The total content of proteins was measured in leaves (1 g) from three individual plants (biological replicates) per treatment, estimated via the Bradford (1976) method and expressed in milligrams per gram of fresh weight (mg g^{-1} of fresh weight).

Statistical Analysis

The analysis of variance (ANOVA) was performed using the SPSS software version 18.0 (SPSS, Chicago, IL, United States), and was followed by pair-wise *post hoc* analyses (Student–Newman–Keuls test) (Sokal and Rohlf, 1969) to determine which means differed significantly at $p < 0.05$ (±std).

RESULTS

Chemical Properties of OMW

In **Table 1** the main chemical properties of OMW are reported. The pH value was within the typical range of OMW (4–6.7) (Incelli et al., 2016), while the chemical oxygen demand (COD) and the biological oxygen demand (BOD) displayed high values (Rinaldi et al., 2003). With respect to inorganic N species, NH_4^+ was prevalent over N oxidized forms (NO_3^- and NO_2). The content of the mineral fraction, in particular of potassium, phosphorus and calcium was medium–high, whereas Na^+ was high. The amount of total suspended particles and total phenolic compounds (TPC) was elevate.

TABLE 1 | Chemical properties of OMW sample.

Parameter	Values	Unit
pH	4.7 ± 0.5	
COD	70 ± 10	g dm^{-3}
BOD	32.5 ± 2.5	g dm^{-3}
Cl^-	5.5 ± 0.5	mg dm^{-3}
SO_4^{2-}	0.10 ± 0.02	mg dm^{-3}
P Tot	180 ± 10	mg dm^{-3}
NH_4^+	125 ± 25	mg dm^{-3}
NO_3^-	3.5 ± 0.5	mg dm^{-3}
NO_2^-	6.0 ± 0.6	mg dm^{-3}
TPC	5.0 ± 1.0	g dm^{-3}
TSP	75 ± 15	g dm^{-3}
Na^+	15 ± 2.5	mg dm^{-3}
K^+	5, 000 ± 1, 000	mg dm^{-3}
Mg^{2+}	7.5 ± 2.5	mg dm^{-3}
Ca^{2+}	20.0 ± 7.3	mg dm^{-3}

COD, chemical oxygen demand; BOD, biological oxygen demand; TPC, Total Phenols Compounds; TSP, Total Suspended Particles. In table, standard deviations are reported (n = 3).

TABLE 2 | Chemical parameters of the organic material from MSW.

Parameter	Values	Unit
Dry leftover	73.0 ± 5.2	%
Moisture	26.9 ± 1.8	%
pH	7.44 ± 0.6	7.44
EC	5, 600 ± 120	$\mu S\ cm^{-1}$
CSC	46 ± 8	cmol(+) kg^{-1}
TOC	161.4 ± 15.6(< 200)	g kg^{-1}
HS	12.26 ± 3.5	g kg^{-1}
TN	17.8 ± 2.9	g kg^{-1}
C/N	9.06 ± 0.35	
P_{olsen}	7.7 ± 1.4	mg kg^{-1}
K_2O	0.68 ± 0.20	g kg^{-1}
Cd	0.10 ± 0.02(< 1.5)	mg kg^{-1}
Cr	18.10 ± 2.14(< 0.5)*	mg kg^{-1}
Hg	0.007 ± 0.001(< 1.5)	mg kg^{-1}
Ni	5.04 ± 1.12(< 100)	mg kg^{-1}
Pb	15.33 ± 2.10(< 140)	mg kg^{-1}
Cu	30.66 ± 10.8(< 230)	mg kg^{-1}
Zn	60 ± 12(< 600)	mg kg^{-1}

*EC, electric conductivity; CSC, cation exchange capacity; TOC, total organic carbon; HS, humic substances; TN, total nitrogen. In table, standard deviations are reported (n = 3). In brackets, the threshold for TOC, TN, P and metals is shown. *The limit in the brackets is referred to hexavalent Cr only.*

Chemical Properties of MSW and Effects of the Bio-Oxidation Treatment on C and N Contents in the Amendments

The organic material used for the composting process was below the limit (<20% TOC) prescribed for organic fertilizers by the Italian regulations (amendments, Italian law 748/1984), and the C/N ratio was slightly below 10. The concentration of all metals was also below the threshold of toxicity (**Table 2**).

The weekly monitoring of both temperature and matrix water content followed the organic material maturation. After 6 months since the beginning of the bio-oxidation process, a higher content in C (plus 13%) and N (plus 11%) was observed in OMWF in comparison with Amd-C (**Figures 1A,B**). Because the increases in C and N were comparable, the C/N ratio was not significantly influenced by the additions of the OMW (**Figure 1C**). Generally, an ideal amendment should have a C/N ratio equal to 10, which is required for the normal microbial growth and the optimal process of humification.

Effect of the Bio-Oxidation Treatment on OMW Phenolic Content

The chromatographic profiles of OMW revealed the presence of multiple peaks corresponding to the following phenolic acids: gallic, hydroxytyrosol, hydroxybenzoic, syringic, ferulic, and verbacoside (**Figure 2A**). Among them, hydroxytyrosol acid was the most abundant. The analysis of extracts from control (Amd-C) (**Figure 2B**) and OMWF (**Figure 2C**) amendments confirmed the capacity of the bio-oxidation process to reduce the content of polyphenols within a short retention time (29 min) in the original OMW. Ferulic acid and verbascoside acid were still present in the OMWF amendment although in little amounts as in OMW, while the same compounds were missing in Amd-C. Quantification of individual phenols identified is reported in **Table 3**. Phytotoxicity tests via germination assays verified the good quality of OMW treated amendment (data not shown).

Fourier Transform Infrared (FT-IR) and Dynamic Light Scattering (DLS) of Humic Acids

The OMWF amendment showed an important enrichment in humic acids (HA) as compared to Amd-C amendment. This result clearly indicates that the amount of total polyphenols in OMW was involved in the generation of humic acids during the bio-oxidative process. FT–IR spectra of HA of Amd-C and OMWF (**Figure 3**) showed bands of absorption corresponding to the major classes of organic compounds typical of HSs

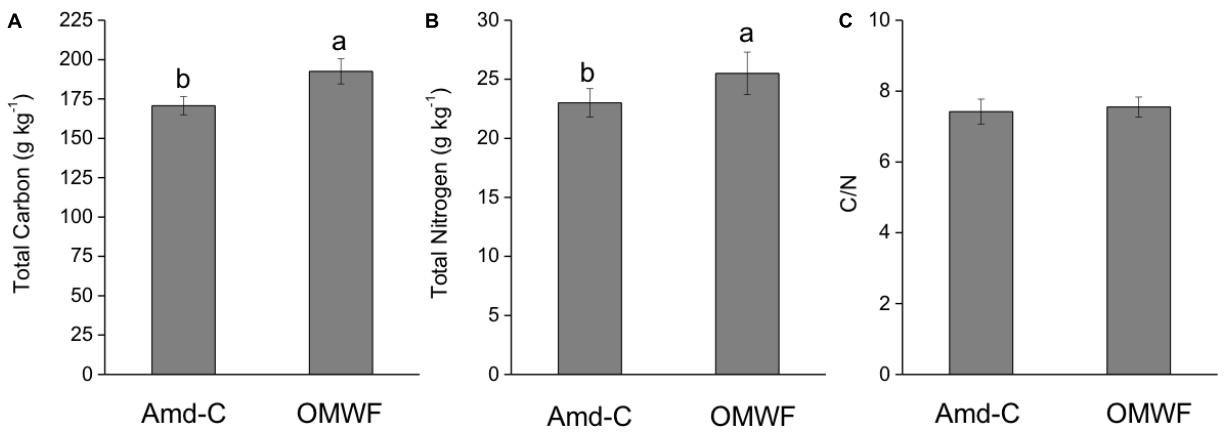


FIGURE 1 | Total carbon **(A)**, total nitrogen **(B)** content, and C/N ratio **(C)** of control amendment (Amd-C) and olive mill water filter plus municipal solid waste (MSW) pre-treated organic material (OMWF) amendment. Different letters above bars indicate significant differences between treatments at $P < 0.05$ according to Student–Newman–Keuls test.

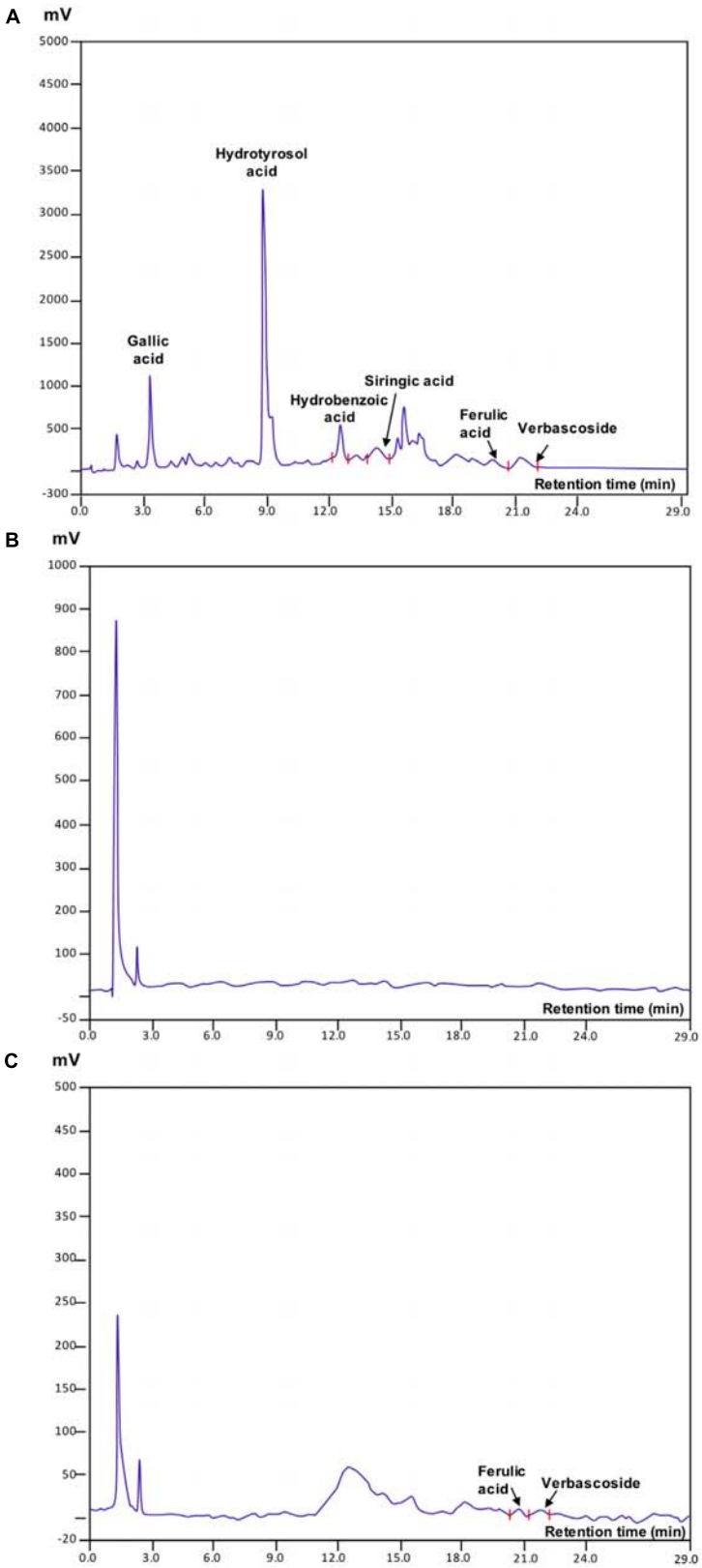


FIGURE 2 | HPLC chromatograms of **(A)** olive mill wastewaters (OMW), and **(B)** control amendment (Amd-C) and **(C)** olive mill water filter plus MSW pre-treated organic material (OMWF) amendment after 6 months of OMW bio-oxidation.

TABLE 3 | Content (mg mL^{-1}) of individual phenolic acids identified in olive mill wastewaters (OMW) before the bio-oxidation process, and in the obtained OMWF amendment.

Identified phenolic compound	OMW	OMWF
Gallic acid	23.62 ± 2.35	<LOD
Hydroxytyrosol acid	373.22 ± 3.98	<LOD
Hydroxybenzoic acid	30.65 ± 3.24	<LOD
Syringic acid	22.60 ± 2.11	<LOD
Ferulic acid	2.73 ± 0.45	0.07 ± 0.01
Verbascoside	17.86 ± 1.10	1.48 ± 0.18

LOD, limit of detection.

(AitBaddi et al., 2003). Indeed, they were characterized by the presence of strong bands attributed to carboxylic acid groups (1710–1715 cm^{-1}), and bands associated to aromatic (1660–1610 cm^{-1}), polysaccharide (1083 cm^{-1}) and aminic compounds (1560–1590 cm^{-1}) (Rao, 1963; Silverstein et al., 1981; Schulz and Baranska, 2007).

The peak at 1360–1580 cm^{-1} was attributed to the stretching of groups (COO−) of carboxylic compounds. In the amendment OMWF, a peak was visible at about 1170–1180 cm^{-1}, which could be attributed to C–H vibrations of bending of the aromatic groups (Rao, 1963; Silverstein et al., 1981; Schulz and Baranska, 2007). In OMWF, an emission band very flared and irregular similar to a shoulder up to 2400 cm^{-1} was also evident and might indicate a different aromatic composition of the sample (Rao, 1963; Silverstein et al., 1981; Schulz and Baranska, 2007).

The particle sizes (D, diameter) and zeta potential (ZP) of HA obtained from Amd-C and OMWF amendments are reported in **Table 4**. Both HA data trends shared a similar behavior, which was characterized by a rapid decrease of particle size when the pH increased from 2 to 4. At higher pH values, the particle size of HA from Amd-C showed a systematic increment, while a weaker increase was observed for HA derived from OMWF. A similar trend was measured for zeta potential. In this case, HA from both amendments exhibited a neutral surface charge at low pH values. While increasing the pH, a progressive decrease in zeta potential was displayed by both series of HA data, which indicated a stronger colloidal stability of HA when the pH was neutral or basic.

Effects of HA From Amd-C and OMWF on Maize Plant Growth and Nutrient Content

The effect of HA extracted from Amd-C and OMWF amendments on maize plant productivity was evaluated in terms of leaf and root dry biomass promotion (**Figures 4A,B**) and nutrient content (**Table 5**). The trend of growth stimulation for leaves was the same as for roots. HA extracted from Amd-C and supplied to plants at 0.5 mg C L^{-1} increased the dry weight (DW) of leaves and roots by about 32 and 68%, respectively, while at higher dosage (1 mg C L^{-1}) they did not produce any significant change in biomass. Conversely, both dosages of HA derived from OMWF amendment determined similar increases in leaf and root growth (plus 35–37%), with values comparable to those measured for plants treated with HA from Amd-C at 0.5 mg C L^{-1}.

With respect to the content in mineral nutrients, plants supplied with HA from Amd-C contained higher concentration of macro- and micro-elements in both leaves and roots compared to the untreated plants (**Table 5**). HA extracted from OMWF amendment exerted a general positive effect on nutrient accumulation when furnished to plants at lower dosage (0.5 mg

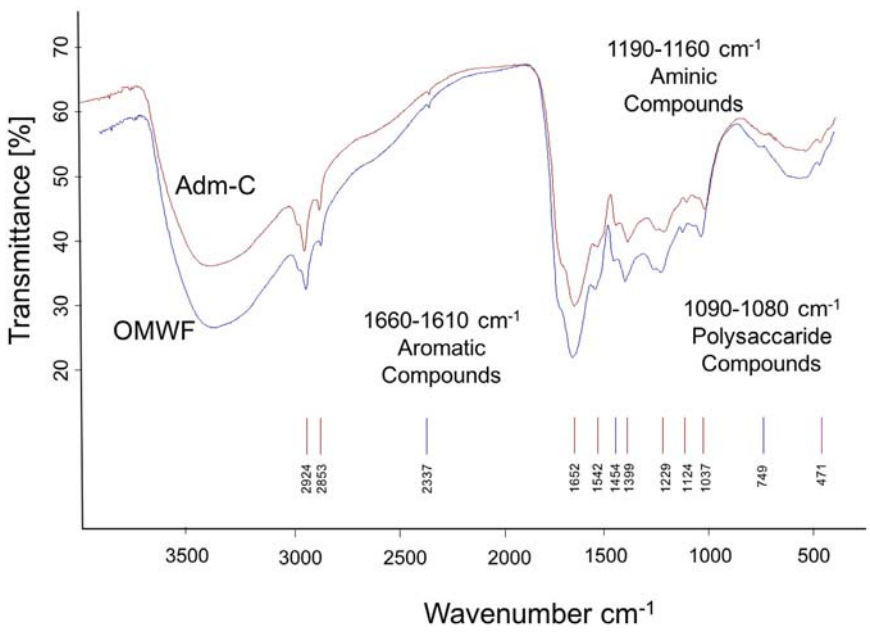


FIGURE 3 | FT-IR spectra of humic acids (HA) extracted from control amendment (Amd-C) and olive mill water filter plus MSW pre-treated OMWF.

TABLE 4 | Diameter of the particles of humic acids and zeta potential at different pH values.

HA from Amd-C					HA from OMWF				
pH	*D* (nm)	PDI/D	ZP (mV)	IC (mS cm^{-1})	pH	*D* (nm)	PDI/D	ZP (mV)	IC (mS cm^{-1})
2	949 ± 45a	0.39 ± 0.03d	−2.68 ± 0.82c	15.10 ± 2.03a	2	869 ± 24a	0.41 ± 0.04c	−3.15 ± 0.23d	14.30 ± 1.40a
4	219 ± 34d	0.53 ± 0.06c	−27.90 ± 1.48b	1.81 ± 0.18b	4	313 ± 18d	0.47 ± 0.04c	−33.30 ± 1.52b	2.24 ± 0.24b
6	358 ± 40c	0.76 ± 0.07b	−30.80 ± 1.60ab	1.71 ± 0.20b	6	478 ± 26b	0.80 ± 0.06a	−27.10 ± 1.83c	2.31 ± 0.21b
8	424 ± 26c	0.70 ± 0.07b	−33.70 ± 1.23a	1.67 ± 0.33b	8	381 ± 25c	0.64 ± 0.06b	−30.66 ± 1.55bc	1.68 ± 0.23c
10	692 ± 40b	1.00 ± 0.08a	−34.90 ± 1.30a	2.02 ± 0.13b	10	419 ± 37bc	0.71 ± 0.03ab	−37.00 ± 2.10a	1.84 ± 0.14b

D, diameter; PDI, Poly Dispersity Index; ZP, zeta potential; IC, ionic conductivity. Different letters along columns indicate significant differences ($p < 0.05$, ±SD, $n = 3$) according to Student–Newman–Keuls test.

C L^{-1}), while they were almost ineffective at higher dosage (1 mg C L^{-1}), as they only increased Mn in leaves and Cu in roots. The concentration of Na in plant tissues was also determined to evaluate whether Na contained in OMW could have been delivered to plants. Interestingly, we found a decrease of Na in leaves of maize plants supplied with HA from both amendments, while in roots Na reduction only occurred when plants were treated with HA from OMWF.

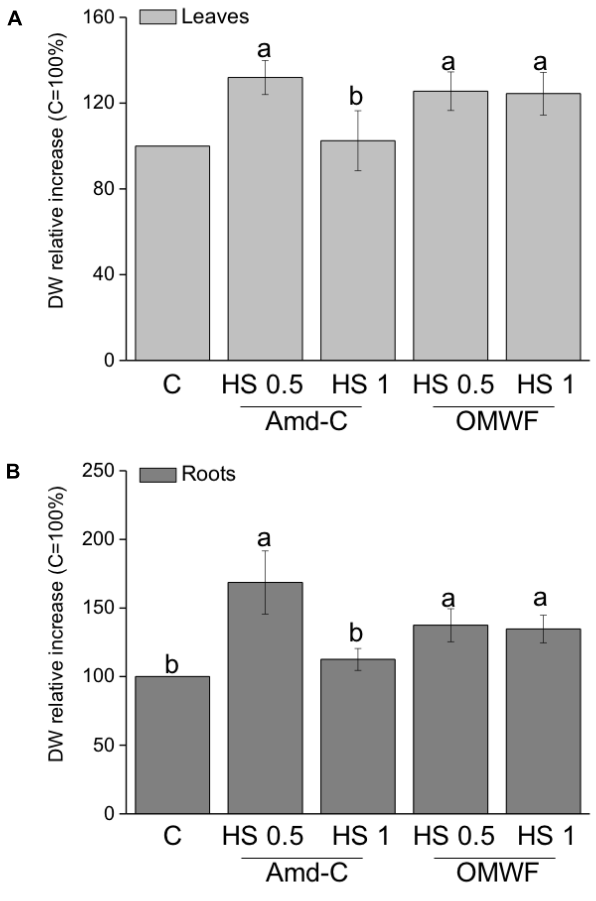


FIGURE 4 | (A) Leaf and **(B)** root dry weight of *Zea mays* plants grown for 12 days in Hoagland modified nutrient solution and supplied for 2 days with HA extracted from control amendment (Amd-C) and olive mill water filter plus MSW pre-treated OMWF at 0.5 or 1 mg C L^{-1}. Data represent the means of three measurements with five plants in each (±*SD*). Values are reported as percentage of leaf and root dry weight variation relative to the control (=100%). Different letters above bars indicate significant differences between treatments at $P < 0.05$ according to Student–Newman–Keuls test.

Effects of HA From Amd-C and OMWF on Enzyme Activity and Protein Content

To assay the effects of HA on plant metabolism, changes in activity of enzymes working in glycolysis and nitrogen assimilation was evaluated in maize plants treated with HA derived from Amd-C and OMWF amendments (**Figures 5A–D**). The activity of NR, GS, PGI, and PK enzymes was differentially regulated by HA from Amd-C and OMWF amendments. Specifically, the activity of NR was significantly and equally enhanced by both dosages of HA extracted from OMWF being about 1.8–1.9-fold higher than the control, but no variation was observed between untreated plants and plants supplied with HA from Amd-C. GS activity was unchanged following HA application to plants, while PGI activity was strongly and similarly up-regulated by HA, regardless of the amendment origin and dosage. PK activity in plants supplied with both dosages of HA from OMWF or with 0.5 mg C L^{-1} of HA produced from Amd-C was higher than in control plants.

The two amendments increased the amount of total proteins at both dosages (**Figure 6**). However, the HA obtained from OMWF determined a more pronounced accumulation of proteins.

DISCUSSION

Findings obtained in this study indicate that humic acids extracted from an amendment obtained combining OMWs with a pre-treated organic material derived from MSW can be used as valuable biostimulants in agricultural practices by virtue of their positive effects on plant biomass production, nutrition and activity of enzymes implied in N metabolism and glycolysis. In support of our statement, previous work showed that biostimulants like HS can elicit morphological changes in plants, primarily a significant development of root biomass and the stimulation of root hair formation, which result in increased plant nutrient uptake and accumulation (Zandonadi et al., 2007, 2010; Nardi et al., 2016; Colla et al., 2017; Rouphael et al., 2017). In addition, HS and other types of biostimulants have been reported to up-regulate the gene expression and activity

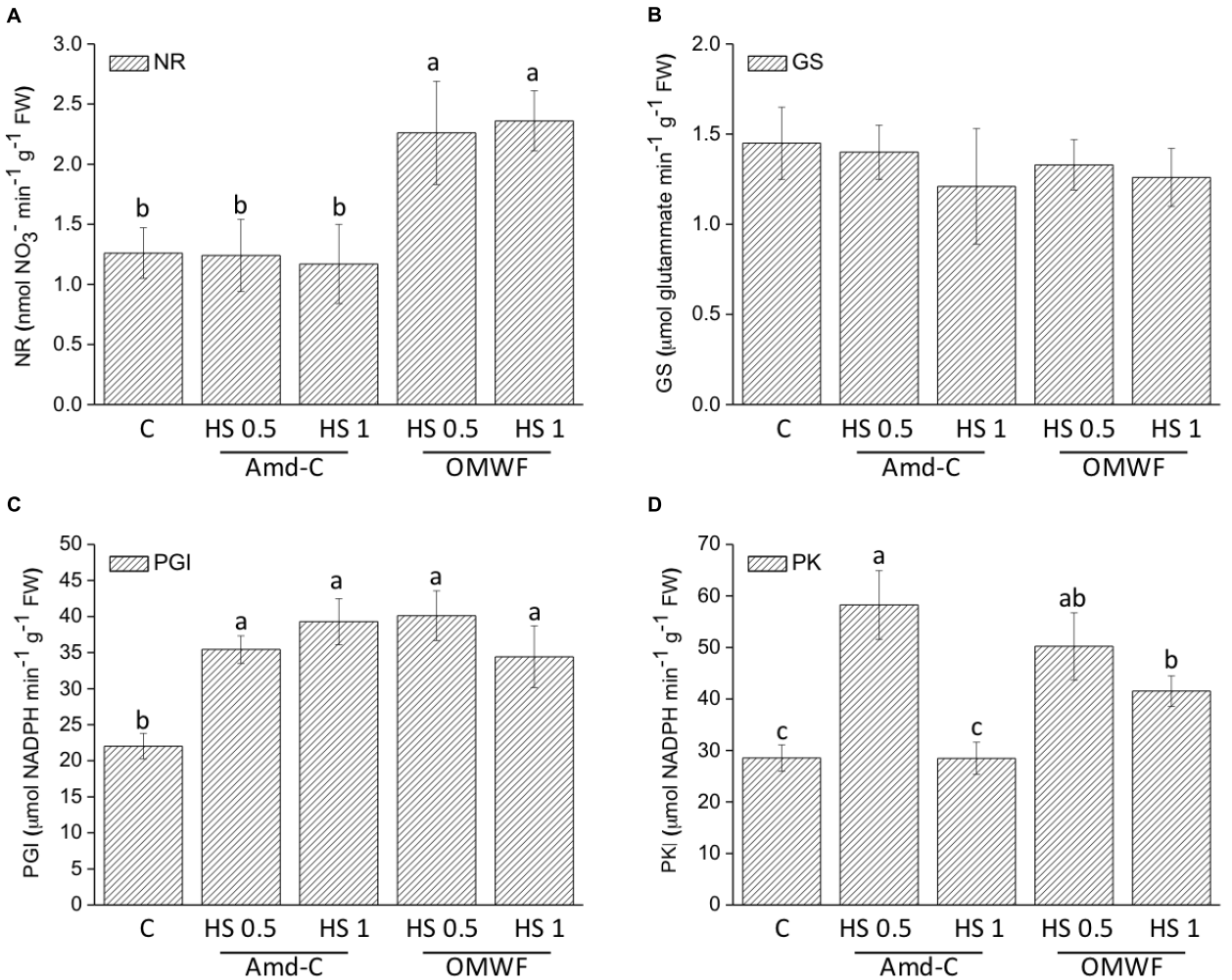


FIGURE 5 | Effect of HA extracted from control amendment (Amd-C) and olive mill water filter plus MSW pre-treated OMWF on the activity of **(A)** nitrate reductase (NR), **(B)** glutamine synthase (GS), **(C)** phosphoglucose isomerase (PGI), and **(D)** pyruvate kinase (PK) enzymes in *Z. mays* plants grown for 12 days in a Hoagland modified nutrient solution and supplied for 2 days with humic acids (HA) extracted Amd-C and OMWF amendments at 0.5 or 1 mg C L^{-1}. Data represent the means of three measurements with five plants in each (±*SD*). Different letters above bars indicate significant differences between treatments at $P < 0.05$ according to Student–Newman–Keuls test.

TABLE 5 | Leaf elemental composition of control maize plants (C, untreated) and plants supplied for 48 h with either HA derived from the amendment control (Amd-C) or HA obtained from amendment OMWF at two dosages (0.5 and 1 mg C L^{-1}).

		Ca	K	Mg	B	Zn	Mn	Cu	Na
Leaves	C	7.66 ± 0.32b	41.61 ± 1.00b	4.24 ± 0.21b	14.1 ± 0.4c	91.9 ± 20.5b	66.2 ± 1.5b	13.3 ± 2.0c	191.7 ± 16.0a
Amd-C	HA 0.5	9.05 ± 0.26a	45.67 ± 1.51a	5.69 ± 0.28a	15.01 ± 0.3b	163.2 ± 15.1a	63.6 ± 2.8b	24.2 ± 3.5b	135.7 ± 17.5b
	HA 1.0	7.90 ± 0.28b	44.40 ± 1.40a	4.58 ± 0.39b	16.19 ± 0.2a	63.6 ± 19.9b	74.9 ± 2.6a	69.8 ± 6.7a	165.5 ± 9.0b
OMWF	HA 0.5	8.75 ± 0.29a	44.01 ± 1.16a	5.11 ± 0.24a	16.07 ± 0.6a	145.8 ± 15.4a	66.8 ± 1.8b	14.3 ± 2.8c	147.2 ± 34.3b
	HA 1.0	7.43 ± 0.34b	39.70 ± 3.29b	4.07 ± 0.31b	14.55 ± 0.6bc	99.5 ± 18.6b	72.3 ± 2.3a	9.9 ± 5.1c	144.6 ± 17.6b
Root	C	5.17 ± 0.39b	15.23 ± 0.30c	4.53 ± 0.30c	30.9 ± 1.8c	344.0 ± 6.3c	265.1 ± 15.7c	262.7 ± 19.8b	68.4 ± 11.2ab
Amd-C	HA 0.5	6.33 ± 0.28a	27.35 ± 1.70a	6.47 ± 0.68a	35.2 ± 2.7c	442.9 ± 25.4a	450.2 ± 27.6a	296.8 ± 25.5b	80.7 ± 11.4a
	HA 1.0	6.47 ± 0.29a	20.30 ± 1.43b	5.90 ± 0.47ab	31.4 ± 2.0c	431.3 ± 16.5a	324.7 ± 16.6b	226.4 ± 33.6b	88.9 ± 10.3a
OMWF	HA 0.5	5.08 ± 0.38b	20.76 ± 1.32b	5.26 ± 0.34b	47.8 ± 3.2a	361.9 ± 8.2b	339.6 ± 28.3b	254.5 ± 31.0b	59.7 ± 11.6b
	HA 1.0	3.69 ± 1.74b	19.24 ± 2.11b	4.45 ± 0.30c	38.5 ± 2.6b	117.6 ± 21.4d	385.1 ± 25.4b	489.2 ± 42.6a	48.0 ± 15.1b

Values are expressed in mg g−1 d.wt. for macroelements, and in mg kg−1 d.wt. for microelements. Data represent the means of five measurements per treatment (±SD). Different letters along the same column indicate significant differences between treatments ($p < 0.05$) according to Student–Newman–Keuls test.

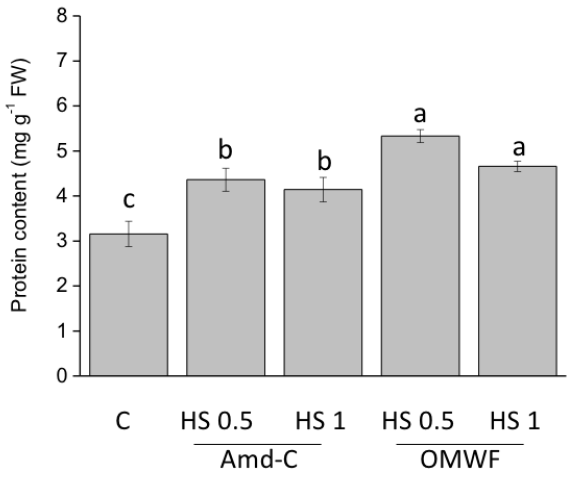


FIGURE 6 | Effect of HA extracted from control amendment (Amd-C) and olive mill water filter plus MSW pre-treated OMWF on total leaf protein content in *Z. mays* plants grown for 12 days in a Hoagland modified nutrient solution and supplied for 2 days with HA extracted Amd-C and OMWF amendments at 0.5 or 1 mg C L^{-1}. Data represent the means of three measurements (±*SD*). Different letters above bars indicate significant differences between treatments at $P < 0.05$ according to Student–Newman–Keuls test.

of enzymes catalyzing key steps of N assimilation and cell respiration processes due to their content in signaling molecules, such as amino acids, peptides, hormone-like substances and phenols (Crawford and Arst, 1993; Hoff et al., 1994; Schiavon et al., 2008; Ertani et al., 2009, 2013, 2014, 2017; Baglieri et al., 2014).

With respect to phenolic compounds in particular, a number of studies has shown that these molecules at low concentrations can trigger positive metabolic and physiological responses in plants (Ertani et al., 2011, 2018). Conversely, at concentrations as high as those normally recorded in OMW, phenols may be responsible for inhibition of soil microbiome activity and induction of several phytotoxic effects, including reduced seed germination, plant growth impairment and drops in productivity (Aggelis et al., 2003; Leonardis et al., 2009; Karpouzas et al., 2010; Leopoldini et al., 2011; García-Sánchez et al., 2012).

The phenolic composition of OMW varies depending on the climate, cultivar type and age, olives processing. Though, the most biologically active phenols commonly occurring in OMW are hydroxybenzoic acid, hydroxytyrosol acid, gallic acid, syringic acid, ferulic acid, caffeic acid, 3,4,5 trimethoxybenzoic acid, 3,4,5 trimethoxyphenyl-acetic acid, verbascoside, tyrosol acid, cyanidin, quercetin, and flavonols (Borja et al., 2006). Similarly to other OMW produced in Italy, the OMW used in this study contained elevate amounts of hydroxytyrosol acid and detectable levels of gallic acid, hydroxybenzoic acid, syringic acid, ferulic acid, and verbacoside (De Marco et al., 2007). These phenolic acids could cause toxicity to plants and soil microorganisms if OMW were spread on soil before being detoxified. However, their content significantly decreased after OMW composting with MSW. Also, based on results retrieved from germination assays (data not shown), the amendment obtained did not display phytotoxic effects. Therefore, our study confirms the effectiveness of the bio-oxidative treatment in reducing the hazardousness of OMW for potential recycling and application in agriculture.

The humic acids produced in the composting process and extracted from control and OMWF amendments were all endowed with a negative superficial charge (at neutral pH) and a particle size close to 500 nm. Spectroscopic analyses highlighted their pronounced aromatic features, with a low proportion of saturated aliphatic hydrocarbons (Colombo et al., 2015). Important differences in structural characteristics between HA from Amd-C and OMWF amendments were evidenced via DLS, which could be ascribed to differences in hydrophobicity and aromaticity of HA observed at pH 6. It is conceivable that at such pH value, HA extracted from OMWF amendment quickly formed micelle-like aggregates because of hydrophobic interactions of aromatic hydrocarbons between particles (Angelico et al., 2014). In support of this hypothesis, HA from OMWF showed a higher particle size (500 nm) at pH 6. On the other hand, the size of HA particles from Amd-C was lower (<360 nm) at the same pH, likely due to intermolecular electrostatic repulsion between the acidic functional groups. The elongate shape of both HA was suggested by the high size observed at pH < 2 (Colombo et al., 2015). The small but significant differences in size and shape between HA from Amd-Control and OMWF could be explained by OMW contribution to the aromatic chemical structures during the bio-oxidative process.

Differences in structural properties between HA obtained from Amd-Control and OMWF could justify the differential their effects on plant biomass, activity of marker enzymes and accumulation of several nutrients. HA have been reported to stimulate plant growth by targeting pivotal steps of plant metabolism, especially N assimilation and cell respiration (Nardi et al., 2007). In this study, HA from Amd-Control enhanced leaf and root biomass of maize plants when supplied at lower dosage (0.5 mg C L^{-1}), but they did not influence the activity of NR and GS enzymes, which catalyze the reduction of nitrate to nitrite and the synthesis of the amino acid glutamine, respectively. However, these HA increased the activity of key enzymes functioning in glycolysis, i.e., PGI at both dosages and PK at lower dosage, thus indicating their capacity to increase cell respiration and induce higher production of ATP molecules to be used for energy-dependent cellular work. HA extracted from OMWF promoted plant biomass production at both dosages, and significantly increased the activity of NR, PGI and PK. Therefore, N assimilation and cell respiration were both metabolic targets of these HA, likely because of their chemical and structural properties, such as prominent hydrophobicity and aromaticity (Jindo et al., 2012; Martinez-Balmori et al., 2014). It is also possible that some compounds contained in detoxified OMW and further included in the HA structure acted as signaling molecules in plants, thus leading to higher activity of both N and C enzymes. The more pronounced effect of HA from OMWF on N assimilation was confirmed by values of protein accumulation in plants.

Treating maize plants with HA extracted from the two amendments promoted nutrient accumulation in plant tissues.

This effect was previously reported for other HS and biostimulants in several studies, and has been related to their capacity to stimulate root growth and development, root hair formation, expression of nutrient transporters and activity of plasma membrane H^+-ATP-ase (Zandonadi et al., 2007, 2010; Ertani et al., 2013, 2017; Nardi et al., 2016; Santi et al., 2017). The lower capacity of HA derived from OMWF to enhance plant nutrition at higher dosage (1 mg C L^{-1}) could be due to the presence of one or more undetermined compounds derived from OMW, which could have modified the root cell membrane permeability, thus preventing the increase of nutrient uptake mediated by HA, despite both ATP synthesis and root growth were stimulated.

CONCLUSION

This study confirms the effectiveness of OMW bio-oxidation with a pre-treated organic material derived from MSW in decreasing the phenolic loading of OMW and producing stabilized organic amendments, in line with the legislative parameters of mixed organic amendments (absence of pathogens, heavy metal concentrations lower the threshold toxicity, no phytotoxicity effects). In addition, humic acids extracted from OMWF amendment could be used as valuable biostimulants in agriculture practices as evinced by their capacity to promote plant growth, activity of marker enzyme and nutrient accumulation significantly.

AUTHOR CONTRIBUTIONS

GP performed the chemical analyses on OMW, MSW, HA, and nutrients in plants. MS wrote the manuscript. SN edited the manuscript. AE performed the analyses on plants and helped in writing the manuscript. GC and CC designed and supervised the experiments.

REFERENCES

Adani, F., Genevini, P. L., Gasperi, F., and Zorzi, G. (1997). Organic matter evolution index (OMEI) as a measure of amendment inefficiency. *Compost Sci. Util.* 5, 53–62. doi: 10.1080/1065657X.1997.10701874

Aggelis, G., Iconomou, D., Christou, M., Bokas, D., Kotzailias, S., Christou, G., et al. (2003). Phenolic removal in a model olive oil mill wastewater using Pleurotusostreatus. *Water Res.* 16, 3897–3904. doi: 10.1016/S0043-1354(03)00313-0

Ahmadi-Esfahani, F. Z. (2006). Constant market shares analysis, uses, limitations and prospects. *Aust. J. Agric. Resour. Econ.* 50, 510–526. doi: 10.1111/j.1467-8489.2006.00364.x

AitBaddi, G., Hafidi, M., Gilard, V., and Revel, J. C. (2003). Characterization of humic acids produced during composting of olive mill wastes: elemental and spectroscopic analyses (FTIR and 13C NMR). *Agronomie* 23, 661–666. doi: 10.1051/agro:2003042

Andreozzi, R., Canterino, M., Di-Somma, I., Lo-Giudice, R., Marotta, R., Pinto, G., et al. (2008). Effect of combined physico-chemical processes on the phytotoxicity of olive mill wastewaters. *Water Res.* 42, 1684–1692. doi: 10.1016/j.watres.2007.10.018

Andreozzi, R., Longo, G., Majone, M., and Modesti, G. (1998). Integrated treatment of olive oil mill effluents (OME): study of ozonation coupled with anaerobic digestion. *Water Res.* 32, 2357–2364. doi: 10.1016/S0043-1354(97)00440-5

Angelico, R., Ceglie, A., Ji-Zheng, J., Liu-Rong, L., Palumbo, G., and Colombo, C. (2014). Particle size, charge and colloidal stability of humic acids coprecipitated with Ferrihydrite. *Chemosphere* 99, 239–247. doi: 10.1016/j.chemosphere.2013.10.092

Arvanitoyannis, I. S., Kassaveti, A., and Stefanatos, S. (2007). Olive oil waste treatment: a comparative and critical presentation of methods, advantages & disadvantages. *Crit. Rev. Food Sci. Nutr.* 47, 187–229. doi: 10.1080/10408390600695300

Baglieri, A., Cadili, V., Mozzetti Monterumici, C., Gennari, M., Tabasso, S., Montoneri, E., et al. (2014). Fertilization of bean plants with tomato plants hydrolysates. Effect on biomass production, chlorophyll content and N assimilation. *Sci. Hortic.* 176, 194–199. doi: 10.1016/j.scienta.2014.07.002

Bonadonna, L., Briancesco, R., Chiaretti, G., Coccia, A. M., Della Libera, S., Marini, R., et al. (2002). *Microbiological Quality of Composting Products: Legislative and Hygienic Measures. Operative Procedures for the Microbiological Analytical Control*. Rome: Istituto Superiore di Sanità.

Borja, R., Sànchez, E., Raposo, F., Rincòn, B., Jiménez, A. M., and Martìn, A. (2006). Study of the natural biodegradation of two-phase olive mill solid waste during its storage in an evaporation pond. *Waste Manag.* 26, 477–486. doi: 10.1016/j.wasman.2005.02.024

Bradford, M. (1976). A rapid and sensitive method for the quantitation of microgram quantities of protein utilizing the principle of protein-dye binding. *Anal. Biochem.* 1976, 248–254. doi: 10.1016/0003-2697(76)90527-3

Bremner, J. M., and Keeney, D. R. (1965). Steam distillation methods for determination of ammonium, nitrate and nitrite. *Anal. Chim. Acta* 32, 485–495. doi: 10.1016/S0003-2670(00)88973-4

Canellas, L. P., Olivares, F. L., Okorokova-Facanha, A. L., and Facanha, A. R. (2002). Humic acids isolated from earthworm amendment enhance root elongations, lateral root emergence, and plasma membrane H+-ATPase activity in maize roots. *Plant Physiol.* 130, 1951–1957. doi: 10.1104/pp.007088

Casa, R., D'Annibale, A., Pieruccetti, F., Stazi, S. R., Giovannozzi, S., and LoCascio, B. (2003). Reduction of the phenolic components in olive-millwaste water by enzymatic treatment and its impact on durum wheat (*Triticum durum* Desf.) germinability. *Chemosphere* 50, 959–966. doi: 10.1016/S0045-6535(02)00707-5

Colarieti, M. L., Toscano, G., and Greco, G. (2006). Toxicity attenuation of olive mill wastewater in soil slurries. *Environ. Chem. Lett.* 4, 115–118. doi: 10.1007/s10311-006-0050-5

Colla, G., Hoagland, L., Ruzzi, M., Cardarelli, M., Bonini, P., Canaguier, R., et al. (2017). Biostimulant action of protein hydrolysates: unraveling their effects on plant physiology and microbiome. *Front. Plant Sci.* 8:2202. doi: 10.3389/fpls.2017.02202

Colombo, C., Palumbo, G., Angelico, R., Cho, H. G., Francioso, O., Ertani, A., et al. (2015). Spontaneous aggregation of humic acid observed with AFM at different pH. *Chemosphere* 138, 821–828. doi: 10.1016/j.chemosphere.2015.08.010

Crawford, N. M., and Arst, H. N. Jr. (1993). The molecular genetics of nitrate assimilation in fungi and plants. *Annu. Rev. Genet.* 27, 115–146. doi: 10.1146/annurev.ge.27.120193.000555

De Marco, E., Savarese, M., Paduano, A., and Sacchi, R. (2007). Characterization and fractionation of phenolic compounds extracted from olive oil mill wastewaters. *Food Chem.* 104, 858–867. doi: 10.1016/j.foodchem.2006.10.005

Di Serio, M. G., Lanza, B., Mucciarella, M. R., Russi, F., Iannucci, E., Marfisi, P., et al. (2008). Effects of olive mill wastewater spreading. *Int. Biodet. Biodeg.* 62, 403–407. doi: 10.1016/j.ibiod.2008.03.006

European Biostimulants Industry Council [EBIC] (2013). *Economic Overview of the Biostimulants Sector in Europe*. Available at: http://www.biostimulants.eu

El-Gohary, F., Tawfik, A., Badawy, M., and El-Khateeb, M. A. (2009). Potentials of anaerobic treatment for catalytically oxidized olive mill wastewater (OMW). *Bioresour. Technol.* 100, 2147–2154. doi: 10.1016/j.biortech.2008.10.051

Ertani, A., Cavani, L., Pizzeghello, D., Brandellero, E., Altissimo, A., Ciavatta, C., et al. (2009). Biostimulant activity of two protein hydrolysates on the growth and nitrogen metabolism in maize seedlings. *J. Plant Nutr. Soil Sci.* 172, 237–244. doi: 10.1002/jpln.200800174

Ertani, A., Francioso, O., Tinti, A., Schiavon, M., Pizzeghello, D., and Nardi, S. (2018). Evaluation of seaweed extracts from *Laminaria* and *Ascophyllum nodosum* spp. as biostimulants in *Zea mays* L. using a combination of chemical, biochemical and morphological approaches. *Front. Plant Sci.* 9:428. doi: 10.3389/fpls.2018.00428

Ertani, A., Pizzeghello, D., Baglieri, A., Cadili, V., Tambone, F., Gennari, M., et al. (2013). Humic-like substances from agro-industrial residues affect growth and nitrogen assimilation in maize (*Zea mays* L.) plantlets. *J. Geochem. Exp.* 129, 103–111. doi: 10.1016/j.gexplo.2012.10.001

Ertani, A., Pizzeghello, D., Francioso, O., Sambo, P., Sanchez-Cortes, S., and Nardi, S. (2014). *Capsicum chinensis* L. growth and nutraceutical properties are enhanced by biostimulants in a long-term period: chemical and metabolomic approaches. *Front. Plant. Sci.* 5:375. doi: 10.3389/fpls.2014.00375

Ertani, A., Schiavon, M., Altissimo, A., Franceschi, C., and Nardi, S. (2011). Phenol-containing organic substances stimulate phenylpropanoid metabolism in *Zea mays*. *J. Plant Nutr. Soil Sci.* 174, 496–503. doi: 10.1002/jpln.201000075

Ertani, A., Schiavon, M., and Nardi, S. (2017). Transcriptome-wide identification of differentially expressed genes in *Solanum lycopersicon* L. in response to an alfalfa-protein hydrolysate using microarrays. *Front. Plant Sci.* 8:1159. doi: 10.3389/fpls.2017.01159

Eyheraguibel, B., Silvestre, J., and Morard, P. (2008). Effects of humic substances derived from organic waste enhancement on the growth and mineral nutrition of maize. *Bioresour. Technol.* 99, 4206–4212. doi: 10.1016/j.biortech.2007.08.082.

Flores, N., Brillas, E., Centellas, F., Rodríguez, R. M., Cabot, P. L., Garrido, J. A., et al. (2018). Treatment of olive oil mill wastewater by single electrocoagulation with different electrodes, and sequential electrocoagulation/electrochemical Fenton-based processes. *J. Hazard. Mater.* 5, 58–66. doi: 10.1016/j.jhazmat.2017.12.059

García-Sánchez, M., Garrido, I., Casimiro, I. J., Casero, P. J., Espinosa, F., García-Romera, I., et al. (2012). Defence response of tomato seedlings to oxidative stress induced by phenolic compounds from dry olive mill residue. *Chemosphere* 89, 708–716. doi: 10.1016/j.chemosphere.2012.06.026

Ghanbari, R., Anwar, F., Alkharfy, K. M., Gilani, A. H., and Saari, N. (2012). Valuable nutrients and functional bioactives in different parts of olive (*Olea europaea* L.) – a review. *Int. J. Mol. Sci.* 13, 3291–3340. doi: 10.3390/ijms13033291

Gigliotti, G., Proietti, P., Said-Pullicino, D., Nasini, L., and Pezzolla, D. (2012). Co-amendmenting of olive husks with high moisture contents: organic matter dynamics and amendment quality. *Int. Biodeter. Biodegr.* 67, 8–14. doi: 10.1016/j.ibiod.2011.11.009

Hamdi, M., Khadir, A., and Garcia, J. L. (1991). The use of *Aspergillus niger* for bioconversion of olive mill wastewater. *Appl. Microbiol. Biotechnol.* 34, 828–831. doi: 10.1007/BF00169359

Hoagland, D. R., and Arnon, D. I. (1950). The water-culture method for growing plants without soil. *Calif. Agric. Exp. Stn. Circ.* 347, 1–32.

Hoff, T., Truong, H. N., and Caboche, M. (1994). The use of mutants and transgenic plants to study nitrate assimilation. *Plant Cell Environ.* 17, 489–506. doi: 10.1111/j.1365-3040.1994.tb00145.x

Incelli, M., Cordiner, S., Tosti, S., Borgognoni, F., Sansovini, M., and Santucci, A. (2016). Trattamento delle acque di vegetazione do oleifici: tecnologie a membrana e processi termochimici. *Chim. Ind.* 3. doi: 10.17374/CI.2016.3.4.1

International Olive Council (2014). *Oil Organization, "World Olive Oil Figures"*. Available at: http://www.internationaloliveoil.org/estaticos/view/131-world-olive-oil-figures

Isidori, M., Lavorgna, M., Nardelli, A., and Parrella, A. (2005). Model study on the effect of 15 phenolic olive mill wastewater constituents on seed germination and vibrofischeri metabolism. *J. Agric. Food Chem.* 53, 8414–8417. doi: 10.1021/jf0511695

Jindo, K., Martim, S. A., Navarro, E. C., Pérez-Alfocea, F., Hernandez, T., Garcia, C., et al. (2012). Root growth promotion by humic acids from composted and non-composted urban organic wastes. *Plant Soil* 353, 209–220. doi: 10.1007/s11104-011-1024-3

Justino, C. I., Pereira, R., Freitas, A. C., Rocha-Santos, T. A., Panteleitchouk, T. S., and Duarte, A. C. (2012). Olive oil mill wastewaters before and after treatment: a critical review from the ecotoxicological point of view. *Ecotoxicology* 21, 615–629. doi: 10.1007/s10646-011-0806-y

Karpouzas, D. G., Ntougias, S., Iskidou, E., Rousidou, C., Papadopoulou, K. K., Zervakis, G. G., et al. (2010). Olive mill wastewater affects the structure of soil bacterial communities,". *Appl. Soil Ecol.* 45, 101–111. doi: 10.1016/j.apsoil.2010.03.002

Khoufi, S., Aloui, F., and Sayadi, S. (2008). Extraction of antioxidants from olive mill wastewater and electro-coagulation of exhausted fraction to reduce its toxicity on anaerobic digestion. *J. Hazard. Mater.* 151, 531–539. doi: 10.1016/j.jhazmat.2007.06.017

Komilis, D. P., Karatzas, E., and Halvadakis, C. P. (2005). The effect of olive mill wastewater on seed germination after various pre-treatment techniques. *J. Environ. Manage.* 74, 339–348. doi: 10.1016/j.jenvman.2004.09.009

Koutsos, T. M., Chatzistathis, T., and Balampekou, E. I. (2018). A new framework proposal, towards a common EU agricultural policy, with the best sustainable practices for the re-use of olive mill wastewater. *Sci Total Environ.* 62, 942–953. doi: 10.1016/j.scitotenv.2017.12.073

Leonardis, A., Macciola, V., and Nag, A. (2009). Antioxidant activity of various phenolextracts of olive-oil mill wastewaters. *Acta Alim.* 38, 77–86. doi: 10.1556/AAlim.2008.0030

Leopoldini, M., Russo, N., and Toscano, M. (2011). The molecular basis of working mechanism of natural polyphenolic antioxidants. *Food Chem.* 125, 288–306. doi: 10.1016/j.foodchem.2010.08.012

Lewis, O. A. M., Watson, E. F., and Hewitt, E. J. (1982). Determination of nitrate reductase activity in barley leaves and roots. *Ann. Bot.* 49, 31–37. doi: 10.1093/oxfordjournals.aob.a086227

Martinez-Balmori, D., Spaccini, R., Aguiar, N. O., Novotny, E. H., Olivares, F. L., and Canellas, L. P. (2014). Molecular characteristics of humic acids isolated from vermicomposts and their relationship to bioactivity. *J. Agric. Food Chem.* 62, 11412–11419. doi: 10.1021/jf504629c

Mechri, B., Echbili, A., Issaoui, M., Braham, M., Elhadj, S. B., and Hammami, M. (2007). Short-term effects in soil microbial community following agronomic application of olive mill wastewaters. *Appl. Soil Ecol.* 36, 216–223. doi: 10.1080/03601234.2013.742398

Mekki, A., Dhouib, A., and Sayadi, S. (2006). Changes in microbial and soil properties following amendment with treated and untreated olive mill wastewater. *Microbiol. Res.* 161, 93–101. doi: 10.1016/j.micres.2005.06.001

Mora, V., Bacaicoa, E., Zamarreño, A. M., Aguirre, E., Garnica, M., Fuentes, M., et al. (2010). Action of humic acid on promotion of cucumber shoot growth involves nitrate-related changes associated with the root-to-shoot distribution of cytokinins, polyamines and mineral nutrients. *J. Plant Physiol.* 167, 633–642. doi: 10.1016/j.envexpbot.2011.10.001

Muscolo, A., Sidari, M., and Nardi, S. (2013). Humic substance: relationship between structure and activity. Deeper information suggests univocal findings. *J. Geochem. Explor.* 129, 57–63. doi: 10.1016/j.gexplo.2012.10.012

Nardi, S., Concheri, G., Dell'Agnola, G., and Scrimin, P. (1991). Nitrate uptake and ATPase activity in oat seedlings in the presence of two humic fractions. *Soil Biol. Biochem.* 23, 833–836. doi: 10.1016/0038-0717(91)90094-Z

Nardi, S., Ertani, A., and Francioso, O. (2017). Soil–root cross-talking: the role of humic substances. *J. Plant Nutr. Soil Sci.* 180, 5–13. doi: 10.1002/jpln.201600348

Nardi, S., Muscolo, A., Vaccaro, S., Baiano, S., Spaccini, R., and Piccolo, A. (2007). Relationship between molecular characteristics of soil humic fractions and glycolytic pathway and krebs cycle in maize seedlings. *Soil Biol. Biochem.* 39, 3138–3146. doi: 10.1016/j.soilbio.2007.07.006

Nardi, S., Panucci, M. R., Abenavoli, M. R., and Muscolo, A. (1994). Auxin-like effect of humic substances extracted from faeces of *Allolobophora Caliginosa* and *Allolobophora Rosea*. *Soil Biol. Biochem.* 23, 833–836. doi: 10.1016/0038-0717(91)90094-Z

Nardi, S., Pizzeghello, D., Muscolo, A., and Vianello, A. (2002). Review "physiological effects of humic substances on higher plants". *Soil Biol. Biochem.* 32, 1527–1536. doi: 10.1016/S0038-0717(02)00174-8

Nardi, S., Pizzeghello, D., Schiavon, M., and Ertani, A. (2016). Plant biostimulants: physiological responses induced by protein hydrolyzed-based products and humic substances in plant metabolism. *Sci. Agric.* 73, 18–23. doi: 10.1590/0103-9016-2015-0006

Nasini, L., Gigliotti, G., Balduccini, M. A., Federici, E., Cenci, G., and Proietti, P. (2013). Effect of solid olive-mill waste amendment on soil fertility and olive (*Olea europaea* L.) tree activity,". *Agric. Ecosyst. Environ.* 164, 292–297. doi: 10.1016/j.agee.2012.10.006

Niaounakis, M., and Halvadakis, C. P. (2006). *Olive Processing Waste Management: Literature Review and Patent Survey*, 2nd Edn. Kidlington: Elsevier Ltd.

Nowotny, I., Schwanz, J., and Rothe, G. M. (1998). Influence of soil acidification and liming on selected enzymes of the carbohydrate metabolism and the contents of two major organic acids of mycorrhizal roots of Norway spruce (*Picea abies* [L.] Karst). *Plant Soil* 199, 41–51. doi: 10.1023/A:1004221910199

Ntougias, S., Gaitis, F., Katsaris, P., Skoulika, S., Iliopoulos, N., and Zervakis, G. I. (2013). The effects of olives harvest period and production year on olive mill wastewater properties evaluation of pleurotus strains as bioindicators of the effluent's toxicity. *Chemosphere* 92, 399–405. doi: 10.1016/j.chemosphere.2013.01.033

Ouzounidou, G., Zervakis, G. I., and Gaitis, F. (2010). Raw and microbiologically detoxified olive mill waste and their impact on plant growth. *Terr. Aquat. Environ. Toxicol.* 4, 21–38.

Oztekin, R., and Sponza, D. T. (2013). Treatment of wastewaters from the olive mill industry by sonication. *J. Chem. Technol. Biotechnol.* 88, 212–225. doi: 10.1002/jctb.3808

Paredes, C., Bernal, M. P., Cegarra, J., and Roig, A. (2002). Bio-degradation of olive mill wastewater sludge by its co-amendmenting with agricultural wastes. *Bioresour. Technol.* 85, 1–8. doi: 10.1016/S0960-8524(02)00078-0

Piccolo, A. (2001). The supramolecular structure of humic substances. *Soil Sci.* 166, 810–832. doi: 10.1097/00010694-200111000-00007

Rao, C. N. R. (1963). *Chemical Application of Infrared Spectroscopy*. New York, NY: Science Academic Press.

Rinaldi, M., Rana, G., and Introna, M. (2003). Olive-mill wastewater spreading in southern Italy: effects on a durum wheat crop. *Field Crops Res.* 84, 319–326. doi: 10.1016/S0378-4290(03)00097-2

Roig, A., Cayuela, M. L., and Sánchez-Monedero, M. A. (2006). An overview on olive mill wastes and their valorisation methods. *Waste Manag.* 26, 960–969. doi: 10.1016/j.wasman.2005.07.024

Rouphael, Y., Colla, G., Giordano, M., El-Nakhel, C., Kyriacou, M. C., and De Pascale, S. (2017). Foliar applications of a legume-derived protein hydrolysate elicit dose-dependent increases of growth, leaf mineral composition, yield and fruit quality in two greenhouse tomato cultivars. *Sci. Hortic.* 226, 353–360. doi: 10.1016/j.scienta.2017.09.007

Sampedro, I., D'Annibale, A., Ocampo, J. A., Stazi, S. R., and Garcia Romera, I. (2007). Solid-state cultures of *Fusarium oxysporum* transform aromatic components of olive-mill dry residue and reduce its phytotoxicity. *Bioresour. Technol.* 98, 3547–3554. doi: 10.1016/j.biortech.2006.11.015

Sanchez Monedero, M. A., Cegarra, J., Garcia, D., and Roing, A. (2002). Chemical and structural evolution of humic acids during organic waste amendmenting. *Biodegradation* 13, 361–371. doi: 10.1023/A:1022888231982

Santi, C., Zamboni, A., Varanini, Z., and Pandolfini, T. (2017). Growth stimulatory effects and genome-wide transcriptional changes produced by protein hydrolysates in maize seedlings. *Front. Plant Sci.* 8:433. doi: 10.3389/fpls.2017.00433

Schaumann, G. E. (2006). Soil organic matter beyond molecular structure Part 1: macromolecular and supramolecular characteristics. *J. Plant. Nutr. Soil Sci.* 169, 145–156. doi: 10.1002/jpln.200521785

Schiavon, M., Ertani, A., and Nardi, S. (2008). Effects of an alfalfa protein hydrolysate on the gene expression and activity of enzymes of TCA cycle and N metabolism in *Zea mays* L. *J. Agric. Food Chem.* 56, 11800–11808. doi: 10.1021/jf802362g

Schulz, H., and Baranska, M. (2007). Identification and quantification of valuable plant substances by IR and Raman spectroscopy. *Vib. Spectrosc.* 43, 13–25. doi: 10.1016/j.vibspec.2006.06.001

Sellami, F., Jarboui, R., Hachicha, S., Medhioub, K., and Ammar, E. (2008). Co-amendmenting of oil exhausted olive-cake, poultry manure and industrial residues of agro-food activity for soil amendment. *Bioresour. Technol.* 99, 1177–1188. doi: 10.1016/j.biortech.2008.01.055

Silva, A. M. T., Nouli, E., Xekoukoulotakis, N. P., and Mantzavinos, D. (2007). Effect of key operating parameters on phenols degradation during H2O2-assisted TiO2 photocatalytic treatment of simulated and actual olive mill wastewaters. *Appl. Catal. B Environ.* 73, 11–22. doi: 10.1016/j.apcatb.2006.12.007

Silverstein, R. M., Bassler, G. C., and Morrill, T. C. (1981). *Spectrometric Identification of Organic Compounds*, 4th Edn. New York, NY John Wiley and Sons.

Sokal, R. R., and Rohlf, F. J. (1969). *Biometry: The Principles and Practice of Statistics in Biological Research*. San Francisco, CA: W. H. Freeman, 776.

Trombetta, A., Accotto, E., Belfiore, G., Piccone, G., Pantusa, S., Nappi, P., et al. (1998). *Metodi di Analisi dei Compost: Determinazioni Chimiche, Fisiche, Biologiche e Microbiologiche: Analisi Merceologiche dei Rifiuti, Volume 6 of Collana Ambiente*. Piemonte: Assessorato All'ambiente, 1–187.

Vaccaro, S., Muscolo, A., Pizzeghello, D., Spaccini, R., Piccolo, A., and Nardi, S. (2009). Effect of a compost and its water-soluble fractions on key enzymes of nitrogen metabolism in maize seedlings. *J. Agric. Food Chem.* 57, 11267–11276. doi: 10.1021/jf901808s

Varanini, Z., and Pinton, R. (2001). "Directve MSWs indirect effects of soil humic substances on plant growth and nutrition," in *The Rizosphere*, eds R. Pinton, Z. Varanini, and P. Nannipieri (Basel: Marcel Dekker), 141–158.

Violante, P. (2000). *Metodi di Analisi Chimica del Suolo*. Milan: Franco Angeli.

Wershaw, R. L. (1999). Molecular aggregation of humic substances. *Soil Sci.* 164, 803–813. doi: 10.1097/00010694-199911000-00004

Zandonadi, D. B., Canellas, L. P., and Façanha, A. R. (2007). Indolacetic and humic acids induce lateral root development through a concerted plasmalemma and tonoplast H+ pumps activation. *Planta* 225, 1583–1595. doi: 10.1007/s00425-006-0454-2

Zandonadi, D. B., Santos, M. P., Dobbss, L. B., Olivares, F. L., Canellas, L. P., Binzel, M. L., et al. (2010). Nitric oxide mediates humic acids-induced root development and plasma membrane H+-ATPase activation. *Planta* 231, 1025–1036. doi: 10.1007/s00425-010-1106-0

Zullo, B. A., Di Stefano, M. G., Cioccia, G., and Ciafardini, G. (2014). Evaluation of polyphenol decay in the oily fraction of olive fruit during storage using a mild sample handling method. *Eur. J. Lipid Sci. Technol.* 116, 160–168. doi: 10.1002/ejlt.201300287

Conflict of Interest Statement: The authors declare that the research was conducted in the absence of any commercial or financial relationships that could be construed as a potential conflict of interest.

frontiers in Plant Science

ORIGINAL RESEARCH
published: 27 July 2018
doi: 10.3389/fpls.2018.01006

Evaluation of Gelatin as a Biostimulant Seed Treatment to Improve Plant Performance

Hiromi T. Wilson, Masoume Amirkhani† and Alan G. Taylor*

Section of Horticulture, School of Integrative Plant Science, New York State Agricultural Experiment Station, Cornell AgriTech, Cornell University, Geneva, NY, United States

The effect of gelatin, used as a biostimulant, was investigated on plant growth in greenhouse studies. Biostimulants are materials that stimulate plant growth, and gelatin, an animal protein hydrolysate, is classified as one type of biostimulant. Gelatin has a unique amino acid composition with a high percentage of proline and hydroxyproline. In a series of experiments gelatin capsules (#3 hard gelatin) containing 7.1 mg nitrogen each, were placed adjacent to seeds of different crop species, at sowing time in individual growing containers and several growth parameters were measured. Different types of hydrolyzed collagen, including granulated gelatin, gelatin hydrolysate, and amino acid mixtures simulating the composition of gelatin were compared on cucumber plant growth. In addition, amino acid mixtures without proline, hydroxyproline, or applied in combination were investigated on cucumber growth. All capsule treatments significantly enhanced crop growth compared to the non-treated control. The treatment with two gelatin capsules placed adjacent to each seed increased shoot dry weight of cucumber, pepper, broccoli, tomato, arugula, and field corn, by 138, 244, 50, 45, 41, and 18 percent, respectively. In an experiment with cucumber alone, there was a positive linear relationship between the number of gelatin capsules from 0 to 3 capsules on plant growth and plant nitrogen content. Cucumber growth and plant nitrogen content was greater from the hydrolyzed collagen treatment compared with the low molecular weight gelatin hydrolysate, a mixture of amino acids or urea and all treatments provided an equivalent amount of nitrogen. Proline and/or hydroxyproline were not responsible for the biostimulant effect. In summary, gelatin provided nitrogen that enhanced plant growth. Moreover, gelatin was an effective biostimulant as the plant growth and nitrogen content was greater from two gelatin capsules compared to amino acid mixture of the same proportion and amount as the gelatin.

Keywords: amino acid, biostimulant, gelatin, hydrolyzed collagen, nitrogen uptake, seed enhancement, vegetable crops

OPEN ACCESS

Edited by:
Giuseppe Colla,
Università degli Studi della Tuscia, Italy

Reviewed by:
Francesco Di Gioia,
University of Florida, United States
Astrit Balliu,
Agricultural University of Tirana, Albania

***Correspondence:**
Masoume Amirkhani
ma862@cornell.edu

†Co-first author

Specialty section:
This article was submitted to Crop and Product Physiology, a section of the journal Frontiers in Plant Science

Received: *06 February 2018*
Accepted: *20 June 2018*
Published: *27 July 2018*

Citation:
Wilson HT, Amirkhani M and Taylor AG (2018) Evaluation of Gelatin as a Biostimulant Seed Treatment to Improve Plant Performance. Front. Plant Sci. 9:1006. doi: 10.3389/fpls.2018.01006

INTRODUCTION

Seed enhancement is a term widely used in the industry to describe beneficial techniques performed to seeds post-harvest, but prior to sowing (Taylor et al., 1998). Seed enhancements include plant biostimulants, a broad class of substances and microorganisms that enhance plant growth. The European Biostimulants Industry Council (EBIC) developed a definition for plant

biostimulants; "substance(s) and/or microorganisms whose function when applied to plants or the rhizosphere is to stimulate natural processes to enhance/benefit nutrient uptake, nutrient efficiency, tolerance to abiotic stress, and crop quality. Biostimulants have no direct action against pests, and therefore do not fall within the regulatory framework of pesticides" (du Jardim, 2012; European Biostimulants Industry Council [EBIC], 2014). Gelatin, an animal based protein hydrolysate (hydrolyzed collagen) is one category of plant biostimulants.

Gelatin is defined as a mixture of peptides and proteins that are generally derived from partial hydrolysis of collagen obtained from connective tissues of animals which can include skin and bones (Gelatin Manufacturers Institute of America, 2012). Gelatin is soluble in water and in most polar solvents. A measure of gelatin strength is termed 'Bloom.' The higher the Bloom number, the stronger the gel. The Bloom number is positively related to average molecular mass (Ward and Courts, 1977; Gelatin Manufacturers Institute of America, 2012). The amino acid contents of protein hydrolysates vary depending on the production method as well as the source of the material. Animal based hydrolysates, such as gelatin, contain a high proportion of proline and glycine, while plant based hydrolysates contain a high proportion of glutamine and arginine (Parrado et al., 2008; Ertani et al., 2013a; Calvo et al., 2014). Gelatin contains 18 amino acids (**Table 1**) and the molecular size distribution for amino acids present in hydrolyzed gelatin ranges from 89.1 to 204.2 g mol^{-1} (Schrieber and Gareis, 2007). About 50% of the total amino acids in gelatin are glycine, proline, and hydroxyproline (**Table 1**). A gelatin molecule is composed of an average of 1,000 amino acids per chain and there are a few disulfide bonds in gelatin molecular structure (Belitz et al., 2004). Hydroxyproline is especially important for its primary role in the structure and maintenance of gelatin. Hydroxyproline is found in the cell wall proteins in plants (Lamport, 1965), and is derived from glycoprotein present in the primary cell walls (Kieliszewski and Shpak, 2001).

Protein hydrolysate and other protein-based product applications were reported to enhance plant growth and yield in field tomato (Parrado et al., 2008), greenhouse tomato (Koukounararas et al., 2013), papaya (Morales-Payan and Stall, 2003), maize seedlings (Ertani et al., 2013a,b), broccoli, and tomato seedlings (Amirkhani et al., 2016, 2017), and hydroponic lettuce (Colla et al., 2013). Protein hydrolysates were also reported to have ameliorating effects on abiotic stress in plants (Ertani et al., 2013a). An alfalfa plant-derived biostimulant increased maize plant biomass under salinity stress, and enhanced K^+ accumulation and reduced Na^+ accumulation in roots and leaves (Ertani et al., 2013b). Combined application of plant-derived protein hydrolysate and beneficial microorganisms improved lettuce root system architecture, chlorophyll synthesis and proline accumulation and enhanced lettuce tolerance to salinity and alkalinity (Rouphael et al., 2017). Perennial ryegrass treated with Macro-Sorb Foliar (FOLIAR^{TM}), an animal membrane hydrolysate, showed membrane stability and increased photosynthetic capacity when subjected to high temperature stress (Kauffman et al., 2007).

Hard gelatin capsules, routinely used in the pharmaceutical industry for medications were developed as a novel approach to deliver single or multiple seeds for greenhouse production (Trias and Takahashi, 2014). Sowing multiple seeds in a single dispersal unit in the same growing container has been used to facilitate sowing different varieties of the same crop. Trias and Takahashi (2014) reported enhanced plant growth from seeds sown in gelatin capsules compared to control seeds. However, in preliminary greenhouse studies, seeds placed inside gelatin capsules initially decreased the germination and seedling emergence rate, followed by enhanced growth (Wilson and Taylor, unpublished). The objective of this study was to investigate the biostimulant effect of the gelatin chemistry on plant growth without the deleterious effect of seed encapsulation. Therefore, capsules were placed adjacent to a seed in all experiments. In addition, only single seeds were placed near each gelatin capsule. The focus of this study was to first characterize the influence of gelatin capsule treatments on plant growth measured as dry weight and leaf area of several crops. Different types of hydrolyzed collagen [with molecular weight distribution (MWD) characterized] amino acids and nitrogen fertilizer were also evaluated as biostimulants with respect to plant dry weight and nitrogen content in cucumber. In these studies, gelatin was investigated both as a source of nitrogen and as a biostimulant enhancing plant nitrogen uptake.

TABLE 1 | List of amino acids and their relative amount in 1 g of gelatin protein (Gelatin Manufacturers Institute of America, 2012).

Amino acid	Amino acid (mg/g) of gelatin protein	Amino acid equivalent of one capsule (mg)
Alanine	113	45
Arginine	90	3.6
Aspartic acid	67	2.7
Glutamic acid	116	4.6
Glycine	272	10.8
Histidine	7	0.3
Hydroxylysine	8	0.3
Hydroxyproline	133	5.3
Isoleucine	16	0.6
Leucine	35	1.4
Lysine	44	1.8
Methionine	6	0.2
Phenylalanine	25	1.0
Proline	155	6.1
Serine	37	1.5
Threonine	24	0.8
Tyrosine	2	0.1
Valine	28	1.1

The calculated amino acid content of one gelatin capsule considering the moisture content of a gelatin capsule at 14% (Buice et al., 1995). Weight of one gelatin capsule is 45 mg, with 7.1 mg nitrogen content and 17.6 mm × 5.5 mm dimension.

MATERIALS AND METHODS

Experiment 1: Capsule Treatments and Above Ground Growth of Selected Vegetable Crops and Field Corn

Six crops: cucumber "Vlaspik" (Seminis, Oxnard, CA, United States), arugula "Astro" (Sakata, Morgan Hill, CA, United States), broccoli "Centura" (Rogers, Boise, United States), tomato "Talladega" (Syngenta, Greensboro, NC, United States), pepper "Boynton Bell" (Harris Moran, Modesto, CA, United States), and field corn "Cornell D2901" (Cornell University, Ithaca, NY, United States) were grown in a controlled greenhouse maintained at 24°C/21°C temperature with 14/10 h photoperiod at New York State Agricultural Experiment Station in Geneva, NY, United States. Five gelatin capsule treatments (Capsule line, Pompano Beach, FL, United States) were evaluated using #3 hard-gel animal based gelatin capsules (Capsule line, Pompano Beach, FL, United States), referred throughout paper as a gelatin capsule or gelatin capsule treatments. One gelatin capsule contains 7.1 mg nitrogen that is equivalent to 40 mg protein (Gelatin Manufacturers Institute of America, 2012). Capsules were placed adjacent to each seed in a 10-cm square pot: control (no capsule), half capsule (0.5), whole capsule (1.0), two capsules (2.0), and three capsules (3.0). The placement of two capsules is illustrated in relation to a cucumber seed (**Figure 1**). Seeds were sown in 10 cm × 10 cm× 12 cm (1,200 cm^3) plastic pots filled with Cornell Peat-Lite Mix A medium routinely used for seedlings or bedding plants. Peat-lite mix A is a combination of 1:2:2 (vol/vol) perlite: sphagnum peat moss: horticultural grade vermiculite with 4 kg of dolomitic limestone, 0.33 kg of nitrogen (N) and potassium (K_2O), and 0.16 kg of phosphate (P_2O_5) per cubic meter of mix (Boodley and Sheldrake, 1972). Five replications of each treatment were placed in a random block design in the greenhouse. Plants were watered as needed every other day throughout the experiment. Plants were harvested 28 days after emergence and plant total leaf area and dry weight were measured. Total leaf area was measured using CI-202 Leaf Area Meter (CID Bio-Science, Camas, WA, United States).

Experiment 2.1: Capsule Treatments and Growth Parameters of Cucumber Plants

Cucumber seeds "Vlaspik" (Seminis, Oxnard, CA, United States) were grown in a controlled greenhouse maintained at 24°C/21°C temperature with 14/10 h photoperiod at New York State Agricultural Experiment Station in Geneva, NY, United States. Four gelatin capsule treatments (Capsule line, Pompano Beach, FL, United States) were evaluated using #3 hard-gel gelatin capsules (Capsule line, Pompano Beach, FL, United States). Capsules were placed adjacent to each seed in a 10 cm square pot: control (no capsule), half capsule (0.5), whole capsule (1.0), two capsules (2.0). The placement of two capsules is illustrated in relation to a cucumber seed (**Figure 1**). Five replications of each treatment were placed in a random block design in the greenhouse, with 15 samples per replication. The plants were harvested 28 days after emergence and seven physiological parameters were measured (plant height, root length, leaf area, shoot fresh/dry weight, and root fresh/dry weight). Total leaf area was measured using CI-202 Leaf Area Meter (CID Bio-Science, Camas, WA, United States).

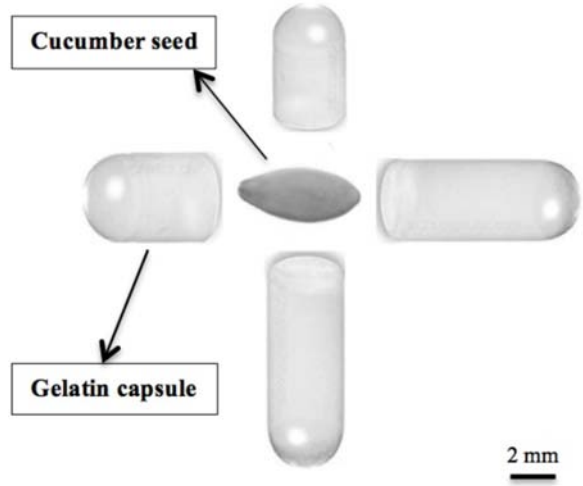


FIGURE 1 | Two gelatin capsules (four halves) placed adjacent to the cucumber seed.

Experiment 2.2: Capsule Treatments and Nitrogen Content of Cucumber Plant Tissue

Dry cucumber plant tissue was ground with a Wiley mill (Thomas Scientific Swedesboro, Swedesboro, NJ, United States) to a particle size of 2 mm. A 100 mg sample was sent to Cornell University Stable Isotope Laboratory (Ithaca, NY, United States) for elemental analysis to determine total nitrogen amount in plant tissue.

Experiment 3: Analysis of Different Hydrolyzed Collagen Types Based on Molecular Weight Distribution

Ten gram samples of three different hydrolyzed collagen types: granulated 220 bloom gelatin, granulated gelatin capsule, and gelatin hydrolysate were submitted to Eastman Gelatine Co. (Peabody, MA, United States) for MWD analysis. MWD of hydrolyzed collagen was performed by high-performance liquid chromatography (HPLC) in the aqueous size exclusion mode (AEC). Gelatin samples were dissolved in the chromatographic eluent along with a phosphate buffer containing sodium dodecyl sulfate (SDS). Molecular weight fractions from experimental samples were separated on a TosoHaas TSK-Gel size exclusion column (TOSOH Co., Japan). The effluent was monitored with a UV detector set at 220 nm. Known molecular weight standards were run to prepare a calibration curve, which was constructed by plotting the log of these molecular weights versus the retention time. The MWDs of unknown gelatin samples were then determined from the linear portion of the calibration curve.

Results were reported in terms of relative area percent of the five molecular weight regions (**Table 2**).

Experiment 4: Analysis of Different Hydrolyzed Collagens on Cucumber Plant Growth and Nitrogen Content

Cucumber seeds were planted in a controlled greenhouse maintained at 24°C/21°C temperature with 14/10 h photoperiod at New York State Agricultural Experiment Station in Geneva, NY, United States. Six treatments were compared; control, two gelatin capsules, hydrolyzed gelatin with bloom value of 220 (bloom 220), gelatin hydrolysate (Eastman Gelatine Co., Peabody, MA, United States), urea (Sigma-Aldrich, St. Louis, MO, United States), and an amino acid mixture that simulated the same composition as gelatin (Sigma-Aldrich, St. Louis, MO, United States) (**Table 1**). Bloom 220 gelatin was chosen as it has the same bloom value as the gelatin capsule. Gelatin hydrolysate was chosen for its small molecular size and its unique characteristic of lack of gelling capability. Two gelatin capsules were placed adjacent to each seed in a 10 cm × 10 cm × 12 cm pot (**Figure 1**). The hydrolyzed bloom 220 gelatin, gelatin hydrolysate, urea and amino acid mixture were weighed to the equivalent weight of two gelatin capsules (90 mg) and applied to the seed as powders in the pot. Each treatment had a total of 75 experimental units arranged in five replications of 15 experimental units per block, and the treatments were placed in a random block design on the greenhouse bench. The plants were harvested 28 days after emergence and leaf area and dry weight were measured. The plant nitrogen content was determined as previously described. The contribution of the gelatin treatments to nitrogen applied per plant was determined by capsule weight with 14% moisture content (Buice et al., 1995) and 17% of gelatin protein is nitrogen (Gelatin Manufacturers Institute of America, 2012).

Experiment 5: Different Amino Acid Mixture Ratios and Cucumber Plant Growth

Proline and hydroxyproline in gelatin were investigated as the active components of the biostimulant response. Amino acid mixtures with different proportions of proline, hydroxyproline, and glycine were mixed to develop a coating powder (Wilson, 2015), which was then applied to cucumber seeds. The amino acid mixtures were designed to assess the effect of amino acids, proline, and hydroxyproline on the plant growth. They were selected because hydrolyzed collagen contains high concentrations of these two amino acids. Five different amino acid mixtures were used for seed coating: an amino acid mixture containing all the amino acids found in hydrolyzed collagen, all amino acids found in hydrolyzed collagen with proline replaced by glycine, all amino acids found in hydrolyzed collagen with hydroxyproline replaced by glycine, and all amino acids found in hydrolyzed collagen with proline and hydroxyproline replaced by glycine. A control contained no amino acid.

TABLE 2 | Five molecular weight regions designated by the size exclusion elution profile and the corresponding molecular weight region of each fraction (Farrugia et al., 1998).

Fraction	Molecular weight region
High molecular weight fraction (HMW)	>250,000
Beta fraction	150,000–250,000
Alpha fraction	50,000–150,000
Sub alpha fraction	20,000–50,000
Low molecular weight fraction	4,000–20,000

Statistical Analysis

Data from experiments 1, 2, 4, and 5 were subjected to one-way analysis of variance (ANOVA). To detect the statistical significance of differences between means, the Tukey's HSD test at a significance level of $\alpha = 0.05$ was performed. Also for experiment 1, capsule treatments were grouped by crop species for analysis. Linear regression was used to study cucumber plant total nitrogen response to increasing nitrogen inputs from gelatin capsules (Experiment 2.2). For all experiments raw data were used for analyses. The statistical analysis was performed using the statistical program JMP Pro 11 (SAS Institute, Cary, NC, United States).

RESULTS

Experiment 1: Capsule Treatments and Above Ground Growth of Selected Vegetable Crops and Field Corn

Enhanced plant growth was observed in the above ground portions of plants in treatments with gelatin capsules placed adjacent to seeds at the time of sowing. Results for total leaf area, and dry weight were reported as cm^2 or g, respectively, and percent change with respect to the control. All of the crops exhibited significant increases in total leaf area with the gelatin capsule treatment. The magnitude of the effect of the capsule treatment on the total leaf area was specific to each crop (**Figure 2**). Cucumber had the most significant increases in total leaf area ($p < 0.0001$) with 25 cm^2 (39% increase) with half capsule treatment, 64 cm^2 (102% increase) with one capsule treatment, and 121 cm^2 (193% increase) with two capsule treatment compared to the control. The maximum total leaf area in cucumber was achieved with the three capsule treatment with 223 cm^2 (356% increase) compared to the control. Arugula and broccoli exhibited a similar increase in total leaf area with capsule treatments compared to cucumber. Tomato showed a significant increase in total leaf area with the addition of capsule treatments ($p < 0.0001$). For tomato there was a 50 cm^2 (123% increase) with one capsule, and maximum increase of 52 cm^2 (126%) with two capsule treatment, and a slight decrease in total leaf area with the three capsule treatment with 48 cm^2 (116% increase). Pepper plants exhibited a similar trend as tomato. Corn did not respond as dramatically as the other crops and an increase of 12 cm^2 (20%) was observed with the two capsule treatment.

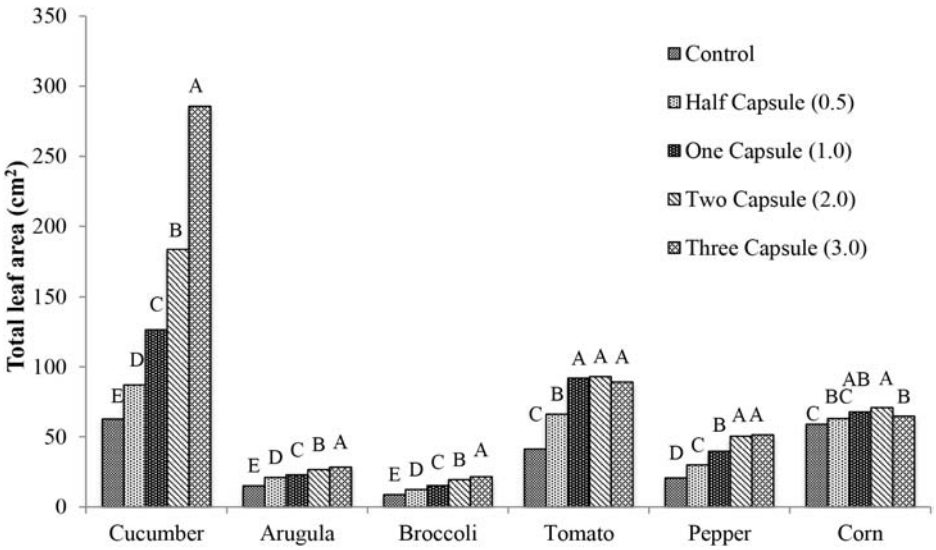


FIGURE 2 | Mean total leaf area 28 days after emergence of six crops: cucumber, arugula, broccoli, tomato, pepper, and corn with five treatments: control, half capsule (0.5), whole capsule (1.0), two capsules (2.0), and three capsules (3.0). Data analyzed by ANOVA ($\alpha = 0.05$) and Tukey's HSD test was used to conduct mean separation comparison. Letters not associated with same letter are significantly different.

Similar to total leaf area, all of the crops exhibited significant increases in dry weight with the capsule treatments ($p < 0.005$) (**Figure 3**) compared to the non-treated control. Cucumber had the most significant increases in dry weight ($p < 0.0001$) with 0.1 g (30% increase) with half capsule treatment, 0.29 g (84% increase) with one capsule treatment, and 0.47 g (138% increase) with the two capsule treatment compared to the control. The maximum dry weight in cucumber was achieved with the three capsule treatment with 0.86 g (251% increase) compared to the control. Arugula and broccoli exhibited similar increases in dry weight with capsule treatments compared to cucumber. Tomato had significant increase in dry weight with the addition of capsule treatments ($p < 0.0001$). There was 0.14 g (56%) maximum increase with one capsule, followed by a slight decrease in dry weight with the two and three capsule treatments with 0.11 g (45%) and 0.10 g (37%) increase, respectively. Pepper had a similar trend as tomato.

Experiment 2.1: Capsule Treatments and Growth Parameters of Cucumber Plants

The effect of capsule treatments on cucumber plant was investigated by measuring seven different growth parameters (plant height, petiole length, total leaf area, shoot/root fresh weight, and shoot/root dry weight) 28 days after emergence. The half capsule treatment had the least effect on plant growth, and the two capsule treatments had the greatest effect on the overall cucumber plant growth compared to the non-treated control. An additive effect of the hard-gel capsule treatment was measured in plant height, petiole length, total leaf area, fresh and dry shoot weight. Data are presented as percent difference of the seven growth parameters compared to the control (**Figure 4**). The effects of the capsule treatments were not consistent for all plant parts. The above ground parts of the plant exhibited a significant increase ($p < 0.05$) with the addition of two capsule treatments. Petiole length increased 37%, total leaf area had a 48% increase, shoot fresh weight had a 45% increase (**Figure 4**). Below ground biomass did not reflect the same growth increase as measured for the above ground parts of the plant. There was no significant difference in growth for the below ground part of the cucumber plant.

Experiment 2.2: Capsule Treatments and Nitrogen Content of Cucumber Plant Tissue

Cucumber plants treated with gelatin capsules exhibited an increase in total nitrogen amount (mg) in plant tissue compared to the control ($p < 0.0001$) (**Figure 5**). The three capsule treatment exhibited the greatest percent increase in total nitrogen amount with 21.7 mg (617% increase), followed by treatment with two capsules 11.3 mg (321% increase), one capsule with 6.3 mg (178% increase), and the half capsule treatment with 1.8 mg (51%). Nitrogen amount recovered from plant tissues was significantly related to the nitrogen supplied by the gelatin treatments ($R^2 = 97.0\%$) (**Figure 5**).

Experiment 3: Analysis of Different Hydrolyzed Collagen Types Based on Molecular Weight Distribution

According to the size exclusion elution profile of the different hydrolyzed collagens, gelatin hydrolysate had the smallest molecular weight, and 100% of its fraction was in the low molecular weight fraction (**Figure 6**). The gelatin capsule had an elution profile similar to bloom 220 gelatin. The gelatin capsule and bloom 220 gelatin, termed granulated hydrolyzed

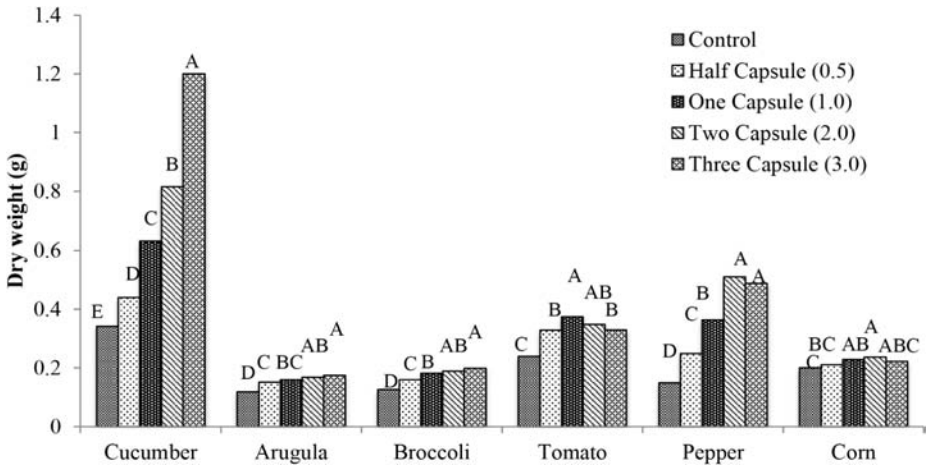


FIGURE 3 | Mean dry weight 28 days after emergence of six crops: cucumber, arugula, broccoli, tomato, pepper, and with five treatments: control, half capsule (0.5), whole capsule (1.0), two capsules (2.0), and three capsules (3.0). Data analyzed by ANOVA ($\alpha = 0.05$) and Tukey's HSD test was used to conduct mean separation comparison. Letters not associated with same letter are significantly different.

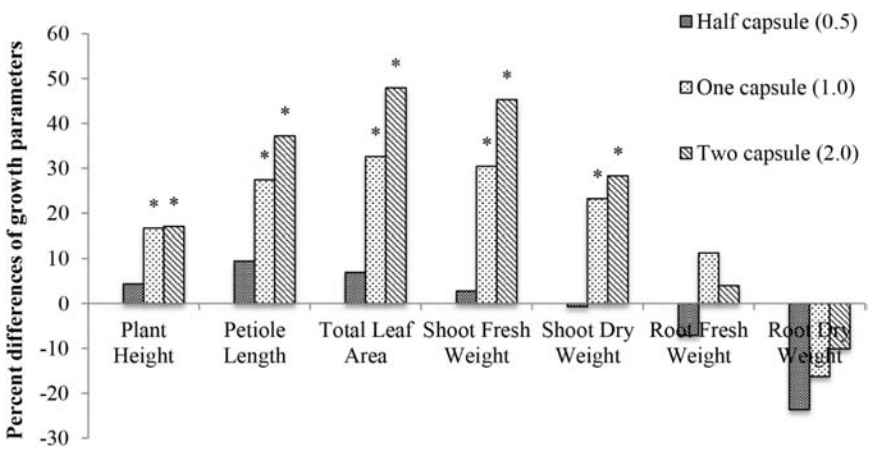


FIGURE 4 | Percent difference of seven growth parameters (plant height, petiole length, total leaf area, shoot fresh weight, shoot dry weight, root fresh weight, and root dry weight) 28 days after emergence of cucumber with three treatments: half capsule (0.5), whole capsule (1.0), and two capsules (2.0) compared to control as a base line. * Denotes significance ($p < 0.05$).

collagen both had high molecular weight (HMW), molecular weight region (MWR) above 250,000 g mol^{-1}, of 25 and 20%, respectively. The beta fraction, MWR between 250,000 and 150,000 g mol^{-1}, was 16% for gelatin capsule, and 14% for granulated hydrolyzed collagen. The alpha fraction, MWR between 150,000 and 50,000 g mol^{-1}, was 35% for gelatin capsule, and 33% for granulated hydrolyzed collagen. The sub alpha fraction, MWR between 50,000 and 20,000 g mol^{-1}, was 16% for gelatin capsule, and 21.1% for the granulated hydrolyzed collagen. The low molecular weight fraction, MWR < 20,000 g mol^{-1}, was 7.5% for gelatin capsule, and 11% for granulated hydrolyzed collagen. There were significant differences ($p < 0.001$) in the molecular distribution of the three hydrolyzed collagens. No significant difference was detected between gelatin capsule and granulated hydrolyzed collagen, while there were significant differences ($p < 0.001$) between gelatin capsule, granulated hydrolyzed collagen and gelatin hydrolysate.

Experiment 4: Analysis of Different Hydrolyzed Collagens on Cucumber Plant Growth and Nitrogen Content

The effects of four types of hydrolyzed collagen on plant growth were investigated by measuring total leaf area and dry weight. Only dry weight data are presented. The two capsules and bloom 220 treatments had the greatest dry weight compared to other treatments (**Figure 7**). The urea and amino acid mixture had similar dry weight, which was significantly greater than the non-treated control. The bloom 220 had the highest N amount per plant sample at 7.7 mg (140% increase), which was not significantly different than the two gelatin capsule treatment

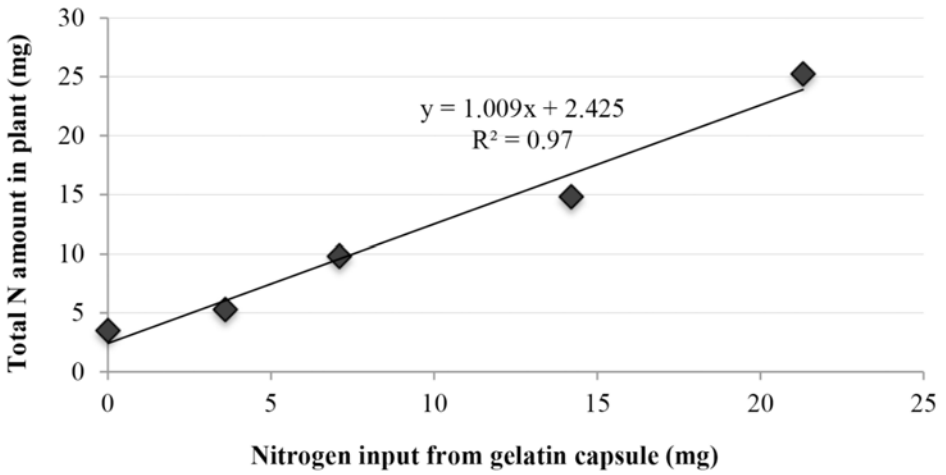


FIGURE 5 | Linear regression with response of cucumber plant total nitrogen concentration (mg) to increasing nitrogen inputs (mg) from gelatin capsules.

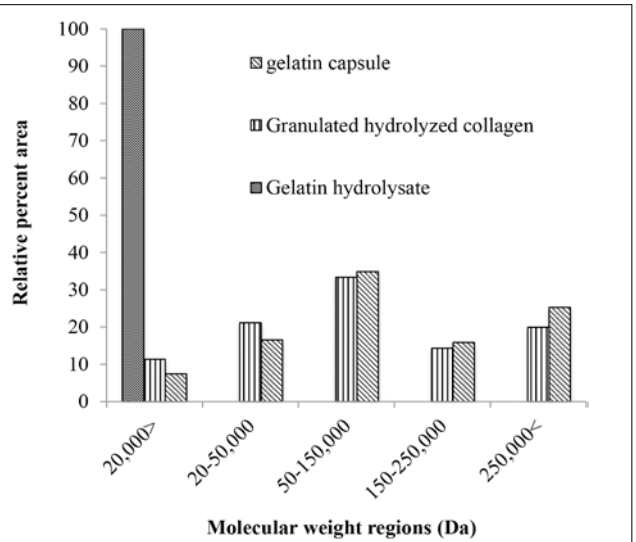


FIGURE 6 | Molecular weight distribution of hydrolyzed collagen by type, reported in relative area percent of the five molecular weight regions the high molecular weight fraction, the beta fraction, the alpha fraction, the sub-alpha fraction, and the low molecular weight fraction of the size exclusion elution profile.

(**Figure 7**). There were no differences in the amount of nitrogen between the urea, gelatin hydrolysate and amino acid mixture treatments.

Experiment 5: Different Amino Acid Mixture Ratios and Cucumber Plant Growth

Proline and hydroxyproline were tested separately or in combination as a biostimulant. Total leaf area and dry weight were not different for the amino acid mixture containing proline, hydroxyproline or their combination, in comparison with the amino acid mixture without proline and/or hydroxyproline (**Figure 8**). Therefore, proline and hydroxyproline were not the primary amino acids responsible for the plant growth promotion from gelatin capsule treatments.

DISCUSSION

Six crops examined in this study (cucumber, arugula, broccoli, tomato, pepper, and corn) exhibited increased total leaf area and dry weight in response to gelatin capsule treatments (**Figures 2**, **3**). Two gelatin capsules placed adjacent to each seed increased dry weight of cucumber, pepper, broccoli, tomato, arugula, and field corn, by 138, 244, 50, 45, 41, and 18 percent, respectively, compared to the non-treated control (**Figure 3**). Therefore, all crops investigated exhibited enhanced plant growth; however, the growth enhancement, measured by the percent increase in dry weight, compared to the non-treated control differed by species. This may be attributed to the uptake mechanism of hydrolyzed collagen that is the main ingredient in the gelatin capsules. Hydrolyzed collagen, a polypeptide, is comprised of amino acids, and the amino acids can be used as a nitrogen source in plant nutrition (Schrieber and Gareis, 2007). Plants acquire nitrogen as nitrate and ammonium and as organic nitrogen in forms such as amino acids and protein from the soil (Nasholm et al., 2009; Tegeder and Rentsch, 2010).

Previous reports have shown that the uptake of amino acids and proteins can vary between plant species (Okamoto and Okada, 2004; Reeve et al., 2008). Okamoto and Okada (2004) investigated utilization of different forms of nitrogen (N) by four gramineous crops including sorghum (*Sorghum bicolor*), rice (*Oryza sativa*), maize (*Zea mays*), and pearl millet (*Pennisetum glaucum*). In their study, seedlings were grown without N, or with 500 mg N kg^{-1} applied to soil as ammonium nitrate, rice bran or a mixture of rice bran and straw. Shoot growth and N uptake in maize and pearl millet were correlated with the inorganic N (ammonium and nitrate) concentration in the soil, suggesting

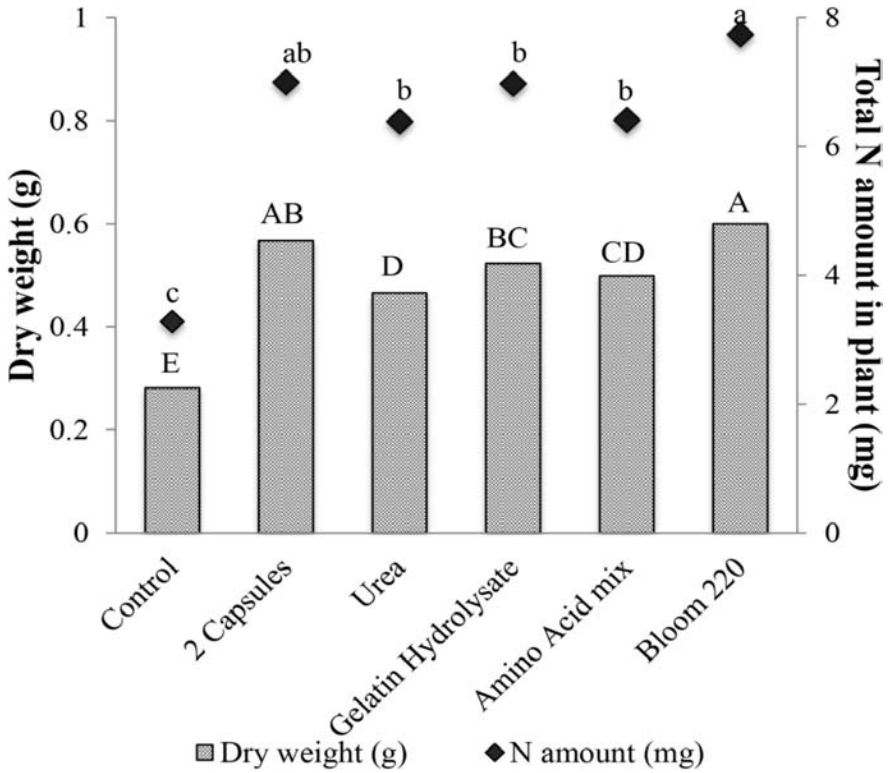


FIGURE 7 | Mean dry weight (g) and total nitrogen amount in plant (mg), 28 days after emergence of cucumber with six treatments: control, two gelatin capsule (2 Capsules), urea, gelatin hydrolysate, amino acid mixture (Amino acid mix), and bloom 220 gelatin (Bloom 220). Data analyzed by ANOVA ($\alpha = 0.05$) and Tukey's HSD test was used to conduct mean separation. Letters (Capital letters for dry weight and small letters for N amount) not associated with same letter are significantly different.

that these species depend on, or have a preference for, inorganic N. In contrast, shoot growth and N uptake patterns in sorghum and rice indicated that these two species could compensate for low inorganic N levels by taking up organic nitrogen (proteins) from the rice bran and straw mixtures. Analysis of N uptake in solution culture experiments confirmed that sorghum and rice roots were more able to absorb N in the form of protein than maize and pearl millet (Okamoto and Okada, 2004). Reeve et al. (2008) conducted a study on differential uptake of amino acid-N by domestic (*Fragaria fragaria*) and wild strawberry (*F. chiloensis* and *F. virginiana*). Exogenous glycine-N, NH_4^+-N, and NO_3^--N was supplied to the three species of strawberry. The domestic strawberry only took up a small portion of N from the amino acids, while the wild species took up significantly more glycine-N and NH_4^+-N. These results show that utilization of organic N in the form of proteins and amino acids can be species specific.

In the study reported here, cucumber exhibited the largest growth response from gelatin capsule treatments than the other crops evaluated in the study. However, the gelatin capsule treatments only increased plant growth in the above ground biomass: plant height, petiole length, total leaf area, and shoot dry weight (**Figure 4**). Different doses of gelatin capsules had no significant effect on root growth compared to non-treated controls (**Figure 4**). The lack of the biostimulant effect on root growth may be related to the source of the protein in gelatin or the concentration and type that was available in the growing medium.

Research has shown that both the protein source and concentration applied can influence root growth and development. Lucini et al. (2018) measured root growth promotion using a plant-based biostimulant that contained lateral root promoting peptides in melon (*Cucumis melo* L.). In their experiments, five rates (0, 0.06, 0.12, 0.24, and 0.48 mL per plant) of a biopolymer biostimulant were applied as a substrate drench. Total root dry matter, maximum root length, and root surface area in biostimulant treated plants were the greatest at 0.24 mL plant^{-1}, but decreased at 0.48 mL plant^{-1}. Lonhienne et al. (2014) investigated externally supplied soluble proteins on root development of *Arabidopsis* and found that addition of low (1.6–5 μM) to intermediate (15–23 μM) concentrations of bovine serum albumen (BSA) protein increased root dry weight, root length and thickness, and root hair length. However, root growth was inhibited at the high concentration of BSA (45 μM).

A linear relationship between the nitrogen supplied by the gelatin capsules and the nitrogen in cucumber tissue was observed (**Figure 5**). Thus the increase in nitrogen in the plant tissue was partially attributed to increased nitrogen supplied by the gelatin capsule treatments. However, the results suggest that growth promotion induced by the gelatin capsules was not caused by the N fertility alone as the treatment with two gelatin

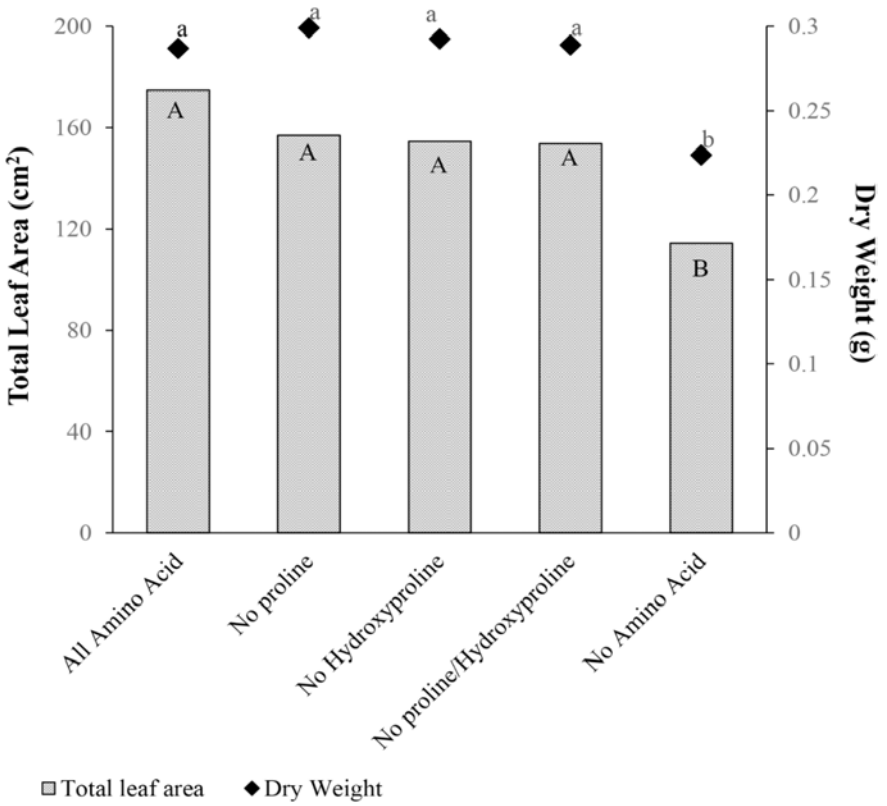


FIGURE 8 | Mean total leaf area (cm^2) and dry weight (g) 28 days after emergence of cucumber with five treatments: amino acid mixture containing all amino acids found in hydrolyzed collagen (All Amino Acid), all amino acids found in hydrolyzed collagen but proline replaced with glycine (No Proline), all amino acids found in hydrolyzed collagen but hydroxyproline replaced with glycine (No Hydroxyproline), and all amino acids found in hydrolyzed collagen but proline and hydroxyproline replaced with glycine (No Proline/Hydroxyproline), and Control (No Amino Acid). Data analyzed by ANOVA ($\alpha = 0.05$) and Tukey's HSD test was conducted for mean separation. Letters (Capital letters for total leaf area and small letters for dry weight) not associated with same letter are significantly different.

capsules had greater dry weight than the amino acid mix with the same proportion and amount of amino acids as the gelatin treatment (**Figure 7**). To determine if the growth promotion by the gelatin capsules was caused by the fertilizer effect from the amino acids in the gelatin, urea was used as a treatment with the equivalent amount of N found in two gelatin capsules. Although urea has the potential to be phytotoxic, no adverse effect in these experiments measured as the growth response and nitrogen per plant were the same from the urea treatment and the amino acid mixture (**Figure 7**). Amirkhani et al. (2016) demonstrated that application of a plant protein to broccoli (*Brassica oleracea* L.) seeds as a seed coating resulted in an unexpected increase in N uptake. Application of soy flour (a plant-based biostimulant source of nitrogen) as a component of a seed-coating blend increased N uptake efficiency. Nitrogen, from the soy flour applied in the seed coatings ranged from 0.024 to 0.073 mg per seed, while the enhanced nitrogen per plant ranged from 1.7 to 8.5 mg. Nitrogen applied in the seed coating only accounted for 1–2% of the increased nitrogen in plants, indicating the soy flour acted as a biostimulant and not as a fertilizer.

The hydrolyzed collagens used in these experiments were grouped by molecular weight (**Table 2**), and their distribution differs between gelatin types (**Figure 6**). Gelatin hydrolysate had the smallest MWD with 100% of the elution in the low molecular weight fraction of <20 kDa (**Figure 6**). Gelatin capsules used in these experiments had an elution profile similar to granulated hydrolyzed collagen, which suggests that they are structurally similar. This speculation is supported by the plant dry weight results from the different hydrolyzed collagens that were evaluated. There were no significant differences in dry weight of cucumber between application of two gelatin capsules, and granulated hydrolyzed collagen (Bloom 220), while the dry weight for gelatin hydrolysate was significantly less than the Bloom 220 treatment (**Figure 7**). One explanation for the difference in plant dry weight between the treatments may be the water solubility of the different hydrolyzed collagens. Both gelatin hydrolysate and the amino acid mix have a high water solubility. As the plants were watered in the greenhouse during the experiments, the treatments that were applied in dry powder form may have dissolved in the water and leached from the containers. The lower solubility collagens may have remained in the container for a longer period of time and thus available for plant growth resulting in a higher plant biomass. Additional research is warranted to investigate water solubility and duration of availability of the different treatments for plant uptake.

The underlying mechanism of plant growth enhancement in response to gelatin capsules remains unclear. Gelatin has a unique composition of amino acids, and is rich in proline and hydroxyproline (**Table 1**). Vaughan and Cusens (1973) showed that application of hydroxyproline enhanced root growth of peas. They proposed that externally supplied hydroxyproline enhanced root extension growth by affecting cell wall synthesis (Vaughan, 1973; Vaughan and Cusens, 1973). However, treatments with no proline and/or hydroxyproline were not significantly different than treatments with all amino acids (**Figure 8**). Therefore, proline and hydroxyproline were not the primary amino acids responsible for the plant growth promotion from gelatin capsule treatments.

Protein hydrolysates have been shown to stimulate carbon and nitrogen metabolism and increase nitrogen assimilation in plants (Schiavon et al., 2008; Ertani et al., 2009). Nitrate reductase, NAD-dependent glutamate dehydrogenase, and malate dehydrogenase increased in maize following application of animal epithelial hydrolysate (Maini, 2006). Alfalfa protein hydrolysate applied to hydroponically grown maize increased the activity of malate dehydrogenase, isocitrate dehydrogenase, and citrate synthase; as well as nitrogen metabolism enzymes, nitrate reductase, nitrite reductase, glutamine synthetase (GS), glutamine oxoglutarate aminotransferase (GOGAT), and aspartate aminotransferase (Schiavon et al., 2008). Ertani et al. (2009) reported that both alfalfa protein hydrolysate and animal connective tissue hydrolysate stimulated plant growth, and also increased nitrate conversion into organic nitrogen by inducing nitrate reductase and GS activities. These results suggest that protein hydrolysates may enhance plant growth by up regulating nitrate assimilation metabolism (Ertani et al., 2009; Calvo et al., 2014; Colla et al., 2017; Rouphael et al., 2017). Research in our lab reported that gelatin capsule treatment increased expression of amino acid and nitrogen transporter genes that may be responsible for root nitrogen uptake enhancement. Wilson et al. (2015) compared non-treated cucumber seeds and gelatin treated cucumber seeds using RNA-seq data. Amino acid permease 3 (AAP3), an amino acid transporter, was upregulated with the gelatin capsule treatment. The AAP3 gene is preferentially expressed in the phloem and has been associated with long distance transport of basic amino acids such as arginine, histidine, and lysine. The increased expression of amino acid transporters AAP3 and amino acid permease 6 (AAP6) in the plants treated with gelatin capsules suggests that amino acid transport in the plants was positively enhanced by the gelatin treatment. Our results provide evidence that proteins of the hydrolyzed collagen present in gelatin capsules provided a sustained source of N and acted as a biostimulant. Further research is needed to fully elucidate the mechanisms involved with the effect of gelatin on plant nutrition and growth.

AUTHOR CONTRIBUTIONS

HW performed the experiments and wrote many parts of the article. MA involved in data analysis, results interpretation, and writing the article. AT defined the scientific hypothesis and set up the experiments protocol and contributed to refinement and development of this paper.

FUNDING

This project was partially supported by a grant from Alliance Seed Capsules, a joint venture of Seed Coating Inc. and Sakata Seed Sudamerica Ltda, and the United States Multistate Research Project, W-2168.

ACKNOWLEDGMENTS

The authors would like to thank Dr. Hilary S. Mayton for her valuable comments, which helped to improve the manuscript quality. The authors would also like to thank Dr. Brent Wilson for his invaluable commitment in providing input for improving the manuscript.

REFERENCES

Amirkhani, M., Netravali, A. N., Huang, W., and Taylor, A. G. (2016). Investigation of soy protein based biostimulant seed coating for broccoli seedling and plant growth enhancement. *HortScience* 51, 1121–1126. doi: 10.21273/HORTSCI10913-16

Amirkhani, M., Netravali, A. N., and Taylor, A. G. (2017). "Improving seedling growth uniformity and seed vigor index by using plant-based protein seed coating in tomato and broccoli," in *Proceedings of the NYSAES Research Symposium*, New York, NY, 6.

Belitz, H. D., Grosch, W., and Scieberle, P. (2004). *Food Chemistry*. Berlin: Springer Verlag. doi: 10.1007/978-3-662-07279-0

Boodley, J. W., and Sheldrake, R. J. (1972). *Cornell Peat-Lite Mixes for Commercial Plant Growing*. Ithaca, NY: Cornell University.

Buice, R. G., Gold, T. B., Lodder, R. A., and Digenis, G. A. (1995). Determination of moisture in intact gelatin capsules by near-infrared spectrophotometry. *Pharm. Res.* 12, 161–163. doi: 10.1023/A:1016219611132

Calvo, P., Nelson, L., and Kloepper, J. W. (2014). Agricultural uses of plant biostimulants. *Plant Soil* 383, 3–41. doi: 10.1007/s11104-014-2131-8

Colla, G., Hoagland, L., Ruzzi, M., Cardarelli, M., Bonini, P., Canaguier, R., et al. (2017). Biostimulant action of protein hydrolysates: unraveling their effects on plant physiology and microbiome. *Front. Plant Sci.* 8:2202. doi: 10.3389/fpls.2017.02202

Colla, G., Svecova, E., Rouphael, Y., Cardarelli, M., Reynaud, H., Canaguier, R., et al. (2013). Effectiveness of a plant-derived protein hydrolysate to improve crop performances under different growing conditions. *Acta Hortic.* 1009, 175–180. doi: 10.17660/actahortic.2013.1009.21

du Jardim, P. (2012). *The Science of Plant Biostimulants-A Bibliographic Analysis. Contract 30-ce0455515/00-96, ad hoc Study on Bio-Stimulants Products*. Available at: http://ec.europa.eu/enterprise/sectors/chemicals/files/fertilizers/final_report_bio_2012_en.pdf

Ertani, A., Cavani, L., Pizzeghello, D., Brandellero, E., Altissimo, A., Ciavatta, C., et al. (2009). Biostimulant activity of two protein hydrolyzates in the growth and nitrogen metabolism of maize seedlings. *J. Plant Nutr. Soil Sci.* 172, 237–244. doi: 10.1002/jpln.200800174

Ertani, A., Pizzeghello, D., Altissimo, A., and Nardi, S. (2013a). Use of meat hydrolyzate derived from tanning residues as plant biostimulant for hydroponically grown maize. *J. Plant Nutr. Soil Sci.* 176, 287–295. doi: 10.1002/jpln.201200020

Ertani, A., Schiavon, M., Muscolo, A., and Nardi, S. (2013b). Alfalfa plant-derived biostimulant stimulate short-term growth of salt stressed *Zea mays* L. plants. *Plant Soil* 364, 145–158. doi: 10.1007/s11104-012-1335-z

European Biostimulants Industry Council [EBIC] (2014). *European Biostimulants Industry Council*. Available at: www.biostimulants.eu

Farrugia, C. A., Farrugia I. V., and Groves, M. J. (1998). Comparison of the molecular weight distribution of gelatin fractions by size-exclusion chromatography and light scattering. *Pharm. Pharmacol. Commun.* 4, 559–562. doi: 10.1111/j.2042-7158.1998.tb00675.x

Gelatin Manufacturers Institute of America (2012). *Gelatin Handbook. GMIA*. Available at: http://www.gelatin-gmia.com/images/GMIA_Gelatin_Manual_2012.pdf

Kauffman, G. L. III., Kneivel, D. P., and Watschke, T. L. (2007). Effects of biostimulants on the heat tolerance associated with photosynthetic capacity, membrane thermostability, and polphenol production of perennial ryegrass. *Crop Sci.* 47, 261–267. doi: 10.2135/cropsci2006.03.0171

Kieliszewski, M. J., and Shpak, E. (2001). Synthetic gene for the elucidation of glycosylation codes for arabinogalactan-proteins and other hydroxyproline-rich glycoproteins. *Cell. Mol. Life Sci.* 58, 1386–1398. doi: 10.1007/PL00000783

Koukounararas, A., Tsouvaltzis, P., and Siomos, A. S. (2013). Effect of root and foliar application of amino dcids on the growth and yield of greenhouse tomato in different fertilization levels. *J. Food Agric. Environ.* 11, 644–648.

Lamport, D. T. A. (1965). The protein components of primary cell walls. *Adv. Bot. Res.* 2, 151–218. doi: 10.1016/S0065-2296(08)60251-7

Lonhienne, T. G. A., Trusov, Y., Young, A., Rentsch, D., Nasholm, T., Schmidt, S., et al. (2014). Effects of externally supplied protein on root morphology and biomass allocation in *Arabidopsis*. *Sci. Rep.* 4:5055. doi: 10.1038/srep05055

Lucini, L., Rouphael, Y., Cardarelli, M., Bonini, P., Baffi, C., and Colla, G. (2018). A vegetal biopolymer-based biostimulant promoted root growth in melon while triggering brassinosteroids and stress-related compounds. *Front. Plant Sci.* 9:472. doi: 10.3389/fpls.2018.00472

Maini, P. (2006). The experience of the first biostimulant, based on amino acids and peptides: a short retrospective review on the laboratory researches and practival results. *Fertil. Agrorum* 1, 29–43.

Morales-Payan, J. P., and Stall, W. M. (2003). Papaya (*Carica papaya*) response to foliar treatments with organic complexes of peptides and amino acids. *Proc. Fla. State Hortic. Soc.* 116, 30–31.

Nasholm, T., Kielland, K., and Ganeteg, U. (2009). Uptake of organic nitrogen by plants. *New Phytol.* 182, 31–48. doi: 10.1111/j.1469-8137.2008.02751.x

Okamoto, M., and Okada, K. (2004). Differential responses of growth and nitrogen uptake to organic nitrogen in four gramineous crops. *J. Exp. Bot.* 55, 1577–1585. doi: 10.1093/jxb/erh167

Parrado, J., Bautista, J., Romero, E. J., Garciìa-Martiìnez A. M., Friaza, V., and Tejada, M. (2008). Production of a carob enzymatic extract: potential use as a biofertilizer. *Bioresour. Technol.* 99, 2312–2318. doi: 10.1016/j.biortech.2007.05.029

Reeve, J. R., Smith, J. L., Carpenter-Boggs, L., and Reganold, J. P. (2008). Soil-based cycling and differential uptake of amino acids by three species of strawberry (*Fragaria* spp.) plants. *Soil Biol. Biochem.* 40, 2547–2552. doi: 10.1016/j.soilbio.2008.06.015

Rouphael, Y., Cardarelli, M., Bonini, P., and Colla, G. (2017). Synergistic action of a microbial-based biostimulant and a plant derived-protein hydrolysate enhances lettuce tolerance to alkalinity and salinity. *Front. Plant Sci.* 8:131. doi: 10.3389/fpls.2017.00131

Schiavon, M., Ertani, A., and Nardi, S. (2008). Effects of an alfalfa protein hydrolysate on the gene expression and activity of enzymes of the tricarboxylic acid (TCA) cycle and nitrogen metabolism in *Zea mays* L. *J. Agric. Food Chem.* 56, 11800–11808. doi: 10.1021/jf802362g

Schrieber, R., and Gareis, H. (2007). *Gelatin Handbook: Theory and Industrial Practice*. Weinheim: Wiley-VCH. doi: 10.1002/9783527610969

Taylor, A. G., Allen, P. S., Bennett, M. A., Bradford, K. J., Burris, J. S., and Misra, M. K. (1998). Seed enhancements. *Seed Sci. Res.* 8, 245–256. doi: 10.1017/S0960258500004141

Tegeder, M., and Rentsch, D. (2010). Uptake and partitioning of amino acid and peptides. *Mol. Plants* 3, 1–15. doi: 10.1093/mp/ssq047

Trias, J., and Takahashi, K. L. (2014). Inventors, Coating Suply Inc. promotion of plant growth using collagen-based gelatin. United States Patent US 20140087924.

Vaughan, D. (1973). Effect of hydroxylproline on the growth and cell-wall protein metabolism of excised root segments of pisum sativum. *Planta* 115, 135–145. doi: 10.1007/BF00387779

Vaughan, D., and Cusens, E. (1973). An effect of hydroxylproline on the growth of excised root segment of pisum sativum under aseptic conditions. *Planta* 112, 243–252. doi: 10.1007/BF00385328

Ward, A. G., and Courts, A. (1977). *The Science and Technology of Gelatin*. New York, NY: Academic Press.

Wilson, H. T. (2015). *Gelatin, a Biostimulant Seed Treatment and Its Impact on Plant Growth, Abiotic Stress, and Gene Regulation*. Ph.D. dissertation, Cornell University, Ithaca, NY, 217.

Wilson, H. T., Xu, K., and Taylor, A. G. (2015). Transcriptome analysis of gelatin seed treatment as a biostimulant of cucumber plant growth. *ScientificWorldJournal* 2015:391234. doi: 10.1155/2015/391234

Conflict of Interest Statement: The authors declare that the research was conducted in the absence of any commercial or financial relationships that could be construed as a potential conflict of interest.

frontiers in Plant Science

PERSPECTIVE
published: 14 August 2018
doi: 10.3389/fpls.2018.01197

High-Throughput Plant Phenotyping for Developing Novel Biostimulants: From Lab to Field or From Field to Lab?

*Youssef Rouphael[1], Lukáš Spíchal[2,3], Klára Panzarová[4], Raffaele Casa[5] and Giuseppe Colla[5]**

[1] Department of Agricultural Sciences, University of Naples Federico II, Portici, Italy, [2] Department of Chemical Biology and Genetics, Centre of the Region Haná for Biotechnological and Agricultural Research, Faculty of Science, Palacký University, Olomouc, Czechia, [3] AgroBioChem, s.r.o., Bystročice, Czechia, [4] Photon Systems Instruments, spol. s.r.o., Drasov, Czechia, [5] Department of Agricultural and Forestry Sciences, University of Tuscia, Viterbo, Italy

Plant biostimulants which include bioactive substances (humic acids, protein hydrolysates and seaweed extracts) and microorganisms (mycorrhizal fungi and plant growth promoting rhizobacteria of strains belonging to the genera *Azospirillum*, *Azotobacter*, and *Rhizobium* spp.) are gaining prominence in agricultural systems because of their potential for improving nutrient use efficiency, tolerance to abiotic stressors, and crop quality. Highly accurate non-destructive phenotyping techniques have attracted the interest of scientists and the biostimulant industry as an efficient means for elucidating the mode of biostimulant activity. High-throughput phenotyping technologies successfully employed in plant breeding and precision agriculture, could prove extremely useful in unraveling biostimulant-mediated modulation of key quantitative traits and would also facilitate the screening process for development of effective biostimulant products in controlled environments and field conditions. This perspective article provides an innovative discussion on how small, medium, and large high-throughput phenotyping platforms can accelerate efforts for screening numerous biostimulants and understanding their mode of action thanks to pioneering sensor and image-based phenotyping techniques. Potentiality and constraints of small-, medium-, and large-scale screening platforms are also discussed. Finally, the perspective addresses two screening approaches, "lab to field" and "field to lab," used, respectively, by small/medium and large companies for developing novel and effective second generation biostimulant products.

Keywords: bioassaying, functional characterization, high-throughput screening, imaging methods, integrative phenotyping, mode of action, morpho-physiological traits, nutrient use efficiency

OPEN ACCESS

Edited by:
Barbara De Lucia,
Università degli Studi di Bari Aldo Moro, Italy

Reviewed by:
Andrea Ertani,
Università degli Studi di Padova, Italy
Spyridon Alexandros Petropoulos,
University of Thessaly, Greece
Georgios Soteriou,
Agricultural Research Institute, Cyprus

****Correspondence:***
Giuseppe Colla
giucolla@unitus.it

Specialty section:
This article was submitted to Plant Breeding, a section of the journal Frontiers in Plant Science

Received: *15 June 2018*
Accepted: *26 July 2018*
Published: *14 August 2018*

Citation:
Rouphael Y, Spíchal L, Panzarová K, Casa R and Colla G (2018) High-Throughput Plant Phenotyping for Developing Novel Biostimulants: From Lab to Field or From Field to Lab? Front. Plant Sci. 9:1197. doi: 10.3389/fpls.2018.01197

PLANT BIOSTIMULANTS: WHAT THEY ARE AND THEIR EFFECTS ON MORPHO-PHYSIOLOGICAL TRAITS OF CROPS

The term "biostimulant" was first introduced by Zhang and Schmidt (1997) in an online article of the Grounds Maintenance Journal describing them as "materials that, in minute quantities, promote plant growth." The biostimulants mentioned were humic acids and seaweed extracts, and their action on plants was proposed to be essentially hormonal. The term was subsequently

adopted by many scientists to denote "substances and/or microorganisms applied to plants with the intention to enhance nutrition efficiency, abiotic stress tolerance and/or crop quality traits, regardless of its nutrients content" (du Jardin, 2015). From a regulatory point of view, there is no agreement worldwide defining plant biostimulants and many countries lack a legal framework. Within the EU, there is an ongoing revision of regulation aiming to establish a common legal framework for biostimulants, currently fragmented across Member States. Under the new regulation, plant biostimulants will be CE marked as fertilizing products stimulating plant nutrition processes independently of the products' nutrient content with the sole aim of improving one or more of the following characteristics of the plant: nutrient use efficiency, tolerance to abiotic stress, and crop quality. Plant biostimulants are defined more by the plant response they elicit than by their makeup, since the category entails diverse substances and microorganisms such as humic acids, protein hydrolysates, seaweed extracts, silicon, mycorrhizal fungi, and nitrogen-fixing bacteria (Colla and Rouphael, 2015). Plant biostimulants can influence phenotypic traits and improve yield by enhancing crop stress-tolerance and nutrient uptake and assimilation. In most species, foliar or root application of plant biostimulants improves leaf pigmentation, photosynthetic efficiency, leaf number and area, shoot and root biomass, as well as fruit number and/or mean weight, especially under adverse environmental conditions (Ertani et al., 2013, 2014; Colla et al., 2015; Lucini et al., 2015, 2018; Rouphael et al., 2017). Precise and accurate assessment of phenotypic variables is critical for unraveling and quantifying the biostimulant activity of various products. High-throughput phenotyping technologies are receiving increasing attention for purposes of product screening and development as efficient means to (1) automated, non-destructive online monitoring of multiple morpho-physiological plant traits; (2) time-series measurements necessary for following the progression of growth, plant performance, and stress responses of individual plants at high-resolution; (3) reduced cost, labor, and time for analyses through automatization, remote sensing, improved data integration, and experimental design. High-throughput phenotyping technologies have been successfully employed in plant breeding (Araus and Cairns, 2014; Tardieu et al., 2017), however, their application in assessing plant biostimulant action has been limited (Petrozza et al., 2014). The current perspective article examines the potential benefits arising from the use of high-throughput phenotyping platforms (**Figure 1**) in biostimulant product screening and discusses current advances in plant phenotyping in the context of developing effective biostimulants.

HIGH-THROUGHPUT PHENOTYPING PLATFORMS TO ASSESS THE BIOSTIMULANT ACTIVITY

Small-Scale Screening Platforms

Screening platforms based on the semi-automated or automated bioassaying of plant/tissues traits using simple read-outs might be useful for identification of new biostimulants as well as for mode of action studies. Such platforms should allow parallel testing of large amounts of samples giving opportunity of high-throughput screening campaigns comparable to the chemical biology pipelines (Humplík et al., 2015). The advantage can lie in the possible miniaturization of the assays and use of simple and fast ways of biological response evaluation (De Diego et al., 2017). Further, because biostimulants represent various

FIGURE 1 | High-throughput plant phenotyping platforms: **(A)** small scale phenotyping platform consisting of XYZ PlantScreen^{TM} growth-chamber with automatic top view RGB imaging (Photon System Instruments, Czechia) for screening biostimulant substances based on the changes on *Arabidopsis* rosette growth in multi-well plates at Palacký University in Olomouc, Czechia; **(B)** medium-scale phenotyping platform PlantScreen^{TM} Modular System (Photon System Instruments, Czechia) with integrated high-resolution RGB, chlorophyll fluorescence, thermal and both VNIR and SWIR hyperspectral imagers for high-precision digital plant phenotyping and plant cultivation of mid-scale size up to large plants in greenhouse or semi-controlled environment; **(C)** Phenomobile for fruit trees and berry bushes developed at the James Hutton Institute (Scotland, United Kingdom), with VNIR and SWIR hyperspectral imagers (Williams et al., 2017; photo courtesy of H. G. Jones); **(D)** Large scale automated field phenotyping system. PlantScreen^{TM} Field System is autonomous mobile platform with multi-functional sensor platform mounted on an XZ-robotic arm with high-resolution visible, chlorophyll fluorescence, thermal infrared, hyperspectral imagers, and 3D laser sensor (Photon System Instruments, Czechia).

types of products including complex mixtures of biologically active compounds, testing should be done in broad concentration ranges offering evaluation of concentration-dependent effects. Importantly, the testing should cover analyses of the performance of a biostimulant in various stress conditions. This can be achieved mainly through bioassays in the platforms located in fully controlled environment allowing setting-up of various stress conditions such as temperature (heat/cold) and light (low/high intensity). The multivariate approach further counts with application of other stresses including low nutrients, salt, drought, or heavy metals. The higher level then represents cross-testing of a biostimulant in a broad concentration range against a concentration range of various stressors, or even their combinations. Such a highly complex screening approach can be highly efficient and lead to identification of novel biostimulants with various modes of action. Hence, the limiting factor of the screening platforms is the real throughput that depends on the level of automation, platform capacity, and the number of variants, which is in turn determined by the number of plants per variant and the number of technical replicates of each variant (De Diego et al., 2017). *Arabidopsis thaliana*, a classical model in plant biology, offers important advantages for phenotype-based high-throughput screening approaches. Bioassays using the *in vitro* grown *Arabidopsis* have high potential to be used in small-scale platforms for screening novel biostimulants applied through the growth medium. Several recently published protocols are based on RGB imaging of *Arabidopsis* shoot (rosette) growth. Miniaturization of the bioassay to the multi-well plates allows increasing the throughput to thousands of samples. *Arabidopsis* grown *in vitro* in 24-well plates were used for screening of growth regulator activity of a library of 10,000 compounds (Rodriguez-Furlán et al., 2016). Moreover, in this work the transferability of the results obtained with the model plant *Arabidopsis* to other crops of commercial interest, such as tomato, lettuce, carrots, has been also demonstrated (Rodriguez-Furlán et al., 2016). Recently, an automated method for high-throughput screening of *Arabidopsis* rosette growth in multi-well plates allowing measurement of 11,000 plants in less than 2 h has been presented by De Diego et al. (2017). In this method, several traits such as changes in the rosette leaf area, relative growth rate, survival rate and homogeneity of the population are scored using fully automated RGB imaging and subsequent image analysis. This method was successfully validated on example of multivariate analysis of rosette growth in different salt concentrations and the interaction with varying nutritional composition of the growth medium (De Diego et al., 2017). Many biostimulant products can directly or indirectly modify the plant hormone homeostasis of a treated plant. Principle of a facile forward chemical screening methodology for intact *Arabidopsis* seedlings harboring the β-glucuronidase (GUS) reporter under plant hormone-responsive promoters can be adapted for semi-automated testing in 96-well plates (Halder and Kombrink, 2015). Several existing transgenic *Arabidopsis* lines can be employed in such a lab-scale assay for multiple analyses of the effect of a biostimulant on the individual signaling pathways of cytokinins (ARR5::GUS), auxins (DR5::GUS), salicylic acid (PR1::GUS), abscisic acid (DC3::GUS), or bacterial elicitors such as flagellin (WRKY29::GUS). Such a complex assay could represent complementary tool for unraveling the mode of action of selected biostimulants. The potential pipeline of a biostimulant testing small-scale screening platform may consist of a sequence of automated assays determining the *Arabidopsis* performance under different growth conditions and the response to different abiotic stress treatments, followed by other species-based bioassays confirming applicability in crops. The next approach can be represented by complex phenotyping of selected variants combining various methods of automated, non-destructive, and simultaneous analyses of plant growth, morphology, and physiology in the medium-large screening platforms.

Medium-Large Screening Platforms

Medium-large screening platforms are fully automated robotic systems usually installed in controlled environment or semi-controlled greenhouse conditions and are designed for automated cultivation, handling, and non-invasive monitoring of plants in throughput for a range of few up to several hundreds of plants. Plants can be dynamically monitored for many morpho-physiological traits related to growth, yield, and performance throughout their development or onset, progression, and recovery from abiotic stress. Biostimulant functional characterization in plants can be thus monitored in high-precision and high-resolution in a given phase of plant development and/or plant response to environmental conditions, depending on the target substance application or type of experimental layout. In terms of dimensions, phenotyping platforms are available for plants ranging from *Arabidopsis,* broadly used as a model plant also in biostimulant research field (Rodriguez-Furlán et al., 2016; De Diego et al., 2017), up to platforms providing technological solutions for screening complex morpho-physiological traits in mature crop plants such as barley, rice, soybean, or vegetable crops. Standard medium-to-large phenotyping platforms integrating one or multiple watering and weighing units ensure that a precise irrigation system with optional controlled nutrient delivery on plant specific basis can be used. This can be a key element for studies when biostimulant action is addressed together in combination with abiotic stress such as salinity or drought stress and/or when specific nutrient regime is applied, and nutrient use efficiency is studied throughout plant development. Integration of automated and programmable spraying unit into the phenotyping pipeline further extends the capacities of the platform by maximizing the standardization of the biostimulant application and/or availability for different modes of applications (e.g., drench vs. spraying). In general, many developmental processes can be actively regulated following biostimulant application. Multiple functions of biostimulant activity on plants can be characterized by growth-promoting features, enhancement of nutrition efficiency, and abiotic stress tolerance (du Jardin, 2015). This broad spectrum of traits can be quantitatively described and qualitatively differentiated by so-called integrative phenotyping in multi-sensoric phenotyping platforms including imaging sensors for visible imaging (RGB imaging) and/or 3D imaging, imaging spectroscopy (hyperspectral imaging), thermal infrared

imaging, and chlorophyll fluorescence imaging. The integrative phenotyping approach based on integration of multiple read-outs from various imaging and non-imaging sensors available in these types of platforms (Humplík et al., 2015) allows to draw more complex images on the possible mode of action of a biostimulant under specific environmental conditions. Range of commercial phenotyping platforms is nowadays available with different specificities and key imaging sensor features. The reader is advised to view recent reviews with overview of available imaging sensors and commercial technologies on the market (Humplík et al., 2015; Rahaman et al., 2015; Mishra et al., 2016). The platforms can be either built within large controlled-environment chambers or implemented inside of greenhouse environments. Implementation of multiple imaging and non-imaging sensors (e.g., environmental sensors) within the phenotyping platforms provides the possibility to design species-specific phenotyping protocols in order to understand: plant growth dynamics and performance via RGB imaging; plant's photosynthetic capacity and ability to harvest light energy by chlorophyll fluorescence imaging, stomatal conductance, and water transpiration rates of plants by measuring leaf and canopy temperature with thermal imaging sensors; biochemical composition of plants by quantification of spectral reflectance profiles with hyperspectral imaging, precise architecture, and shape of the plants by 3D imaging. Above all, standardized data management routines and sophisticated image analysis algorithms are implemented within the general phenotyping pipelines (Tardieu et al., 2017). Altogether by using advanced data analysis algorithms and statistical analysis for the multi-dimensional phenotype data that are resulting from integrative phenotyping approach, the broad spectrum of morpho-physiological traits can be clustered and the traits correlating with the given phase of biostimulant application or the stress response can be identified. The so far above-described phenotyping approaches can be successfully used for in-depth characterization of biostimulants action in a range of plants species, however, the read-out refers solely to above-ground morpho-physiological features. The below-ground features referring to root system architecture and its function are not analyzed as routinely as shoot features but certainly should not be neglected. Range of automated and semi-automated phenotyping platforms are currently available for quantitative and dynamic analysis of root growth and architecture (Paez-Garcia et al., 2015). However, in most cases, and especially for crop species of bigger size, range of technical limitations must still be overcome. Major challenges for root phenotyping remain in providing high throughput level tools with relevant growing conditions and with appropriate spatial and time resolution of image acquisition and this in both time and cost-effective manner.

Field Phenotyping Systems

Many of the effects of biostimulants are related to improvements of the functioning of root systems and their interaction with the soil environment and to improved mechanisms of tolerance to environmental stresses (Calvo et al., 2014). Therefore, it is clear that controlled environments do not always provide a realistic context for their assessment. Soil characteristics, rainfall, temperature, and weather, along with the presence of diseases, insect pests and weeds, interact with the mechanisms of action of biostimulants, thereby influencing their efficacy across years. Additionally, crop physiological processes acting at the canopy scale, when plants are grown together in the field, have their own specific mechanisms, such as root mutual relationships and competitive effects that interact with those influenced by biostimulants in the single plant, when grown alone in a pot. In recent years there has been impressive progress in the development of approaches for open-field phenotyping (Araus and Cairns, 2014; Shakoor et al., 2017), and the accuracy of proximal or remote sensing systems for ground-based to aerial platforms is dramatically increasing. The use of such systems opens the way to a spectacular increase in the capability of screening large number of genotypes in the field, with non-destructive, repeated, objective observations, without the requirement of an extensive labor force. It is not only for plant breeding that these systems could be used, but also for physiological and agronomic studies, including the assessment of biostimulants. Sensors can be deployed on the ground, on fixed or mobile platforms, so that the distance to the target ranges from less than one to a few ten meters. Fixed platforms, in which the sensors do not move, include towers (Naito et al., 2017), tripods (Friedli et al., 2016), and wireless sensor networks (WSN) (Jones et al., 2018). Mobile ground platforms range from tractor-based systems (Enciso et al., 2017; Salas Fernandez et al., 2017), to manually driven buggies (Deery et al., 2014), or autonomous mobile rovers (Madec et al., 2017), to fixed rails (Virlet et al., 2017), or wires (Kirchgessner et al., 2017). Alternatively, phenotyping systems can be carried by unmanned aerial vehicles (UAV) (Sankaran et al., 2015; Yang et al., 2017) or blimps/balloons, in which case the distance from the target is generally of the order of 30–150 m, so they could be considered as remote sensing systems. There are advantages and disadvantages for each platform type, extensively discussed in previous reviews (Deery et al., 2014; Shakoor et al., 2017; Yang et al., 2017). In general, ground-based systems have a higher spatial resolution (i.e., ground sampling distance) and the possibility of assembling multiple-sensor arrays, combining, for example, hyperspectral, thermal, and lidar sensors. Conversely UAVs are limited by a small payload of just one or two instruments. On the other hand, ground platforms can be slow to move, so that environmental conditions may change by the time they move from one plot to another. This is a disturbing effect for some spectral (Virlet et al., 2017) and thermal sensing systems (Deery et al., 2014), which are sensitive to the effect of varying solar irradiance, for example, in the case of sky conditions with scattered clouds. Additionally, fixed ground-based systems constrain the possibility of changing the experimental plot area, sometimes preventing a sound agronomic practice of crop rotation. They also pose strong limitations to conventional soil preparation (i.e., tillage), because the platform only covers a fixed small land area, where conventional agricultural machinery cannot be used (Kirchgessner et al., 2017; Virlet et al., 2017). Thus, for biostimulants assays, mobile systems should be preferred. In general, the suitability of the platform will vary in relation to the objectives of the study and on the plant variables that need

to be estimated, as well as on the accuracy required in their estimation. In the case of biostimulants, the variables of interest that can be monitored from current field phenotyping systems, are those related to canopy structure and growth, photosynthesis, water relations, and leaf biochemistry. These variables should be generally estimated with an accuracy better than 10%, in order to be able to discriminate the effect of biostimulants (Calvo et al., 2014). Ground-based lidar or terrestrial 3D laser scanning systems seem to provide the most accurate and versatile tool for canopy structure and functioning assessment (Deery et al., 2014; Kjaer and Ottosen, 2015; Friedli et al., 2016), better than for example, RGB structure from motion techniques (Madec et al., 2017). Infrared thermography, when due attention is paid to ancillary measurements and/or reference surfaces, allows the assessment of transpiration and stomatal functioning (Jones et al., 2018). Close-range imaging spectroscopy (Mishra et al., 2017) seems to be the most promising tool for the assessment of tissue biochemistry, though technical issues exist, related to the data acquisition configuration, for example, for line scanners and for conversion into absolute reflectance (Deery et al., 2014), as well as for heavy data processing (Virlet et al., 2017). For the assessment of photosynthetic functioning and stress responses, fluorescence imaging has great potential, despite technical limitations of some techniques in field conditions (e.g., illumination) (Shakoor et al., 2017; Virlet et al., 2017). The possibility to assess root structure and functioning is not available in current systems (Pauli et al., 2016) although it would be extremely interesting for biostimulant assessment. In this context, the mapping of soil properties, for example, by geoelectrical sensors or/and hyperspectral bare soil data (Casa et al., 2013), rather than root structure *per se* provides a potentially powerful ally to direct root detection.

BIOSTIMULANT DEVELOPMENT PROCESS: FROM LAB TO FIELD OR FROM FIELD TO LAB?

Biostimulant activity is modulated by interacting factors such as plant genotype, growing conditions, dose, and application time. Crops in open field are faced with multiple/combined abiotic stresses difficult to reproduce in controlled environment. Moreover, the performance of microbial biostimulants depends on native soil microflora, physical, and chemical conditions of the soil and climatic factors. For these reasons, biostimulants screened in controlled environment do not always perform as expected under field conditions. An effective approach would be to screen substances/microorganisms for biostimulant activity under real field conditions and then use small-medium phenotyping platforms in controlled-environment experiments to understand their mode of action on model plants like *Arabidopsis*. Although this approach seems most appropriate for identifying effective biostimulant products, many companies initiate the screening process in controlled environment to shorten the time needed to identify new bioactive substances and beneficial microorganisms and to narrow the number of products later tested in real field conditions. This "lab to field" approach is mostly used by SMEs to reduce the cost of field testing for product development. On the contrary, a "field to lab" approach is especially adopted by big companies using large-scale field testing to develop efficient biostimulants under real growing conditions. For instance, Albaugh, LLC, and Italpollina United States, Inc. recently announced a long-term strategic collaboration to deliver biological seed treatment solutions for boosting crop yields in a sustainable way. In 2015–2017, the Alliance tested more than 50 seed treatments (vegetal-based protein hydrolysates, *Rhizoglomus irregulare* BEG72, *Funelliformis mossae* BEG234, and *Trichoderma atroviride* MUCL 45632) across 330 field trials in more than 100 locations in United States (Bonini et al., 2017). The best performing products were also tested in trials under controlled environment to investigate their mode of action using a 'multi-omics' approach. This collaboration has resulted in the launch of several biological seed treatments (BIOST®VPH100; BIOST®Mycorrhizae 100; BIOST®Trichoderma 100) for growers of field crops such as canola, corn, cotton, rice, sorghum, soybeans, sugarbeets, and wheat[1].

AUTHOR CONTRIBUTIONS

YR and GC had the original idea to write the perspective article and GC coordinated the manuscript preparation. YR and GC wrote the section "Plant Biostimulants: What They Are and Their Effects on Morpho-Physiological Traits of Crops" and "Biostimulant Development Process: From Lab to Field or From Field to Lab?" LS wrote the section "Small-Scale Screening Platforms." KP wrote the section "Medium-Large Screening Platforms." RC wrote the section "Field Phenotyping Systems." All authors contributed significantly to improve the final version of the article.

FUNDING

The authors gratefully acknowledge MIUR (Ministry for Education, University and Research) for financial support (Law 232/216, Department of Excellence), the Ministry of Education, Youth and Sports of the Czechia (Grant LO1204 from the National Program of Sustainability) and funding by the European Union's Horizon 2020 research and innovation program under grant agreement no. 675006.

[1] http://www.albaughllc.com/north-america/seed-treatment/biost-platform/

REFERENCES

Araus, J. L., and Cairns, J. E. (2014). Field high-throughput phenotyping: the new crop breeding frontier. *Trends Plant Sci.* 19, 52–61. doi: 10.1016/j.tplants.2013.09.008

Bonini, P., Long, D. H., Canaguier, R., Colla, G., and Leman, J. (2017). *Seed Treatments with Endophytic Fungi and Biostimulant Compounds Enhance Yield of Corn and Soybean. the 3rd World Congress on the Use of Biostimulants in Agriculture, 27-30 November 2017*. Miami, FL: Book of Abstracts, 147.

Calvo, P., Nelson, L., and Kloepper, J. W. (2014). Agricultural uses of plant biostimulants. *Plant Soil* 383, 3–41. doi: 10.1007/s11104-014-2131-8

Casa, R., Castaldi, F., Pascucci, S., Basso, B., and Pignatti, S. (2013). Geophysical and hyperspectral data fusion techniques for in-field estimation of soil properties. *Vadose Zone J.* 12:4. doi: 10.2136/vzj2012.0201

Colla, G., and Rouphael, Y. (2015). Biostimulants in horticulture. *Sci. Hortic.* 196, 39-48. doi: 10.1016/j.scienta.2015.10.044

Colla, G., Rouphael, Y., Bonini, P., and Cardarelli, M. (2015). Coating seeds with endophytic fungi enhances growth, nutrient uptake, yield and grain quality of winter wheat. *Int. J. Plant Prod.* 9, 171–190.

De Diego, N., Fürst, T., Humplík, J. F., Ugena, L., Podlešáková, K., and Spíchal, L. (2017). An automated method for high-throughput screening of Arabidopsis rosette growth in multi-well plates and Its validation in stress conditions. *Front. Plant Sci.* 8:1702. doi: 10.3389/fpls.2017.01702

Deery, D., Jimenez-Berni, J., Jones, H., Sirault, X., and Furbank, R. (2014). Proximal remote sensing buggies and potential applications for field-based phenotyping. *Agronomy* 4, 349–379. doi: 10.3390/agronomy4030349

du Jardin, P. (2015). Plant biostimulants: definition, concept, main categories and regulation. *Sci. Hortic.* 196, 3–14. doi: 10.1016/j.scienta.2015.09.021

Enciso, J., Maeda, M., Landivar, J., Jung, J., and Chang, A. (2017). A ground based platform for high throughput phenotyping. *Comp. Elect. Agric.* 141, 286–291. doi: 10.1016/j.compag.2017.08.006

Ertani, A., Pizzeghello, D., Francioso, O., Sambo, P., Sanchez-Cortes, S., and Nardi, S. (2014). *Capsicum chinensis* L. growth and nutraceutical properties are enhanced by biostimulants in a long-term period: chemical and metabolomic approaches. *Front. Plant Sci.* 5:375. doi: 10.3389/fpls.2014.00375

Ertani, A., Schiavon, M., Muscolo, A., and Nardi, S. (2013). Alfalfa plant-derived biostimulant stimulate short-term growth of salt stressed Zea mays L. plants. *Plant Soil* 364, 145–158.

Friedli, M., Kirchgessner, N., Grieder, C., Liebisch, F., Mannale, M., and Walter, A. (2016). Terrestrial 3D laser scanning to track the increase in canopy height of both monocot and dicot crop species under field conditions. *Plant Methods* 12:9. doi: 10.1186/s13007-016-0109-7

Halder, V., and Kombrink, E. (2015). Facile high-throughput forward chemical genetic screening by in situ monitoring of glucuronidase-based reporter gene expression in Arabidopsis thaliana. *Front. Plant Sci.* 6:13. doi: 10.3389/fpls.2015.00013

Humplík, J. F., Lazár, D., Husičková, A., and Spíchal, L. (2015). Automated phenotyping of plant shoots using imaging methods for analysis of plant stress responses – a review. *Plant Methods* 11:29. doi: 10.1186/s13007-015-0072-8

Jones, H. G., Hutchinson, P. A., May, T., Jamali, H., and Deery, D. M. (2018). A practical method using a network of fixed infrared sensors for estimating crop canopy conductance and evaporation rate. *Biosyst. Eng.* 165, 59–69. doi: 10.1016/j.biosystemseng.2017.09.012

Kirchgessner, N., Liebisch, F., Yu, K., Pfeifer, J., Friedli, M., Hund, A., et al. (2017). The ETH field phenotyping platform FIP: a cable-suspended multi-sensor system. *Funct. Plant Biol.* 44, 154–168.

Kjaer, K. H., and Ottosen, C. O. (2015). 3D laser triangulation for plant phenotyping in challenging environments. *Sensors* 15, 13533–13547. doi: 10.3390/s150613533

Lucini, L., Rouphael, Y., Cardarelli, M., Bonini, P., Baffi, C., and Colla, G. (2018). A vegetal biopolymer-based biostimulant promoted root growth in melon while triggering brassinosteroids and stress-related compounds. *Front. Plant Sci* 9:472. doi: 10.3389/fpls.2018.00472

Lucini, L., Rouphael, Y., Cardarelli, M., Canaguier, R., Kumar, P., and Colla, G. (2015). The effect of a plant-derived protein hydrolysate on metabolic profiling and crop performance of lettuce grown under saline conditions. *Sci. Hortic.* 182, 124–133. doi: 10.1016/j.scienta.2014.11.022

Madec, S., Baret, F., De Solan, B., Thomas, S., Dutartre, D., Jezequel, S., et al. (2017). High-throughput phenotyping of plant height: comparing unmanned aerial vehicles and ground lidar estimates. *Front. Plant Sci.* 8:2002. doi: 10.3389/fpls.2017.02002

Mishra, K. B., Mishra, A., Klem, K., and Govindjee (2016). Plant phenotyping: a perspective. *Ind. J. Plant Physiol.* 21, 514–527. doi: 10.1007/s40502-016-0271-y

Mishra, P., Asaari, M. S. M., Herrero-Langreo, A., Lohumi, S., Diezma, B., and Scheunders, P. (2017). Close range hyperspectral imaging of plants: a review. *Biosyst. Eng.* 164, 49–67. doi: 10.1016/j.biosystemseng.2017.09.009

Naito, H., Ogawa, S., Valencia, M. O., Mohri, H., Urano, Y., Hosoi, F., et al. (2017). Estimating rice yield related traits and quantitative trait loci analysis under different nitrogen treatments using a simple tower-based field phenotyping system with modified single-lens reflex cameras. *ISPRS J. Photog. Remote Sens.* 125, 50–62. doi: 10.1016/j.isprsjprs.2017.01.010

Paez-Garcia, A., Motes, C. M., Scheible, W. R., Chen, R., Blancaflor, E. B., and Monteros, M. J. (2015). Root traits and phenotyping strategies for plant improvement. *Plants* 4, 334–355. doi: 10.3390/plants4020334

Pauli, D., Chapman, S. C., Bart, R., Topp, C. N., Lawrence-Dill, C. J., Poland, J., et al. (2016). The quest for understanding phenotypic variation via integrated approaches in the field environment. *Plant Physiol.* 172, 622–634. doi: 10.1016/j.isprsjprs.2017.01.010

Petrozza, A., Santaniello, A., Summerer, S., Di Tommaso, G., Di Tommaso, D., Paparelli, E., et al. (2014). Physiological responses to Megafol treatments in tomato plants under drought stress: a phenomic and molecular approach. *Sci. Hortic.* 174, 185–192. doi: 10.1016/j.scienta.2014.05.023

Rahaman, M. M., Chen, D., Gillani, Z., Klukas, C., and Chen, M. (2015). Advanced phenotyping and phenotype data analysis for the study of plant growth and development. *Front. Plant Sci.* 6:619. doi: 10.3389/fpls.2015.00619

Rodriguez-Furlán, C., Miranda, G., Reggiardo, M., Hicks, G. R., and Norambuena, L. (2016). High throughput selection of novel plant growth regulators: assessing the translatability of small bioactive molecules from Arabidopsis to crops. *Plant Sci.* 245, 50–60. doi: 10.1016/j.plantsci.2016.01.001

Rouphael, Y., Colla, G., Giordano, M., El-Nakhel, C., Kyriacou, M. C., and De Pascale, S. (2017). Foliar applications of a legume-derived protein hydrolysate elicit dose dependent increases of growth, leaf mineral composition, yield and fruit quality in two greenhouse tomato cultivars. *Sci. Hortic.* 226, 353–360. doi: 10.1016/j.scienta.2017.09.007

Salas Fernandez, M. G., Bao, Y., Tang, L., and Schnable, P. S. (2017). A high-throughput, field-based phenotyping technology for tall biomass crops. *Plant Physiol.* 174, 2008–2022. doi: 10.1104/pp.17.00707

Sankaran, S., Khot, L. R., Espinoza, C. Z., Jarolmasjed, S., Sathuvalli, V. R., Vandemark, G. J., et al. (2015). Low-altitude, high-resolution aerial imaging systems for row and field crop phenotyping: a review. *Eur. J. Agron.* 70, 112–123. doi: 10.1016/j.eja.2015.07.004

Shakoor, N., Lee, S., and Mockler, T. C. (2017). High throughput phenotyping to accelerate crop breeding and monitoring of diseases in the field. *Curr. Opin. Plant Biol.* 38, 184–192. doi: 10.1016/j.pbi.2017.05.006

Tardieu, F., Cabrera-Bosquet, L., Pridmore, T., and Bennett, M. (2017). Plant phenomics, from sensors to knowledge. *Curr. Biol.* 27, R770–R783. doi: 10.1016/j.cub.2017.05.055

Virlet, N., Sabermanesh, K., Sadeghi-Tehran, P., and Hawkesford, M. J. (2017). Field scanalyzer: an automated robotic field phenotyping platform for detailed crop monitoring. *Funct. Plant Biol.* 44, 143–153. doi: 10.1071/fp16163

Williams, D., Britten, A., McCallum, S., Jones, H. G., Aitkenhead, M., Karley, A., et al. (2017). A method for automatic segmentation and splitting of hyperspectral images of raspberry plants collected in field conditions. *Plant Methods* 13:74. doi: 10.1186/s13007-017-0226-y

Yang, G., Liu, J., Zhao, C., Li, Z., Huang, Y., Yu, H., et al. (2017). Unmanned aerial vehicle remote sensing for field-based crop phenotyping: current status and perspectives. *Front. Plant Sci.* 8:1111. doi: 10.3389/fpls.2017.01111

Zhang, X., and Schmidt, R. E. (1997). The impact of growth regulators on alpha-tocopherol status of water-stressed *Poa pratensis* L. *Int. Turfgrass Soc. Res. J.* 8, 1364–2137.

Conflict of Interest Statement: The authors declare that the research was conducted in the absence of any commercial or financial relationships that could be construed as a potential conflict of interest.

frontiers in Plant Science

ORIGINAL RESEARCH
published: 15 August 2018
doi: 10.3389/fpls.2018.01191

The Selenium Supplementation Influences Olive Tree Production and Oil Stability Against Oxidation and Can Alleviate the Water Deficiency Effects

Roberto D'Amato[1†], Mauro De Feudis[1†], Paul E. Hasuoka[2], Luca Regni[1], Pablo H. Pacheco[2], Andrea Onofri[1], Daniela Businelli[1] and Primo Proietti[1]*

[1] DSA3, Department of Agricultural, Food and Environmental Sciences, University of Perugia, Perugia, Italy, [2] Instituto de Química de San Luis, INQUISAL, Centro Científico-Tecnológico de San Luis (CCT-San Luis), Consejo Nacional de Investigaciones Científicas – Universidad Nacional de San Luis, Chacabuco y Pedernera, San Luis, Argentina

OPEN ACCESS

Edited by:
Giuseppe Colla,
Università degli Studi de lla Tuscia, Italy

Reviewed by:
Georgios Liakopoulos,
Agricultural University of Athens, Greece
Juan Jose Rios,
Centro de Edafología y Biología Aplicada del Segura (CEBAS), Spain
Beatrice Pezzarossa,
Istituto per lo Studio degli Ecosistemi (ISE), Italy

***Correspondence:**
Pablo H. Pacheco
ppacheco@unsl.edu.ar

[†] First authors

Specialty section:
This article was submitted to Plant Nutrition, a section of the journal Frontiers in Plant Science

Received: 15 May 2018
Accepted: 25 July 2018
Published: 15 August 2018

Citation:
D'Amato R, De Feudis M, Hasuoka PE, Regni L, Pacheco PH, Onofri A, Businelli D and Proietti P (2018) The Selenium Supplementation Influences Olive Tree Production and Oil Stability Against Oxidation and Can Alleviate the Water Deficiency Effects. Front. Plant Sci. 9:1191. doi: 10.3389/fpls.2018.01191

Foliar fertilization with selenium (Se) may well be beneficial in increasing the nutritional and qualitative values of food in Se-deficient regions such as the Mediterranean Basin, and may contribute to an increase in drought resistance in plants. The present study has considered detachment force, flesh firmness, pigmentation, fresh and dry weight, and oil content of olive drupes from Se fertilized olive orchards (*Olea europaea* L.) under drought stress and well-watered conditions. This study has also evaluated the total Se, Se amino acid, phenol, carotenoid and chlorophyll contents of EVOO, plus its oxidative stability against oxidation. While there was no change in the ripening indexes and the production of olives generally, Se application did increase the total Se, Se methionine, phenol, and carotenoid and chlorophyll contents. The higher concentration of these (bio) chemical compounds in EVOO obtained from Se fertilized plants might well suggest enhanced antioxidant activity. Consequently, EVOO obtained from Se fertilized trees possesses a higher nutritional value and, as indicated by the greater oxidative stability against oxidation, longer shelf life. In addition, under water deficient conditions, a higher fresh olive weight corresponds to a higher level of phenol, carotenoid and chlorophyll, and the chlorophyll-to-carotenoid ratio in Se fertilized trees would appear to confirm the positive role of selenium in alleviating damage caused by drought stress conditions.

Keywords: seleno amino acids, olive oil stability, drought stress, antioxidant compounds, *Olea europaea* L., phenols

INTRODUCTION

Selenium (Se) is essential for humans since it integrates GPx an enzyme that plays an important role in cerebral and reproductive functions (Navarro-Alarcón and López-Martínez, 2000; Rayman, 2000), prevention of cancer and cardiovascular disease (Rayman, 2000; Roman et al., 2014), and the detoxification of heavy metals. Nutritionists encourage Se-enriched food consumption, especially

Abbreviations: ANOVA, analysis of variance; DS, drought stressed; EVOO, extra-virgin olive oil; GPx, glutathione peroxidase; HCl, hydrochloric acid; HNO_3, nitric acide; H_2O_2, hydrogen peroxide; LC–ICP MS, liquid chromatography–inductively coupled plasma mass spectrometry; MI, maturity index; MeOH, methanol; NDS, non-drought stressed; NT, not Se-treated plants; ROS, reactive oxygen species; RPC–ICP MS, reversed-phase chromatography inductively coupled plasma mass spectrometry; Se, selenium; SeCys, selenocysteine; SeMeSeCys, selenomethylselenocysteine; SeMet, selenomethionine; T, Se-treated plants.

in areas where soil Se levels are low, which is the case in certain parts of Europe (Sager, 2006). As a result, interest in Se-enrichment in food is steadily increasing (Rayman, 2000; Finley, 2007; Businelli et al., 2015; D'Amato et al., 2017; Fontanella et al., 2017).

Olive oil is a key component in the traditional Mediterranean diet, and the olive tree (*Olea europaea* L.) is one of the oldest and most important tree species in the Mediterranean Basin accounting for 98% of the world's olive cultivation (Banias et al., 2017). In fact, European countries provide 80% of the olive oil produced worldwide, and Italy is the second largest olive oil producer after Spain (Banias et al., 2017). The importance of EVOO in these regions coupled with a low level of Se in the area (Spadoni et al., 2007) has forced researchers to develop new methods of increasing the Se concentration of olive oil. Previous studies have demonstrated Se enrichment in EVOO obtained from Se fertilized olive trees (Proietti et al., 2013; D'Amato et al., 2014). Once administrated because of its chemical similarity to sulfur (S) Se is generally taken up by the plant via sulfate transporters and then metabolized via the S assimilation pathway (Sors et al., 2005), which may incorporate this micronutrient into organic forms such as SeCys and SeMet. Recently, interest in seleno-amino acids has increased because they integrate the active centers of several selenoenzymes, which are involved in biological synthesis and plant metabolism, and are used for protein structure determinations (Iwaoka et al., 2008). For example, the study performed by Torres et al. (2014) on EVOO obtained from olive groves in Argentina grown on soil containing Se, has shown the presence of seleno-amino acids such as SeMeSeCys and selenocysteine. Data concerning the effect of these Se compounds on EVOO is scarce, but Zalejska-Fiolka (2000) who demonstrated the anti-oxidative properties of α-tocopherol, SeMet and methionine in olive oil, noted greater anti-oxidative properties in SeMet than in α-tocopherol.

A key element to EVOO quality might well be the drought periods that generally occur in the Mediterranean area during the spring and summer periods (Spadoni et al., 2007; Proietti et al., 2013). Indeed, during water limitation, a reduction of net photosynthesis and an increased production of ROS occurs in plants (Kaushik and Roychoudhury, 2014), and this might undermine EVOO stability against oxidation. In this context, Se appears to be involved in the mechanism of water-stress tolerance, via ROS detoxification (Feng et al., 2013). This aspect helps to preserve membrane integrity, and enzyme and protein stability (Chaves and Oliveira, 2004; Proietti et al., 2013) with possible positive effects on EVOO biochemical properties. While membrane integrity is essential to avoiding oil deterioration, the proteins present in the oil bodies of the mesocarp, which pass into the oil during the extraction process, contribute to its shelf life (Zamora et al., 2001).

Despite the importance of Se in EVOO in the Mediterranean diet, and in general for overall human health, there is still limited information concerning the effects of the micronutrient on olive oil quality. This present work has aimed to evaluate the effects of foliar Se fertilization on the antioxidant compounds that are involved in olive oil stability against oxidation, and, as a consequence, on human nutrition. Furthermore, since there is an increasing drought problem in the Mediterranean area, we have examined the effect of Se on EVOO obtained from both DS and well-watered olive trees.

MATERIALS AND METHODS

Reagents and Standards

Sodium selenate (cod. S0882-25g), sodium sulfate anhydrous, and SeMet, Se-MeSeCys, and SeCys standards were purchased from Sigma-Aldrich (St. Louis, United States). The wetting agent Alba Milagro was provided by Alba Milagro International S.p.A (Milano, Italy). Acetone, n-hexane, water and methanol Optima grade were purchased from Fisher Scientific (Fair Lawn, NJ, United States). HNO_3 (65% v/v), H_2O_2 (30% v/v), ultrapure grade HCl (37% v/v), methanol and mercaptoethanol were purchased from Carlo Erba Reagents (Milano, Italy).

Experimental Setup

A 2-year field study (2014 and 2015) was conducted on a 17-year-old olive orchard (*O. europaea* L. cv Leccino) located at 43°02′ N, 12°43′ E with an altitude of 220 m (Central Italy). The field consisted of 18 rows, 78 m long, with a north-south orientation. There was a distance of 6.0 m between the rows and 3.0 m between each plant. In order to perform the experiment, two plots (one per year) with six rows per plot were selected. The olive trees, annually pruned, were trained to a monocone system (about 4 m high). Each spring, the olive orchard was fertilized using 16.5 t ha^{-1} of cow manure with the following composition: moisture 51.2%, organic matter 18.1%, total nitrogen (N) 0.68%, total phosphorus (P_2O_5) 0.30%, available potassium (K_2O) 0.35%, available calcium (Ca) 0.70% and total Se 0.018 mg kg^{-1}. The soil, derived from calcareous marl, was classified as Typic Haploxerept (Soil Survey Staff, 2014). It was initially characterized by a loam texture (sand 41%, silt 34%, clay 25%) with alkaline pH (8.1), and a concentration of 10.0 g kg^{-1} of total organic C, 2.0 g kg^{-1} of total N, 5.5 mg kg^{-1} of available P and 190 mg kg^{-1} of exchangeable K and 0.010 mg kg^{-1} of total Se. The location was characterized by a mean annual rainfall of 831 mm (1921–2015) with the wettest month being November (106 mm on average) and the driest one July (37 mm on average). The mean annual air temperature was 13.2°C (1951–2015), ranging from 23.2°C in July to 4.0°C in February. In 2014 the mean annual air temperature was 17.0°C, with August being the warmest month (27.7°C) and January the coldest one (7.5°C), while rainfall was 1074.6 mm (614 mm from April to October). In 2015 the mean annual air temperature was 17.0°C with August being the warmest month (28.1°C) and January the coldest one (7.2°C), while rainfall was 758.6 mm (377 mm from April to October). The environmental parameters of the study site in 2014 and 2015 have been reported in **Table 1**.

For the DS trees no water was provided, while for the NDS trees irrigation was carried out in the morning, from late July to mid-September, using two drippers per tree, with a flow rate of 4 L h^{-1} each. With the aim of obtaining DS and NDS plants, each plot was divided into two sub-plots of three rows each.

In 2014, the NDS trees were supplied with water at the rate of 0.19 m^3 $plant^{-1}$ in three interventions (2nd July, 28th July, and 10th August) for a total of 110 m^3 ha^{-1}, while in 2015 they were supplied with water at the rate of 0.26 m^3 $plant^{-1}$ in four interventions (4th July, 23rd July, 13th August, 25th August) for a total of 147 m^3 ha^{-1}. Irrigation was implemented when leaf water potential—measured with pressure chambers (Scholander chamber) reached −2 MPa (Gómez-del-Campo, 2013).

With the aim of obtaining Se fertilized (T) and NT plants under DS and NDS conditions, nine trees were Se-fertilized along each row in such a way that three T plants alternated with three NT ones. Between the T and NT treated trees there was one border tree. On 29th April each T tree was sprayed with 5 L per plant of a solution containing a Se concentration of 100 mg L^{-1}, obtained by dissolving 239.26 g of sodium selenate in water, plus 0.5% of Albamilagro wetting agent.

Plant Material Sampling, Yield, Fruit Ripeness Indexes and EVOO Extraction

To avoid any treatment interference, sampling was carried out on trees located in the center of each treatment area. As a consequence, three trees per treatment were selected ($n = 3$).

In both years of the study, at the beginning of November (harvest time), detachment force, flesh firmness, pigmentation, fresh and dry weight, and oil content were determined on 50 olives per tree, picked from the three selected plants per treatment.

Detachment force was measured using a Carpo hand dynamometer. Flesh firmness was determined by an Effe.gi dynamometer DT 05 with a 1.0 mm diameter tip. Fruit pigmentation was evaluated using the MI according to the Agronomic Station of Jaen method (Uceda, 2008) based on the evaluation of skin and pulp color.

The fresh and dry weight of the fruit was determined by weighing the olives before and after drying at 90°C for 48 h. Oil content was determined using a SpectraAlyzer ZEUTEC NIR: Near Infra Red.

Then, randomly selected fruit of three trees per treatment were harvested using pneumatic combs and net and weighed to estimate the yield.

Within 6 h of the harvest, 3.0 kg of olives from each sample were used to extract EVOO using a lab scale system. Fruit were crushed by a hammer mill the resulting paste was malaxed at 22°C for 20 min, and the oil separated by centrifugation. To remove water and impurities, the oil was filtered with cotton wool and sodium sulphate anhydrous, and then stored in glass bottles in the dark at 15°C until analysis.

Preparation of Standard Solutions

Acid stock solutions of SeMet, SeMetSeCys, and SeCys standards, were prepared by dissolving the respective substances in 0.1 M HCl with 20% MeOH, except for SeMet, which was prepared in 0.5% 2-mercaptoethanol (0.3 mg g^{-1}). Stock solutions were prepared once and stored at −20°C. Dilutions were made with 0.004% (m/v) aqueous solution of 2-mercaptoethanol to avoid oxidation of SeMet. Working standard solutions were prepared by appropriate dilution with ultrapure water, adjusted to pH with HCl or sodium hydroxide when it was required according to analysis.

Oil Digestion and Protein Extraction

For total Se, 0.5 g of olive oil sample was treated with 7 mL concentrated HNO_3 and 1 mL H_2O_2, and digested in a microwave oven (Milestone Inc., ETHOS One, Sorisole, Italy). Digestion was carried out at a ramp temperature of up to 200°C for 10 min, and a final hold time of 10 min. The employed microwave power was up to 1000 W. Total Se determination was performed with an ICP-MS (ELAN DRC-e; Perkin-Elmer SCIEX, Thornhill, Canada).

The modified method described by Martín-Hernández et al. (2008) was performed for protein extraction. 10 mL of cold n-hexane/acetone (1:1, v/v) (2°C) was added to 5 g of olive oil. The mixture was shaken vigorously, kept at 2°C for 1 h, and shaken every 10 min. The mixture was then centrifuged, and the supernatant was discarded. The precipitate was washed twice with 1 mL of cold n-hexane/acetone solution (1:1). After each washing, the mixture was centrifuged, and the supernatant was discarded. At both stages centrifugation lasted 10 min at 7000 rpm (6.026 × g) at 2°C in a refrigerated centrifuge [Boeco U-320 R; Boeckel + Co (GmbH + Co), Hamburg, Germany]. After the centrifugation stage, the supernatant was discarded and the pellet obtained was re-dissolved with water:methanol (80:20). This solution was centrifuged for 5 min at 3500 rpm (3.013 × g), followed by freezing at −18°C for 1 h to obtain a clear solution for analysis.

EVOO Analysis

To achieve seleno-amino acid determination, the pellet obtained was treated for protein hydrolysis assisted by microwave (Reiz and Li, 2010), to this end 0.05% (v/v) phenol was added to avoid any amino acid oxidation by acids used during digestion. Mild conditions were used: 15% HCl (v/v) was added for a period of 5.5 min at a power of 900 W. Afterward, this solution was nitrogen evaporated at room temperature to avoid any volatilization of seleno-amino acids. The residue was then dissolved in 1 mL of 0.02 M HCl and filtered through a membrane filter before injection (200 μL) on LC–ICP MS. Seleno-amino acid determination was performed by coupling the chromatographer (Series 200; Perkin-Elmer, Thornhill, Canada) to ICP MS. Hydrolyzed fractions were analyzed for seleno-amino acids using reverse phase chromatography (RPC). The selected isotope for mass monitoring by ICP-MS was ^{82}Se, in order to avoid interference by polyatomic argon. The RPC–ICP MS conditions for separation of seleno-species by reverse phase chromatography are summed up in **Supplementary Table S1**.

Total Phenol, Carotenoid and Chlorophyll Contents, and Oxidative Stability

Phenol content was determined using the Folin-Ciocalteu reagent according to the Folin–Ciocalteu method (Singleton et al., 1998) and the results were expressed as μg gallic acid equivalent g^{-1} oil.

TABLE 1 | Environmental parameters of the study site in 2014 and 2015.

	Years	
	2014	**2015**
Mean annual air temperature (°C)	17.0	17.0
Mean temperature in the warmest month (°C)	27.7 (August)	28.1 (August)
Mean temperature in the coldest month (°C)	7.5 (January)	7.2 (January)
Total annual rainfall (mm)	1074.6	758.6
Rainfall from April to October (mm)	614	377

Total chlorophyll and total carotenoids were extracted dissolving 0.5 g of oil sample in 25 mL of 95% diethyl ether. The solution was filtered through a double layer of cheese cloths and the absorbance of the extract was measured with a Varian Cary 210 spectrophotometer at 662, 646, and 470 nm (Lichtenthaler and Wellburn, 1983).

The stability against oxidation, expressed as the oxidation induction time (hours), was estimated with a Rancimat 679 apparatus (Metrohm Co., Herisau, Switzerland) using 5.0 g of oil heated at 120°C with an air flow of 20 L h^{-1} passing through the sample. The volatile compounds were collected in a conductivity cell filled with distilled water. The time needed for the appearance of a sudden water conductivity rise caused by the adsorption of volatiles derived from the oil oxidation was registered as the induction time in hours.

Statistical Analysis

A two-way ANOVA was conducted to analyze the effects of Se fertilization and irrigation on the selected EVOO parameters. The assumption of normality and homoscedasticity of the data was verified by graphical analysis of residuals, and transformed where necessary. Means were compared to Tukey's *post hoc* test ($P < 0.05$) and the statistical analyses were performed using R software (R Development Core Team, 2011).

RESULTS

Ripening Indexes and Production of Olives

While in 2014 no differences were observed between the treatments, in 2015 a higher fruit yield and detachment force, and lower pigmentation was found in the NDS plants than in the DS plants (**Table 2**). Furthermore, in 2015 the T trees under drought stress showed a higher fresh weight fruit yield than the NT ones.

Carotenoid and Chlorophyll Content, and Chlorophyll-to-Carotenoid Ratio of EVOO

Whereas Se application increased EVOO carotenoid content under both drought stress and well-watered conditions, a higher carotenoid concentration was observed in the EVOO obtained from the NT trees under DS than under NDS conditions (**Table 3**). Unlike carotenoids, the EVOO chlorophyll content was lower under NDS compared to DS condition, but similarly to carotenoids, Se application increased their amount in the EVOO (**Table 3**).

In both years, while under drought stress the EVOO chlorophyll-to-carotenoid ratio was increased with Se application, conversely, in well-watered conditions the ratio decreased with Se application (**Table 3**). Furthermore, while in NT samples the chlorophyll-to-carotenoid ratio increased with irrigation, the opposite was observed in EVOO obtained from T trees.

Phenol Content and Stability Against Oxidation of EVOO

The promoting effect of Se treatment to EVOO phenol content and stability against oxidation was more pronounced under DS compared to NDS conditions, especially in the second year.

Total Se, SeMet, SeCys, and SeMetSeCys Contents of EVOO

In both years, while Se application increased the Se concentration in EVOO samples, irrigation reduced its concentration (**Table 4**).

While SeCys and SeMetSeCys were not detected in any olive oil samples, SeMet was found in all samples obtained from T trees with the exception of the EVOO obtained from the NDS plants in 2014. In addition, in 2015 a higher SeMet content was found under NDS than under DS conditions (**Table 4**).

DISCUSSION

The weather conditions during the two growing seasons were quite different. Compared to the 1921–2015 period, the mean annual rainfall in 2014 was +29%, whereas in 2015 it was −11%.

In 2015, according to Proietti et al. (2013) and D'Amato et al. (2014), Se treatments reduced the negative effects of low water availability on the olive yield per tree. In fact, because of its stimulating effect on root water uptake, Se increased the tolerance of plants to drought stress by regulating the water status (Djanaguiraman et al., 2005). The lower pigmentation and higher detachment force and pulp firmness of olive drupes from the NDS trees as compared to those from the DS ones might be due to the higher fruit yield (crop load) induced by irrigation. This fact might have caused a slowing down in fruit ripening due to a reduced availability of assimilates per drupe (Proietti et al., 2013). The absence of any differences in 2014 could be attributed to the more abundant rainfall and to a lower crop load (an off year) which possibly masked the DS effect. Conversely, Se treatment did not influence the fruit ripening process.

As expected, the foliar application of Se increased the Se content in EVOO samples. These results are consistent with previous studies which, after foliar Se treatments, found enhanced Se concentrations in several agricultural crops such as rice (Li et al., 2008), winter wheat (Ducsay and Ložek, 2006), carrot (Kápolna et al., 2009), peach and pear (Pezzarossa et al., 2012) as well as in agri-food industry products such as EVOO and wine (D'Amato et al., 2014; Fontanella et al., 2017). The higher quantities of total Se in EVOO obtained from DS

TABLE 2 | Fruit yield tree^{-1} expressed both as fresh and dry weights, color, pulp firmness, detachment force and oil content of olive (cv Leccino) orchard fertilized (T) and unfertilized (NT) with Se under well-watered (NDS) and drought stress (DS) conditions.

Year	Irrigation	Treatment	Fruit yield tree^{-1}		Color	Pulp firmness	Detachment force	Oil content
			kg fresh weight	kg dry weight	0–7	N	N	%
2014	DS	NT	23.2 (1.2)a	11.0 (0.5)a	4.4 (0.3)a	3.4 (0.2)a	4.6 (0.4)a	17.8 (1.6)a
2014	DS	T	22.4 (1.0)a	10.6 (0.8)a	5.0 (0.3)a	3.7 (0.3)a	4.6 (0.1)a	17.5 (1.5)a
2014	NDS	NT	21.8 (1.1)a	10.5 (0.7)a	4.6 (0.4)a	3.4 (0.1)a	4.4 (0.2)a	17.4 (1.4)a
2014	NDS	T	21.2 (0.9)a	11.0 (0.6)a	4.5 (0.3)a	3.1 (0.3)a	4.6 (0.6)a	17.9 (1.4)a
2015	DS	NT	28.8 (0.8)c	17.7 (0.9)b	4.8 (0.1)a	4.0 (0.2)a	4.7 (0.2)b	16.9 (1.6)a
2015	DS	T	33.6 (1.2)b	18.6 (1.1)b	5.0 (0.2)a	3.9 (0.2)a	4.8 (0.3)b	17.3 (1.3)a
2015	NDS	NT	38.1 (1.1)a	20.7 (0.7)a	3.5 (0.1)b	4.8 (0.2)a	5.8 (0.4)a	17.0 (1.7)a
2015	NDS	T	37.7 (0.9)a	20.6 (0.9)a	3.9 (0.1)b	4.9 (0.1)a	5.9 (0.3)a	17.0 (1.4)a

Values are means with SEs in parentheses (n = 3). In each column and for each year, different letters indicate significant differences among treatments at $P < 0.05$ using Tukey-HSD test.

TABLE 3 | Carotenoids, chlorophylls, phenols contents, chlorophylls-to-carotenoids ratio (Chlor/Carot), and stability against oxidation (Stability) in extra virgin olive oils obtained from olive (cv Leccino) orchard fertilized (T) and unfertilized (NT) with Se under well-watered (NDS) and drought stress (DS) conditions.

Year	Irrigation	Treatment	Carotenoids	Chlorophylls	Phenols	Stability	Chlor/Carot
				μg g^{-1}		h	
2014	DS	NT	10.6 (0.3)b	14.2 (0.2)c	287 (6)b	12.4 (0.2)b	1.34 (0.04)bc
2014	DS	T	13.0 (0.2)a	19.6 (0.3)a	343 (9)a	14.3 (0.2)a	1.52 (0.04)b
2014	NDS	NT	7.7 (0.1)c	14.3 (0.3)c	257 (7)c	11.0 (0.3)c	1.87 (0.05)a
2014	NDS	T	13.7 (0.4)a	15.8 (0.4)b	274 (4)bc	11.7 (0.2)bc	1.16 (0.05)c
2015	DS	NT	11.7 (0.3)c	15.6 (0.2)c	246 (5)c	11.8 (0.1)b	1.34 (0.03)b
2015	DS	T	14.0 (0.3)b	21.7 (0.3)a	296 (4)a	13.6 (0.1)a	1.55 (0.01)a
2015	NDS	NT	9.2 (0.1)c	14.5 (0.2)d	245 (6)c	10.0 (0.1)c	1.58 (0.02)a
2015	NDS	T	15.5 (0.4)a	18.5 (0.2)b	262 (3)b	11.1 (0.2)b	1.19 (0.02)c

Values are means with SEs in parentheses (n = 3). In each column and for each year, different letters indicate significant differences among treatments at $P < 0.05$ using Tukey-HSD test.

TABLE 4 | Total Se, SeCys, SeMet, and SeMeSeCys contents in extra virgin olive oils obtained from olive (*Olea europaea* L. cv Leccino) orchard fertilized (T) and unfertilized (NT) with Se under well-watered (NDS) and drought stress (DS) conditions.

Year	Irrigation	Treatment	Total Se	SeCys	SeMet	SeMetSeCys
				μg kg^{-1}		
2014	DS	NT	149 (9)c	n.d.	n.d.	n.d.
2014	DS	T	378 (8)a	n.d.	4.83 (0.14)	n.d.
2014	NDS	NT	16 (1)d	n.d.	n.d.	n.d.
2014	NDS	T	189 (6)b	n.d.	n.d.	n.d.
2015	DS	NT	164 (8)b	n.d.	n.d.	n.d.
2015	DS	T	529 (7)a	n.d.	4.17 (0.11)b	n.d.
2015	NDS	NT	12 (1)c	n.d.	n.d.	n.d.
2015	NDS	T	171 (6)b	n.d.	5.39 (0.06)a	n.d.

Values are means with SEs in parentheses (n = 3). In each column and for each year, different letters indicate significant differences among treatments at $P < 0.05$ using Tukey-HSD test.
n.d., not detected.

plants than from irrigated ones, might well be attributed to the ability of Se to increase the drought stress tolerance of plants (Proietti et al., 2013). The ability of Se to improve photosynthesis and protect PSII in fruit crops (peach and pear) was reported also by Feng et al. (2015). Since Se has this positive effect on plants that are exposed to stress conditions, there might be the risk of the plants tending to accumulate Se. The findings of this study are in accordance to those of Nawaz et al. (2016), which found a higher total Se content in wheat shoots grown under drought stress conditions, than wheat shoots grown under well-watered conditions. However, because of the high water solubility of Se, we cannot exclude a possible higher loss of Se during the

EVOO extraction process in the NDS olive drupes, than in the DS ones.

The higher carotenoid and chlorophyll contents in the T treatments than in the NT ones might indicate the stimulatory effect of Se on the biosynthesis of the photosynthetic pigments (Malik et al., 2012; Hashem et al., 2013). This aspect is interesting in relation to the EVOO stability against oxidation and to its nutritional value. Indeed, while in EVOO carotenoids and chlorophylls play an important role in oxidative processes due to their antioxidant nature in the dark, and their pro-oxidant activity in the light (Tovar et al., 2002), in humans carotenoids prevent cardio-vascular disease, show anti-cancer activity and provide protection against UV radiation (Tapiero et al., 2004). In contrast to Gómez-Rico et al. (2007) and Tovar et al. (2002), the carotenoid concentration of EVOO obtained from the NT trees decreased with irrigation, which may be due to the lowered stress conditions of the irrigated plants (Munné-Bosch and Alegre, 2000). In fact, it is well known that plants tend to accumulate carotenoids when exposed to some stress factors such as drought (Reddy et al., 2004). However, we cannot exclude that the lower content of carotenoids, as well as chlorophylls, might be due to the delay of the fruit ripening processes (Beltrán et al., 2005; D'Amato et al., 2014; Nasini and Proietti, 2014). For the T treatments, the lack of differences in carotenoid concentration between NDS and DS plants might be due the role of Se in alleviating the drought stress conditions (Nawaz et al., 2015).

Since changes of the chlorophyll-to-carotenoid ratio can indicate water deficient conditions (Jaleel et al., 2009; Ramakrishna and Ravishankar, 2011), the higher ratio in EVOO from the NDS-NT group as compared to the DS-NT one, and from the DS-T group as compared to the DS-NT one, might confirm the capacity of Se to protect plants from drought stress damage (Hasanuzzam et al., 2010; Proietti et al., 2013; Ahmad et al., 2016). In fact, Se has protective effects on chloroplast enzymes with a consequent increase in photosynthetic pigment biosynthesis (Pennanen et al., 2002). However, the higher chlorophyll-to-carotenoid ratio could be also partly attributed to the delay in the ripening process, due to a higher yield per tree (Nasini et al., 2013).

Under well-watered conditions, the lower chlorophyll-to-carotenoid ratio of EVOO from NT plants compared to those from T ones could be due to an inhibitory effect of Se on the production of an enzyme involved in chlorophyll biosynthesis (Fargašová et al., 2006).

The lower phenol content in EVOO from irrigated rather than non-irrigated trees could be attributed to the different water availability. Indeed, it is well-known that the level of phenols is higher in oil from drought-stressed plants than from irrigated ones (Jose Motilva et al., 2000; Servili et al., 2007). Furthermore, as already reported by D'Amato et al. (2014), Se treatment increases EVOO phenol content. However, the higher levels of phenols in EVOO from Se-treated trees may be due to a secondary effect caused by the inhibition of enzymatic phenol oxidation by strong antioxidant-active Se compounds (D'Amato et al., 2014). Since the high phenol content in EVOO could be considered a positive aspect because the higher the phenol content, the greater the oxidative stability, the increase of phenol content obtained from the DS and T treatments resulted in an increase of EVOO oxidative stability (Tovar et al., 2002; Ayton et al., 2007; Gouveia et al., 2013).

The higher stability in the T than in the NT EVOO could be also due to the greater content of both chlorophyll and SeMet which are involved in the anti-oxidative processes that take place in the olive oil (Zalejska-Fiolka, 2000; Vacca et al., 2006). The enhanced amount of Se in the form of SeMet could be interesting for both human health and olive oil quality. Indeed, while in humans SeMet is the only Se amino acid that can form proteins and provide several positive effects on human health (Schrauzer, 2000; Navarro-Alarcon and Cabrera-Vique, 2008) in EVOO SeMet is involved in the anti-oxidative processes (Zalejska-Fiolka, 2000; Vacca et al., 2006) which promote its stability.

Our findings are in contrast to Torres et al. (2004) who found the presence of SeMeSeCys in seven EVOO samples from different Argentinian regions. In the present study only selenomethionine has been detected in EVOO from Se fertilized plants.

Ultimately, Se-biofortification may well be of interest to areas with poor cultivars or cold, rainy weather patterns, which would normally lead to the production of EVOO with an unfavorable phenol content.

CONCLUSION

Olive tree (*O. europaea* L.) is the most important evergreen tree in the Mediterranean basin and EVOO is regarded as a key component of the traditional Mediterranean diet. The present study demonstrated that Se fertilization through foliar application can be adopted in olive groves to improve some qualitative and nutritional properties of EVOO. Indeed, our findings suggested how Se application, besides to enhance the Se content, increased the concentration of the oil antioxidant compounds. The greater amounts of these molecules, such as chlorophylls, carotenoids, phenols, and SeMet, brings advantages for the EVOO itself because enable to increase the EVOO oxidative stability and, as consequence, its shelf-life. Furthermore, in a drought scenario, this work confirmed the important role of Se to alleviate the negative effect of water deficiency on fruit production and olive oil stability against oxidation. As a consequence, this study highlighted how Se fertilization could be a suitable technique in areas characterized by low Se contents and subjected to frequent drought events to obtain high quality EVOOs.

AUTHOR CONTRIBUTIONS

PP, LR, and RD designed the experiments. PHP, PH, RD, and LR performed the analytical assays and analyzed the data. PP, AO, MDF, RD, DB, and LR wrote the manuscript. PP, DB, MDF, RD, LR, and AO revised the manuscript.

FUNDING

This research was partially supported by the Fondazione Cassa di Risparmio di Perugia (Project: "Selenolivo," project code: 2015.0347.021, Scientific and Technological Research).

SUPPLEMENTARY MATERIAL

The Supplementary Material for this article can be found online at: https://www.frontiersin.org/articles/10.3389/fpls.2018.01191/full#supplementary-material

REFERENCES

Ahmad, R., Waraich, E. A., Nawaz, F., Ashraf, M. Y., and Khalid, M. (2016). Selenium (Se) improves drought tolerance in crop plants - a myth or fact? *J. Sci. Food Agric.* 96, 372–380. doi: 10.1002/jsfa.7231

Ayton, J., Mailer, R. J., Haigh, A., Tronson, D., and Conlan, D. (2007). Quality and oxidative stability of Australian olive oil according to harvest date and irrigation. *J. Food Lipids* 14, 138–156. doi: 10.1111/j.1745-4522.2007.00076.x

Banias, G., Achillas, C., Vlachokostas, C., Moussiopoulos, N., and Stefanou, M. (2017). Environmental impacts in the life cycle of olive oil: a literature review. *J. Sci. Food Agric.* 97, 1686–1697. doi: 10.1002/jsfa.8143

Beltrán, G., Aguilera, M. P., Del Rio, C., Sanchez, S., and Martinez, L. (2005). Influence of fruit ripening process on the natural antioxidant content of Hojiblanca virgin olive oils. *Food Chem.* 89, 207–215. doi: 10.1016/j.foodchem.2004.02.027

Businelli, D., D'Amato, R., Onofri, A., Tedeschini, E., and Tei, F. (2015). Se-enrichment of cucumber (*Cucumis sativus* L.), lettuce (*Lactuca sativa* L.) and tomato (*Solanum lycopersicum* L. Karst) through fortification in pre-transplanting. *Sci. Hortic.* 197, 697–704. doi: 10.1016/j.scienta.2015.10.039

Chaves, M. M., and Oliveira, M. M. (2004). Mechanisms underlying plant resilience to water deficits: prospects for water-saving agriculture. *J. Exp. Bot.* 55, 2365–2384. doi: 10.1093/jxb/erh269

D'Amato, R., Proietti, P., Nasini, L., Del Buono, D., Tedeschini, E., and Businelli, D. (2014). Increase in the selenium content of extra virgin olive oil: quantitative and qualitative implications. *Grasas Aceites* 65:e025. doi: 10.3989/gya.097313

D'Amato, R., Proietti, P., Onofri, A., Regni, L., Esposto, S., Servili, M., et al. (2017). Biofortification (Se): Does it increase the content of phenolic compounds in virgin olive oil (VOO)? *PLoS One* 12: e0176580. doi: 10.1371/journal.pone.0176580

Djanaguiraman, M., Devi, D. D., Shanker, A. K., Sheeba, J. A., and Bangarusamy, U. (2005). Selenium - An antioxidative protectant in soybean during senescence. *Plant Soil* 272, 77–86. doi: 10.1007/s11104-004-4039-1

Ducsay, L., and Ložek, O. (2006). Effect of selenium foliar application on its content in winter wheat grain. *Plant Soil Environ.* 52, 78–82. doi: 10.17221/3349-PSE

Fargašová, A., Pastierová, J., and Svetková, K. (2006). Effect of Se-metal pair combinations (Cd, Zn, Cu, Pb) on photosynthetic pigments production and metal accumulation in *Sinapis alba* L. seedlings. *Plant Soil Environ.* 52, 8–15. doi: 10.17221/3340-PSE

Feng, R., Wei, C., and Tu, S. (2013). The roles of selenium in protecting plants against abiotic stresses. *Environ. Exp. Bot.* 87, 58–68. doi: 10.1016/j.envexpbot.2012.09.002

Feng, T., Chen, S. S., Gao, D. Q., Liu, G. Q., Bai, H. X., Li, A., et al. (2015). Selenium improves photosynthesis and protects photosystem II in pear (*Pyrus bretschneideri*), grape (*Vitis vinifera*), and peach (*Prunus persica*). *Photosynthetica* 53, 609–612. doi: 10.1007/s11099-015-0118-1

Finley, J. W. (2007). Increased intakes of selenium-enriched foods may benefit human health. *J. Sci. Food Agric.* 87, 1620–1629. doi: 10.1002/jsfa.2943

Fontanella, M. C., D'Amato, R., Regni, L., Proietti, P., Beone, G. M., and Businelli, D. (2017). Selenium speciation profiles in biofortified sangiovese wine. *J. Trace Elem. Med. Biol.* 43, 87–92. doi: 10.1016/J.JTEMB.2016.11.015

Gómez-del-Campo, M. (2013). Summer deficit irrigation in a hedgerow olive orchard cv. Arbequina: relationship between soil and tree water status, and growth and yield components. *Spanish J. Agric. Res.* 11, 547–557. doi: 10.5424/sjar/2013112-3360

Gómez-Rico, A., Salvador, M. D., Moriana, A., Pérez, D., Olmedilla, N., Ribas, F., et al. (2007). Influence of different irrigation strategies in a traditional *Cornicabra* cv. olive orchard on virgin olive oil composition and quality. *Food Chem.* 100, 568–578. doi: 10.1016/j.foodchem.2005.09.075

Gouveia, J. B., Vasconcelos, P., Ferreira, T. C., and Villalobos, F. J. (2013). Efecto de diferentes regímenes de riego sobre los índices de calidad del aceite de oliva de cv. "*Cobrançosa*"?; effect of different irrigation regimes on the quality attributes of monovarietal virgin olive oil from cv. "*Cobrançosa*". *Grasas Aceites* 64, 41–49. doi: 10.3989/gya.070512

Hasanuzzam, M., Hossain, M. A., and Fujita, M. (2010). Selenium in higher plants: physiological role, antioxidant metabolism and abiotic stress tolerance. *J. Plant Sci.* 5, 354–375. doi: 10.3923/jps.2010.354.375

Hashem, H. A., Hassanein, R. A., Bekheta, M. A., and El-Kady, F. A. (2013). Protective role of selenium in canola (*Brassica napus* L.) plant subjected to salt stress. *Egypt. J. Exp. Biol.* 9, 199–211.

Iwaoka, M., Ooka, R., Nakazato, T., Yoshida, S., and Oishi, S. (2008). Synthesis of selenocysteine and selenomethionine derivatives from sulfur-containing amino acids. *Chem. Biodivers.* 5, 359–374. doi: 10.1002/cbdv.200890037

Jaleel, C. A., Manivannan, P., Wahid, A., Farooq, M., Hameed, J. A.-J., and Ramamurthy, S. (2009). Drought stress in plants?: a review on morphological characteristics and pigments composition. *Int. J. Agric.* 11, 100–105.

Jose Motilva, M., Jess Tovar, M., Paz Romero, M., Alegre, S., and Girona, J. (2000). Influence of regulated deficit irrigation strategies applied to olive trees (*Arbequina* cultivar) on oil yield and oil composition during the fruit ripening period. *J. Sci. Food Agric.* 80, 2037–2043. doi: 10.1002/1097-0010(200011)80:14<2037::AID-JSFA733>3.0.CO;2-0

Kápolna, E., Hillestrøm, P. R., Laursen, K. H., Husted, S., and Larsen, E. H. (2009). Effect of foliar application of selenium on its uptake and speciation in carrot. *Food Chem.* 115, 1357–1363. doi: 10.1016/j.foodchem.2009.01.054

Kaushik, D., and Roychoudhury, A. (2014). Reactive oxygen species (ROS) and response of antioxidants as ROS-scavengers during environmental stress in plants. *Front. Environ. Sci.* 2:53. doi: 10.3389/fenvs.2014.00053

Li, H.-F., McGrath, S. P., and Zhao, F.-J. (2008). Selenium uptake, translocation and speciation in wheat supplied with selenate or selenite. *New Phytol.* 178, 92–102. doi: 10.1111/j.1469-8137.2007.02343.x

Lichtenthaler, H. K., and Wellburn, A. R. (1983). Determinations of total carotenoids and chlorophylls a and b of leaf extracts in different solvents. *Biochem. Soc. Trans.* 11, 591–592. doi: 10.1042/bst0110591

Malik, J. A., Goel, S., Kaur, N., Sharma, S., Singh, I., and Nayyar, H. (2012). Selenium antagonises the toxic effects of arsenic on mungbean (*Phaseolus aureus* Roxb.) plants by restricting its uptake and enhancing the antioxidative and detoxification mechanisms. *Environ. Exp. Bot.* 77, 242–248. doi: 10.1016/J.ENVEXPBOT.2011.12.001

Martín-Hernández, C., Bénet, S., and Obert, L. (2008). Determination of proteins in refined and nonrefined oils. *J. Agric. Food Chem.* 56, 4348–4351. doi: 10.1021/jf7036888

Munné-Bosch, S., and Alegre, L. (2000). Changes in carotenoids, tocopherols and diterpenes during drought and recovery, and the biological significance of chlorophyll loss in *Rosmarinus officinalis* plants. *Planta* 210, 925–931. doi: 10.1007/s004250050699

Nasini, L., Gigliotti, G., Balduccini, M. A., Federici, E., Cenci, G., and Proietti, P. (2013). Effect of solid olive-mill waste amendment on soil fertility and olive (*Olea europaea* L.) tree activity. *Agric. Ecosyst. Environ.* 164, 292–297. doi: 10.1016/j.agee.2012.10.006

Nasini, L., and Proietti, P. (2014). "Olive harvesting," in *The Extra-Virgin Olive Oil Handbook*, ed. C. Peri (Chichester: John Wiley & Sons), 87–105. doi: 10.1002/9781118460412.ch8

Navarro-Alarcon, M., and Cabrera-Vique, C. (2008). Selenium in food and the human body: a review. *Sci. Total Environ.* 400, 115–141. doi: 10.1016/j.scitotenv.2008.06.024

Navarro-Alarcón, M., and López-Martínez, M. C. (2000). Essentiality of selenium in the human body: relationship with different diseases. *Sci. Total Environ.* 249, 347–371. doi: 10.1016/S0048-9697(99)00526-4

Nawaz, F., Ahmad, R., Ashraf, M. Y., Waraich, E. A., and Khan, S. Z. (2015). Effect of selenium foliar spray on physiological and biochemical processes and chemical constituents of wheat under drought stress. *Ecotoxicol. Environ. Saf.* 113, 191–200. doi: 10.1016/j.ecoenv.2014.12.003

Nawaz, F., Tahir, M. N., Ashraf, M. Y., Naeem, M., Zulfiqar, B., Salahuddin, M., et al. (2016). Selenium supplementation affects physiological and biochemical processes to improve fodder yield and quality of maize (*Zea mays* L.) under water deficit conditions. *Front. Plant Sci.* 7:1438. doi: 10.3389/FPLS.2016.01438

Pennanen, A., Xue, T., and Hartikainen, H. (2002). Protective role of selenium in plant subjected to severe UV irradiation stress. *J. Appl. Bot.* 76, 66–76.

Pezzarossa, B., Remorini, D., Gentile, M. L., and Massai, R. (2012). Effects of foliar and fruit addition of sodium selenate on selenium accumulation and fruit quality. *J. Sci. Food Agric.* 92, 781–786. doi: 10.1002/jsfa.4644

Proietti, P., Nasini, L., Del Buono, D., D'Amato, R., Tedeschini, E., and Businelli, D. (2013). Selenium protects olive (*Olea europaea* L.) from drought stress. *Sci. Hortic.* 164, 165–171. doi: 10.1016/j.scienta.2013.09.034

R Development Core Team (2011). *R: A Language and Environment for Statistical Computing*. Available at: http://www.R-project.org [accessed May 8, 2018]

Ramakrishna, A., and Ravishankar, G. A. (2011). Influence of abiotic stress signals on secondary metabolites in plants. *Plant Signal. Behav.* 6, 1720–1731. doi: 10.4161/psb.6.11.17613

Rayman, M. P. (2000). The importance of selenium to human health. *Lancet* 356, 233–241. doi: 10.1016/S0140-6736(00)02490-9

Reddy, A. R., Chaitanya, K. V., and Vivekanandan, M. (2004). Drought-induced responses of photosynthesis and antioxidant metabolism in higher plants. *J. Plant Physiol.* 161, 1189–1202. doi: 10.1016/J.JPLPH.2004.01.013

Reiz, B., and Li, L. (2010). Microwave-assisted acid and base hydrolysis of intact proteins containing disulfide bonds for protein sequence analysis by mass spectrometry. *J. Am. Soc. Mass Spectrom.* 21, 1596–1605. doi: 10.1016/j.jasms.2010.04.012

Roman, M., Jitaru, P., and Barbante, C. (2014). Selenium biochemistry and its role for human health. *Metallomics* 6, 25–54. doi: 10.1039/C3MT00185G

Sager, M. (2006). Selenium in agriculture, food, and nutrition. *Pure Appl. Chem.* 78, 111–133. doi: 10.1351/pac200678010111

Schrauzer, G. N. (2000). Selenomethionine: a review of its nutritional significance, metabolism and toxicity. *J. Nutr.* 130, 1653–1656. doi: 10.1093/jn/130.7.1653

Servili, M., Esposto, S., Lodolini, E., Selvaggini, R., Taticchi, A., Urbani, S., et al. (2007). Irrigation effects on quality, phenolic composition, and selected volatiles of Virgin Olive Oils Cv. Leccino. *J. Agric. Food Chem.* 55, 6609–6618. doi: 10.1021/jf070599n

Singleton, V. L., Orthofer, R., and Lamuela-Raventós, R. M. (1998). Analysis of total phenols and other oxidation substrates and antioxidants by means of folin-ciocalteu reagent. *Methods Enzymol.* 299, 152–178. doi: 10.1016/S0076-6879(99)99017-1

Soil Survey Staff (2014). *Keys to Soil Taxonomy by Soil Survey Staff*, 12th Edn. Available at: http://www.nrcs.usda.gov/Internet/FSE_DOCUMENTS/nrcs142p2_051546.pdf

Sors, T. G., Ellis, D. R., Gun, N. N., Lahner, B., Lee, S., Leustek, T., et al. (2005). Analysis of sulfur and selenium assimilation in Astragalus plants with varying capacities to accumulate selenium. *Plant J.* 42, 785–797. doi: 10.1111/j.1365-313X.2005.02413.x

Spadoni, M., Voltaggio, M., Carcea, M., Coni, E., Raggi, A., and Cubadda, F. (2007). Bioaccessible selenium in Italian agricultural soils: Comparison of the biogeochemical approach with a regression model based on geochemical and pedoclimatic variables. *Sci. Total Environ.* 376, 160–177. doi: 10.1016/j.scitotenv.2007.01.066

Tapiero, H., Townsend, D. M., and Tew, K. D. (2004). The role of carotenoids in the prevention of human pathologies. *Biomed. Pharmacother.* 58, 100–110. doi: 10.1016/j.biopha.2003.12.006

Torres, S., Cerutti, S., Raba, J., Pacheco, P., and Silva, M. F. (2014). Preconcentration of seleno-amino acids on a XAD resin and determination in regional olive oils by SPE UPLC-ESI-MS/MS. *Food Chem.* 159, 407–413. doi: 10.1016/j.foodchem.2014.03.045

Tovar, M. J., Romero, M. P., Alegre, S., Girona, J., and Motilva, M. J. (2002). Composition and organoleptic characteristics of oil from *Arbequina* olive (*Olea europaea* L) trees under deficit irrigation. *J. Sci. Food Agric.* 82, 1755–1763. doi: 10.1002/jsfa.1246

Uceda. (2008). "Maduración," *El Cultivo del Olivo*, 6a Edn, eds D. Barranco, R. Fernández-Escobar, and L. Rallo (New York NY: Ediciones Mundi-Prensa).

Vacca, V., Del Caro, A., Poiana, M., and Piga, A. (2006). Effect of storage period and exposure conditions on the quality of Bosana extra-virgin olive oil. *J. Food Qual.* 29, 139–150. doi: 10.1111/j.1745-4557.2006.00062.x

Zalejska-Fiolka, J. (2000). Antioxidative properties of alpha-tocopherol, methionine and selenomethionine in olive oil. *Riv. Ital. Sostanze Grasse* 77, 543–547.

Zamora, R., Alaiz, M., and Hidalgo, F. J. (2001). Influence of cultivar and fruit ripening on olive (*Olea europaea*) fruit protein content, composition, and antioxidant activity. *J. Agric. Food Chem.* 49, 4267–4270. doi: 10.1021/jf0104634

Conflict of Interest Statement: The authors declare that the research was conducted in the absence of any commercial or financial relationships that could be construed as a potential conflict of interest.

frontiers in Plant Science

ORIGINAL RESEARCH
published: 22 August 2018
doi: 10.3389/fpls.2018.01233

Protein Hydrolysate Stimulates Growth in Tomato Coupled With N-Dependent Gene Expression Involved in N Assimilation

*Francesco Sestili[1], Youssef Rouphael[2], Mariateresa Cardarelli[3], Anna Pucci[1], Paolo Bonini[4], Renaud Canaguier[5] and Giuseppe Colla[1]**

[1] Department of Agricultural and Forestry Sciences, University of Tuscia, Viterbo, Italy, [2] Department of Agricultural Sciences, University of Naples Federico II, Portici, Italy, [3] Consiglio per la Ricerca in Agricoltura e l'Analisi dell'Economia Agraria, Centro di Ricerca Orticoltura e Florovivaismo, Pontecagnano, Italy, [4] NGA Laboratory, Tarragona, Spain, [5] Nixe, Valbonne, France

OPEN ACCESS

Edited by:
Angeles Calatayud, Instituto Valenciano de Investigaciones Agrarias, Spain

Reviewed by:
Mamoru Okamoto, The University of Adelaide, Australia
Andrea Ertani, Università degli Studi di Padova, Italy
Sheng Shu, Nanjing Agricultural University, China

***Correspondence:**
Giuseppe Colla
giucolla@unitus.it

Specialty section:
This article was submitted to Crop and Product Physiology, a section of the journal Frontiers in Plant Science

Received: *14 February 2018*
Accepted: *06 August 2018*
Published: *22 August 2018*

Citation:
Sestili F, Rouphael Y, Cardarelli M, Pucci A, Bonini P, Canaguier R and Colla G (2018) Protein Hydrolysate Stimulates Growth in Tomato Coupled With N-Dependent Gene Expression Involved in N Assimilation. Front. Plant Sci. 9:1233. doi: 10.3389/fpls.2018.01233

Plant-derived protein hydrolysates (PHs) have received increased attention in the last decade because of their potential to improve yield, nutritional quality as well as tolerance to abiotic stressors. The current study investigated the effects and the molecular mechanisms of a legume-derived PH under optimal and sub-optimal nitrogen (N) concentrations (112 and 7 mg L^{-1}, respectively) in tomato (*Solanum lycopersicum* L.). Growth and mineral composition of tomato plants treated with PHs by foliar spray or substrate drench were compared to untreated plants. In addition, the expression was determined of genes encoding ammonium and nitrate transporters and seven enzymes involved in N metabolism: nitrate reductase (*NR*), nitrite reductase (*NiR*), glutamine synthetase 1 (*GS1*), glutamine synthetase 2 (*GS2*), ferredoxin-dependent glutamate synthase (*GLT*), NADH-dependent glutamate synthase (*GLS*), and glutamate dehydrogenase (*GDH*). The root and total plant dry weight, SPAD index and leaf nitrogen content were higher by 21, 17, 7, and 6%, respectively, in plants treated by a substrate drench in comparison to untreated tomato plants, whereas foliar application of PH gave intermediate values. PH-treated plants grown with lower N availability showed reduced expression of *NR* and *NiR* as well as of nitrate and ammonium transporter transcripts in both leaf and root tissues in comparison with untreated plants; this was especially pronounced after application of PH by substrate drench. Conversely, the transcript level of an amino acid transporter gene was up-regulated in comparison with untreated plants. At high N regime, the transcript levels of the ammonium and amino acid transporters and also *NR*, *NiR*, and *GLT* were significantly up-regulated in root after PH foliar and substrate drench applications compared with untreated plants. An up-regulation was also observed for *GS1*, *GS2*, and *GDH* transcripts in leaf after substrate drench. These results highlighted the potential benefits of using legume PH in vegetable production systems to increase growth and N-nutritional status of plants.

Keywords: ammonium and nitrate transporters, biostimulants, N metabolism, amino acids, peptides, substrate drench application, *Solanum lycopersicum* L.

INTRODUCTION

Nitrate (NO_3^-) constitutes the most important and available form of nitrogen (N) taken up readily in large quantities by vegetable crops to secure maximal productivity (Colla et al., 2010, 2011). However, the high production cost of N fertilizers as well as its high mobility and facility to leach into groundwater made N use a major environmental threat throughout the world (Tilman et al., 2002).

Any improvement in crop management practices that increases N capture efficiency should reduce the environmental pollution without affecting the reliability and stability of agricultural crop yield (Jannin et al., 2013; Santi et al., 2017). Many attempts have been proposed to enhance N use efficiency in vegetables by means of traditional breeding programs and genetic engineering; however, the commercial success of these cultivars has been very limited (Colla et al., 2010, 2011). More recently, the use of plant biostimulants which include beneficial microorganisms (i.e., mycorrhizal fungi and plant growth promoting rhizobacteria) as well as natural substances or compounds (i.e., humic acids, seaweed extracts, and protein hydrolysates) has been introduced as an efficient, safe and environmentally friendly approach to ensure high yield and improve the quality in a sustainable manner (i.e., by enhancing nutrient use efficiency) (Colla and Rouphael, 2015; du Jardin, 2015; Rouphael et al., 2015, 2017c; Colla et al., 2017b).

Plant-derived protein hydrolysates (PHs) have gained prominence globally as natural plant biostimulants in vegetable cropping systems (Ertani et al., 2014, 2015; Colla et al., 2015). Plant-derived PHs are mainly produced by enzymatic hydrolysis of plant biomass such as legume seeds, alfalfa hay, and plant by-products (Ertani et al., 2013; Colla et al., 2015, 2017b). Particularly, PHs coming from agricultural organic waste are gaining interest among the biostimulant enterprises and scientists, since they could be considered an efficient solution to the problem of plant by-product disposal, turning them into economic benefits for the growers (Pecha et al., 2012; Santi et al., 2017). Plant-derived PHs are a source of free amino acids and soluble peptides, and can also contain carbohydrates, phenols and limited amounts of plant nutrients (Calvo et al., 2014; Colla et al., 2015). Foliar or root applications of plant-derived PHs may activate several molecular and physiological mechanisms, in a wide range of horticultural commodities, that stimulate seedling and plant growth (Colla et al., 2014; Amirkhani et al., 2016), improve yield and nutritional quality (Colla et al., 2017a; Rouphael et al., 2017b) and mitigate the impact of a wide range of abiotic stresses such as salinity (Lucini et al., 2015), alkalinity (Rouphael et al., 2017a), and thermal stress (Botta, 2013). Recent review papers (Calvo et al., 2014; Halpern et al., 2015; Colla et al., 2015, 2017b) aiming to elucidate the mechanisms regulating these positive effects indicate that these products could affect crops by stimulating N metabolism through the regulation of key enzymes involved in N assimilation, and interfering with hormone-like activity (Schiavon et al., 2008; Ertani et al., 2009; Colla et al., 2014). PHs have been also shown to modulate the crop root system architecture (in particular the number of lateral roots), thus affecting the efficiency and uptake with which PH-treated plants explore the soil and capture nutrients (Ertani et al., 2013; Colla et al., 2015, 2017b; Nardi et al., 2016).

Inorganic N is absorbed by the roots of higher plants and it is rapidly turned into ammonium through the coordinated action of two key enzymes (Nitrate Reductase-NR and Nitrite Reductase-NiR). The first enzyme is the limiting factor of nitrate assimilation as it reduces nitrate in nitrite that is the substrate for the next reaction catalyzed by NiR that leads to the production of ammonium. This latter is then incorporated into glutamine by glutamine synthetase (GS). This step is a crucial checkpoint of plant growth, as it allows the first incorporation of the mineral nitrogen (Hirel et al., 2007). Two GS isozymes (cytosolic GS1, and plastidic GS2) have been identified in higher plants (Bernard and Habash, 2009). Their different organ- and cell-specific expression suggests a distinct function. GS1 is located usually in the cytosol of vascular tissues, involved in N recycling, and plays also a role in N mobilization in germinating kernels (Zhang et al., 2017). GS2 is mainly expressed in leaf mesophyll and re-assimilates the ammonium released during the process of photorespiration (amino acid turnover) or nitrate reduction (Husted et al., 2002).

The enzyme glutamate synthases (GOGATs) catalyze the conversion of glutamine and 2-oxoglutarate to glutamic acid that is nitrogen donor to other amino acids in subsequent transamination reactions (Bernard and Habash, 2009). Two forms of GOGAT, ferredoxin- (Fd-GOGAT or GLT), and NADH-dependent (NADH-GOGAT or GLS), have been identified in higher plants. The first is the predominant enzyme for glutamate synthesis in photosynthetic tissues; GLS is the major enzyme in non-photosynthetic tissues (Lancien et al., 2007). Glutamate dehydrogenase (GDH) is an important branch-point enzyme between carbon and nitrogen metabolism, because it catalyzes the reversible oxidative deamination of glutamate to 2-oxoglutarate and ammonia (Tercé-Laforgue et al., 2013).

Although the stimulation of NO_3^- assimilation enzymes (NR, NiR, GS, and GOCAT) in both leaf and root tissues of maize seedlings after the application of an alfalfa-PH has been documented (Schiavon et al., 2008); however, the molecular mechanism(s) that may elucidate the mode of action of commercial legume-derived PHs under sub-optimal N conditions remain unknown.

It is well established that root systems respond to N limitation in the soil solution by two important adaptive responses (i) up-regulation of the high-affinity transport system (HATS) for NO_3^- ($<$0.5 mM of external nitrate) and (ii) stimulation of lateral root growth (Remans et al., 2006). In their work, Remans et al. (2006) demonstrated that high-affinity nitrate transporter 2.1 (NRT2.1) plays a key role in the coordination of the root development, acting on lateral root initiation under low nitrate regime; whereas high-affinity nitrate transporter 2.3 (NRT2.3) was involved in the root-to-shoot long distance transport and nitrate uptake (Fu et al., 2015). Because PHs contain amino acids and peptides, the expression of a key gene encoding the amino acid transporter AAT1 (previously named Solyc11g0084401.1; Snowden et al., 2015) could provide further insight into the effects of PHs on amino acid turnover and allocation.

Based on these considerations, the aim of the current study was to assess the morphological, compositional and molecular

changes in tomato plants grown under optimal and sub-optimal N conditions in response to PH application (foliar spray or substrate drench) in order to unravel the molecular mechanisms that may elucidate its mode of action.

MATERIALS AND METHODS

Plant Material and Growth Conditions

The experiment was conducted in the 2015 summer growing season in a polyethylene greenhouse at the experimental farm of Tuscia University (latitude 42° 25′ N, longitude 12° 08′, altitude 310 m). The tomato (*Solanum lycopersicum* L. cv. Console F1, Società Agricola Italiana Sementi-SAIS, Cesena, Italy) seedlings were transplanted on June 8 at the four true leaf stage into plastic pots (diameter 14 cm and height 12 cm) containing 1.5 L of quarziferous sand with a particle size between 0.4 to 0.8 mm. Plastic pots were arranged in single rows on 16 cm wide and 5 m-long troughs at a plant density of 11 plants m^{-2} (30 cm between pots and 30 cm between troughs). The daily air temperature inside the greenhouse was maintained between 18 and 30°C by forced ventilation and day/night air relative humidity was 55/85%.

Treatments, Experimental Design, and Nutrient Solution Management

Six treatments were compared, which derived by the factorial combination of two N levels in the nutrient solution (low, 7 mg L^{-1}; high, 112 mg L^{-1}) and three biostimulant application treatments (untreated, foliar spray, or substrate drench). The treatments were arranged in a randomized complete-block design with three replications per treatment, amounting to a total of 18 experimental plots with 15 plants each.

The commercial legume-derived protein hydrolysate Trainer® (Italpollina S.p.A., Rivoli Veronese, Italy) was used in the current greenhouse experiment. Trainer® is a commercial biostimulant obtained through enzymatic hydrolysis of proteins from legume seeds; it contains 50 g kg^{-1} of N as free amino acids, and soluble peptides (Colla et al., 2017a; Rouphael et al., 2017b). The aminogram of the product was (g kg^{-1}): Ala (12), Arg (18), Asp (34), Cys (3), Glu (54), Gly (12), His (8), Ile (13), Leu (22), Lys (18), Met (4), Phe (15), Pro (15), Thr (11), Trp (3), Tyr (11), and Val (14) (Rouphael et al., 2018).

The commercial biostimulant Trainer® was applied in both foliar spray and substrate drench treatments at a concentration of 2.5 ml L^{-1}. The Trainer® concentration was adopted based on the company recommendations. The PH-treated plants were uniformly sprayed (foliar spray treatment) or applied at a rate of 30 ml per plant (substrate drench treatment) two times during the experiment on 16 and 23 June (9 and 16 days after transplanting, respectively). A 5-L stainless style sprayer "Vibi Sprayer" (Volpi, Piadena, Italy) was used in the foliar spray treatment. In both application dates, the PH treatments (foliar spray and substrate drench) were performed at 10:00 with an average air temperature inside the greenhouse of 24°C and relative humidity of 65%.

Nutrient solution was applied through the drip irrigation system and delivered at a rate of 2 L min^{-1}. The composition of the basic nutrient solution used in the current study was: 32 mg L^{-1} S, 31 mg L^{-1} P, 117 mg L^{-1} K, 24 mg L^{-1} Mg, 1.12 mg L^{-1} Fe, 0.5 mg L^{-1} Mn, 19.0 μg L^{-1} Cu, 104.6 μg L^{-1} Zn, 216.0 μg L^{-1} B, and 28.8 μg L^{-1} Mo. The two N levels in the nutrient solution were obtained by adding calcium ammonium nitrate (14.2% nitrate and 1.3% ammonium) to the basic nutrient solution at 22.6 mg L^{-1} (7 mg L^{-1} N) or 720.0 mg L^{-1} (112 mg L^{-1} N). Moreover, in the low nitrogen solution, calcium chloride ($CaCl_2$) was added at 831 mg L^{-1} to balance the calcium concentration (160 mg L^{-1}) in both nutrient solutions.

Biomass Production, Partitioning, and SPAD Index

On June 26 (19 days after transplanting; 72 h after the second biostimulant application), five plants per experimental unit were sampled and separated in leaves, stems and roots. All plant tissues were dried at 60°C for 72 h until they reach a constant weight to determine dry biomass production and partitioning. The number of leaves per plant was also counted.

On the same date, the soil plant analysis development (SPAD) index was measured on fully expanded leaves by means of a portable chlorophyll meter SPAD-502 (Konica Minolta, Japan). Ten healthy and fully expanded leaves were randomly measured and averaged to a single SPAD value for each experimental plot.

Nitrogen Analysis

The dried leaf tissues, sampled from the first fully expanded leaves at 48 and 72 h after the second biostimulant application (18 and 19 days after transplanting) were ground in a Wiley Mill to pass through a 841 μm screen; then 1 g of dried leaf samples were analyzed for total nitrogen, nitrate, and ammonium.

Nitrogen (total N) concentration was assessed after mineralization with sulfuric acid (96%, Carlo Erba Reagents, Milan, Italy) in the presence of potassium sulfate and a low concentration of copper by the Kjeldahl method (Bremner, 1965).

Mineral N in the form of nitrate ($N\text{-}NO_3$) and ammonium ($N\text{-}NH_4$) was determined spectrophotometrically (Helios Beta Spectrophotometer, Thermo Electron Corporation, United Kingdom) using the salicylic-sulfuric acids and the salicylate-hypochlorite methods, respectively (Cataldo et al., 1975; Anderson and Ingram, 1989).

Collection of Samples, RNA Extraction, and Purification

Two terminal leaflets were sampled from the first fully expanded leaves as well as fine roots of two plants per experimental plot at 6 h after the second biostimulant application, and immediately frozen in liquid nitrogen and stored at −80°C for molecular analysis. Samples of fresh leaves and roots were frozen and then grinded in liquid nitrogen.

Total RNA was isolated from homogenized leaf and root tissues according to the manufacturer's instructions of the Spectrum Total Plant RNA Kit (Sigma-Aldrich, St. Louis, MO, United States) and re-suspended in 50 μl of DEPC-treated water. RNA concentration and quality were evaluated using a

Multiskan GO Microplate Spectrophotometer (Thermo Fisher Scientific, Madison, WI, United States) and by agarose gel electrophoresis.

Quantitative Real-Time PCR (qRT-PCR)

One microgram of the extracted RNA was used as template for the synthesis of cDNA, following the protocol of the QuantiTect Reverse Transcription Kit (Qiagen, Hilden, Germany). qRT-PCR was performed in a CFX 96 Real-Time PCR Detection System device (Bio-Rad, Hercules, CA, United States); each reaction was carried out in a volume of 15 μl, containing 7.5 μl of SsoAdvanced™ SYBR® Green supermix (Bio-Rad, Hercules, CA, United States), 1 μl of cDNA and 0.5 mM of each primer. qRT-PCR conditions were: an initial denaturation at 94°C for 30 s, followed by 40 cycles at 94°C for 5 s, 60°C for 30 s and melt curve analysis ranging from 65 to 95°C with 0.5°C per 5 s increments. Relative levels of transcript abundance were estimated as described in Sestili et al. (2010). Three biological samples per treatment were analyzed with three technical replicates per sample; each qRT-PCR data point represented the mean of three biological samples. A list of all genes analyzed throughout this study along with the corresponding primer pairs is provided in **Supplementary Table S1**.

Statistical Analysis of Data

Analysis of variance of the experimental data set was assessed using SPSS 13 for Windows, 2001 (SPSS Inc., United States). To separate treatment means within each measured parameter, Duncan's multiple-range test was performed at $P \leq 0.05$.

RESULTS AND DISCUSSION

Growth Responses and Nitrogen Concentration in Tomato Plants

In the current study, SPAD index (i.e., greenness readings), the dry weight of stems, root and total dry weight were influenced by N level in the nutrient solution and biostimulant treatments with no significant N level × biostimulant interaction, whereas the leaf number per plant and leaf dry weight were only affected by N regime (**Table 1**). When averaged over biostimulant application, a significant difference between the two N concentrations in the nutrient solution was recorded, with the highest values of leaf number, SPAD index, and total dry weight recorded at high N level (**Table 1**). Concerning the influence of the commercial legume-derived PH application on growth responses, the root and total dry weight as well as SPAD index were higher by 21, 17, and 7%, respectively, in substrate drench treatment in comparison to untreated tomato plants with no significant difference between the two modes of application (foliar spray and substrate drench; **Table 1**).

A presumed mode of action behind the stimulation of biomass production in response to substrate drench application of PH could involve the increased presence of *signaling molecules* such as small peptides which are typical compounds of PHs. The former elicitors in the commercial legume-derived PH which are easily perceived by both plant tissues (leaf and root) (Matsumiya and Kubo, 2011) may have generated a signal transduction pathway through modulation of endogenous phytohormone biosynthesis (Ryan et al., 2002; Cavani and Ciavatta, 2007; Ertani et al., 2017). Our results are consistent with the findings of

TABLE 1 | Effect of nitrogen level in the nutrient solution and biostimulant mode of application on leaf number, soil plant analysis development (SPAD) index, dry weight of leaves, stems, roots, and total biomass of tomato plants at 19 days after transplanting.

Source of variance	Leaf number (no. plant^{-1})	SPAD index	Dry biomass (g plant^{-1})			
			Leaves	Stems	Root	Total
Nitrogen level	***	***	***	***	***	***
Biostimulant	*ns*	**	ns	***	**	**
Nitrogen level × Biostimulant	ns	ns	ns	ns	ns	ns
Nitrogen level (mg L^{-1})						
7	10.4 ± 0.3 b	46.0 ± 0.5 b	4.94 ± 0.20 b	3.58 ± 0.12 b	1.22 ± 0.06 b	9.74 ± 0.31 b
112	11.9 ± 0.3 a	56.0 ± 0.9 a	7.76 ± 0.27 a	6.36 ± 0.19 a	2.04 ± 0.06 a	16.15 ± 0.47 a
Biostimulant						
No application	11.0 ± 0.3	49.5 ± 1.3 b	6.02 ± 0.51	4.52 ± 0.40 b	1.49 ± 0.15 b	12.01 ± 1.02 b
Foliar spray	11.3 ± 0.5	50.7 ± 1.9 ab	6.33 ± 0.49	4.89 ± 0.46 ab	1.60 ± 0.13 ab	12.82 ± 1.03 ab
Substrate drench	11.3 ± 0.5	52.8 ± 1.8 a	6.71 ± 0.54	5.50 ± 0.48 a	1.80 ± 0.13 a	14.00 ± 1.12 a
Nitrogen level × Biostimulant						
7 mg L^{-1} N without biostimulant	10.2 ± 0.3	45.7 ± 0.7	4.82 ± 0.20	3.07 ± 0.12	1.09 ± 0.07	9.10 ± 0.73
7 mg L^{-1} N with foliar spray	10.8 ± 0.5	44.6 ± 0.5	4.94 ± 0.60	3.70 ± 0.14	1.16 ± 0.11	9.67 ± 0.35
7 mg L^{-1} N with substrate drench	10.3 ± 0.7	47.7 ± 0.9	5.06 ± 0.14	3.97 ± 0.18	1.41 ± 0.09	10.44 ± 0.34
112 mg L^{-1} N without biostimulant	11.8 ± 0.4	53.3 ± 1.3	7.09 ± 0.44	5.97 ± 0.34	1.89 ± 0.12	14.92 ± 0.72
112 mg L^{-1} N with foliar spray	11.8 ± 0.3	56.8 ± 1.0	7.83 ± 0.49	6.09 ± 0.28	2.04 ± 0.09	15.96 ± 0.88
112 mg L^{-1} N with substrate drench	12.2 ± 0.7	57.9 ± 1.8	8.35 ± 0.40	7.03 ± 0.20	2.19 ± 0.08	17.57 ± 0.58

*ns, **,***: non-significant or significant at $P \leq 0.01$, and $P \leq 0.001$, respectively. Different letters within each column indicate significant differences according to Duncan's multiple-range test (P = 0.05). All data are expressed as mean ± standard error, n = 3.*

several research groups (Ertani et al., 2009; Matsumiya and Kubo, 2011; Colla et al., 2014; Ugolini et al., 2015), who observed that foliar spray or substrate drench applications of plant-derived PHs exhibited auxin- and/or gibberellin-like activities as demonstrated by laboratory bioassays (Ertani et al., 2009; Colla et al., 2014), thus stimulating plant growth and yield.

Another putative mechanism behind the biostimulant activity of legume-derived PH on crop performance is the stimulation of the root system architecture in particular the increase in root hair length and density (Matsumiya and Kubo, 2011), which may improve N use efficiency, leading to an increase in total biomass when N is limiting plant growth. These findings are in line with previous studies testing the stimulation action of plant-derived PHs on root and shoot biomass (Ertani et al., 2009; Colla et al., 2014). For instance, Ertani et al. (2009) demonstrated that short-term application (48 h) of PHs derived from enzymatic hydrolysis of alfalfa plants (applied at 0.01, 0.1, or 1 mL L^{-1}) elicited dose-dependent increase of root dry mass (from 20 to 42%) in corn compared to the untreated control. These results were also consistent with those of Colla et al. (2014) who reported that treating tomato cuttings with 6 ml L^{-1} of the legume-derived PH increased root density and length in comparison to untreated plants, inducing a "*nutrient acquisition response*" that favors N uptake and translocation.

Total N in leaf tissue was influenced by N level in the nutrient solution and biostimulant treatments with no significant N level × biostimulant interaction, whereas the mineral N in the form of nitrate ($N\text{-}NO_3$, at 48 and 72 h after the second biostimulant application) and ammonium ($N\text{-}NH_4$, at 48 h after the second biostimulant application) incurred significant N level × biostimulant interaction (**Table 2**). A significant correlation ($p < 0.01$) was also observed between SPAD index and total leaf N content (Pearson's coefficient 0.961). No significant differences among biostimulant applications were observed for leaf nitrate content under low N regime. However, under high N level the highest nitrate concentration was observed with substrate drench (48 h) and with both foliar and root application (72 h) (**Table 2**). The positive effect of amino acids on the uptake and assimilation of nitrates under high N regime (70–140 g L^{-1}) was previously described in other vegetable crops (radish and pepper) grown hydroponically (Liu and Lee, 2012). Our results also showed that the highest concentration of ammonium (at 48 h) was recorded with foliar spray application under high N regime (**Table 2**). Similarly to the effects on biomass production and partitioning, the total N as well as the nitrate and ammonium concentrations at high N level were significantly higher than those obtained from tomato plants grown at low N regime (**Table 2**). A different behavior was observed under low N regime with an increase of total N concentration after biostimulant application without any significant effect on nitrate concentration (**Table 2**). Because the only sources of nitrogen for plant uptake were the mineral fertilizer and the biostimulant, we hypothesized that the increase of the total N concentration in leaves of plants grown under low N fertilization regime may be due to the plant uptake of organic N (amino acids and peptides) coming from the biostimulant.

Irrespective of the N level in the nutrient solution, our results showed that substrate drench application of legume-derived

TABLE 2 | Effect of nitrogen level in the nutrient solution and biostimulant mode of application on nitrate, ammonium, and total nitrogen of leaves in tomato plants.

Source of variance	$N\text{-}NO_3$ (mg·kg^{-1} FW)		$N\text{-}NH_4$ (mg·kg^{-1} FW)		Total N (g·kg^{-1} DW)
	48 h	72 h	48 h	72 h	
Nitrogen level	***	***	*	***	***
Biostimulant	**	***	ns	ns	**
Nitrogen level × Biostimulant	*	***	*	ns	ns
Nitrogen level (mg L^{-1})					
7	78.0 ± 3.3 b	68.2 ± 4.5 b	23.5 ± 1.4 b	21.8 ± 1.0 b	14.3 ± 0.3 b
112	135.8 ± 11.7 a	196.7 ± 23.4 a	31.6 ± 4.7 a	36.9 ± 2.2 a	38.3 ± 0.5 a
Biostimulant					
No application	86.7 ± 7.9 b	95.6 ± 7.5 b	24.3 ± 1.8	28.3 ± 3.6	25.6 ± 3.8 b
Foliar spray	104.5 ± 12.5 b	149.3 ± 39.5 a	33.5 ± 7.0	29.9 ± 3.3	26.0 ± 3.7 ab
Substrate drench	129.5 ± 21.3 a	152.3 ± 42.7 a	24.9 ± 2.4	29.9 ± 4.9	27.2 ± 3.5 a
Nitrogen level × Biostimulant					
7 mg L^{-1} N without biostimulant	69.3 ± 2.3 d	82.3 ± 2.9 bc	26.5 ± 2.1 b	20.9 ± 2.2	13.3 ± 0.3
7 mg L^{-1} N with foliar spray	79.0 ± 4.0 cd	63.1 ± 1.3 c	21.2 ± 3.0 b	23.1 ± 1.7	13.8 ± 0.3
7 mg L^{-1} N with substrate drench	85.6 ± 6.2 cd	59.1 ± 9.1 c	22.8 ± 1.6 b	21.5 ± 2.0	15.8 ± 0.2
112 mg L^{-1} N without biostimulant	104.1 ± 2.6 bc	108.9 ± 9.6 b	22.0 ± 2.4 b	35.7 ± 2.2	37.9 ± 1.0
112 mg L^{-1} N with foliar spray	129.9 ± 10.7 b	235.5 ± 19.1 a	45.7 ± 9.2 a	36.8 ± 2.2	38.2 ± 0.9
112 mg L^{-1} N with substrate drench	173.4 ± 17.3 a	245.6 ± 18.2 a	27.0 ± 4.6 b	38.3 ± 6.8	38.7 ± 0.7

Nitrate and ammonium were measured on first fully expanded leaves at 18 (48 h after biostimulant application) and 19 days (72 h after biostimulant application) after transplanting, while leaf N content was determined at 19 days after transplanting. All data are expressed as mean ± standard error, n = 3. ns,,**,***: non-significant or significant at P ≤ 0.05, P ≤ 0.01, and P ≤ 0.001, respectively, Different letters within each column indicate significant differences according to Duncan's multiple-range test (P = 0.05).*

PH Trainer® elicited significant increase (+6.2%) of total leaf N content compared to untreated plants, whereas foliar spray treatment exhibited intermediate values (**Table 2**). Our findings on the beneficial effect of legume-derived PH application were in agreement with those of Colla et al. (2013, 2014) who reported that the leaf or root application of commercial PH-biostimulant stimulated N metabolism and incurred significant increase in leaf N content in maize seedling and tomato plantlets grown under controlled environments amounting to 18 and 22%, respectively. Furthermore, the higher SPAD index values observed in tomato plants treated with PH-biostimulant (substrate drench) could be also considered a mechanism by which PH application can promote N use efficiency. In fact, SPAD index is widely considered as a key indicator of chlorophyll and N content which have been often associated with a better crop performance (Colla et al., 2017a; Ertani et al., 2017).

Transcript Levels of Nitrate, Ammonium, and Amino Acids Transporters

The transcript levels of the key genes encoding for nitrate, ammonium, and amino acid transporters were investigated to provide novel insights on the effect of PHs either as signaling molecules or N source.

In higher plants two distinct systems of nitrate uptake were reported: the low-affinity transport system, responsible for uptake in presence of high nitrate concentration (>1 mM) and the HATS, involved in nitrate uptake in presence of low nitrate concentration (between 1 μM and 1 mM) (Little et al., 2005). In tomato, five nitrate transporter (*NRT*) genes inducible by nitrate were described: two *NRT1* and three *NRT2* (Ono et al., 2000; Hildebrandt et al., 2002). The expression of several *NRT2* genes was up-regulated by nitrogen starvation, suggesting a role of these transporters in the stimulation of the HATS for NO_3^- (Forde, 2000; Williams and Miller, 2001; Remans et al., 2006).

The expression analysis was carried out on two genes encoding high-affinity nitrate transporters belonging to *NRT2* family: *NRT2.1* and *NRT2.3* (Remans et al., 2006; Fu et al., 2015). Remans et al. (2006) demonstrated that *NRT2.1* plays a key role in the coordination of the root development, acting on lateral root initiation under low nitrate regime; whereas *NRT2.3* is involved in nitrate uptake and long-distance transport from root to shoot (Fu et al., 2015).

Our analyses confirmed that these genes are only expressed in root and are undetected in leaves (**Figures 1A,B**, **2A,B**). Moreover, foliar applications of PH did not produce significant effects on the transcript levels of the genes encoding *NRT2.1* and *NRT2.3* in roots of tomato plants grown under low N level in the nutrient solution (**Figure 1B**). Conversely, both genes were drastically down-regulated at 6 h after the substrate drench application (**Figure 2B**); this different response of transcript levels between foliar and substrate drench application of PH may be due to the time needed by foliarly applied PH to reach the root system through the phloematic transport.

Little et al. (2005) reported that *NRT2.1* is involved in the repression of lateral root initiation under high sucrose/low nitrate growth conditions. The suppression of *NRT2.1* produced a phenotype able to initiate a large number of lateral roots in *Arabidopsis* (Malamy and Ryan, 2001). In the current study, the PH treated-tomato plants had a significant increase of root biomass (**Table 1**) compared to untreated ones; this phenotype can be correlated with the drastic repression of *NRT2.1* transcript. Transcript analyses of *NRT2.1* and *NRT2.3* in plants grown under high N concentration in the nutrient solution confirmed the repressor effect of the biostimulant (**Figures 1B**, **2B**). In this case both methods of PH applications (foliar spray and substrate drench) led to a drastic reduction of transcripts for both genes (**Figures 1B**, **2B**).

AMT ammonium transporters are integral membrane proteins that mediate the uptake of NH_4^+, a suitable nitrogen form for root uptake due to the reduced state of the nitrogen (Loqué and von Wirén, 2004). Although distinct *AMT* family members exist, we focused on *AMT1.2*, which encodes a high

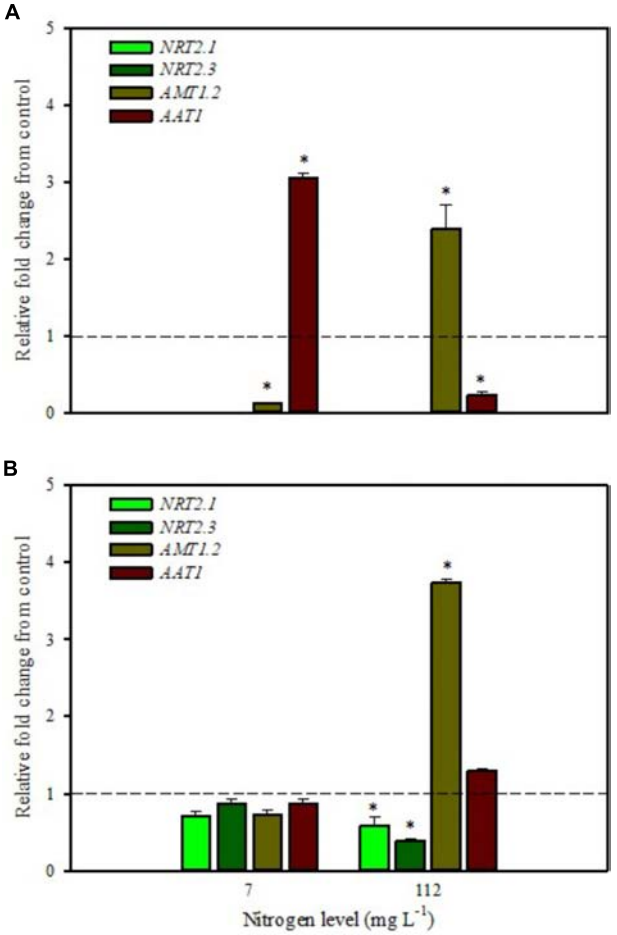


FIGURE 1 | Gene expression of nitrate transporters (*NRT2.1* and *NRT2.3*), ammonium transporter (*AMT1.2*), and amino acid transporter (*AAT1*) in leaves **(A)** and roots **(B)** of tomato plants grown under low (7 mg L^{-1}) and high nitrogen supply (112 mg L^{-1}) after 6 h from foliar spray with a legume-derived protein hydrolysate. The values are reported as relative fold change from control, which was normalized to 1; values >1 represent up-regulation and <1 down-regulation. Vertical bars indicate ± standard error of means; *$P < 0.05$ compared with control.

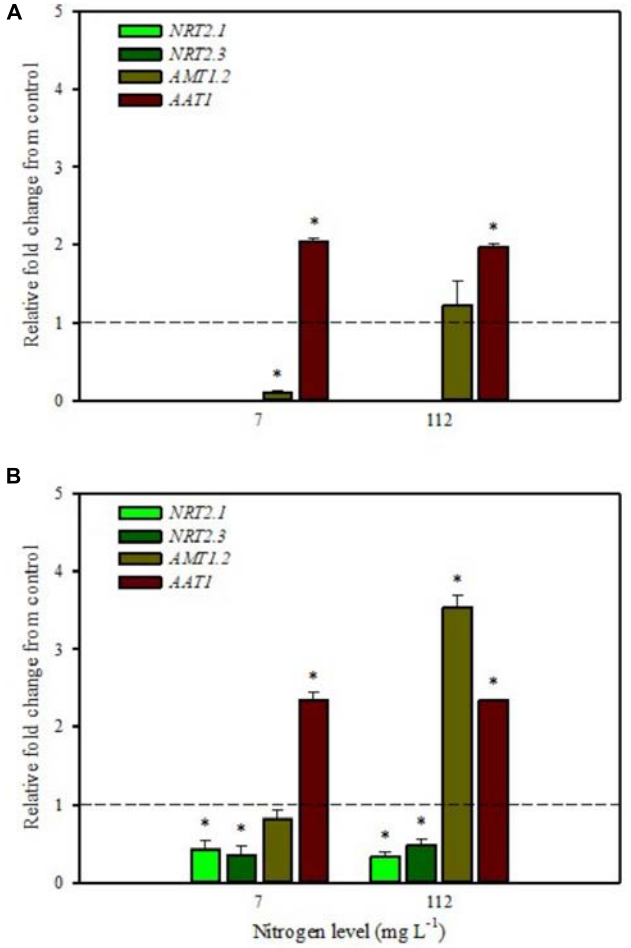


FIGURE 2 | Gene expression of nitrate transporters (*NRT2.1* and *NRT2.3*), ammonium transporter (*AMT1.2*), and amino acid transporter (*AAT1*) in leaves **(A)** and roots **(B)** of tomato plants grown under low (7 mg L^{-1}) and high nitrogen supply (112 mg L^{-1}) after 6 h from substrate drench with a legume-derived protein hydrolysate. The values are reported as relative fold change from control, which was normalized to 1; values >1 represent up-regulation and <1 down-regulation. Vertical bars indicate ± standard error of means; *$P < 0.05$ compared with control.

affinity transporter that is expressed in leaf and root tissue. This gene was strongly down-regulated after 6 h from foliar spray and substrate drench applications in leaves of tomato plants grown under low N supply (**Figures 1A**, **2A**).

The expression analysis, carried out on plants grown under high N conditions, showed a different regulation of the *AMT1.2* gene: it was strongly up-regulated in leaf and root at 6 h after foliar application of PH and only in root at 6 h after substrate drench treatment (**Figures 1A,B**, **2B**). The results suggested that both biostimulant application methods favored the ammonium translocation between apoplast and symplast cells under high N regime. von Wirén et al. (2000) demonstrated that the transcript level of *AMT1.2* was inducible by NH_4^+ and suggested that it could be involved in the retrieval of ammonium, thus compensating ammonium uptake from roots due to amino acid catabolism.

To elucidate the effects of PH application on amino acid turnover and allocation, the expression of a key gene encoding for an amino acid transporter was investigated. *AAT1* is a member of amino acid transporter family SL1.00sc07184_335.1.1, that is homologous to a member of the *Avt* family of vacuolar transporters belonging to the amino acid/auxin permease family isolated from *Saccharomyces cerevisiae* (*Avt1p*, GenBank No. NP_012534.1) (Snowden et al., 2015). The amino acid transporter encoded by *AAT1* is involved in the transport of glutamic acid, aspartic acid and isoleucine.

AAT1 transcript was strongly induced in leaves (up to more than threefold) after foliar application of PH in plants grown under low N regime (**Figure 1A**); a positive regulation was also observed in roots and leaves after substrate drench application of PH (**Figures 2A,B**). No difference of *AAT1* transcript abundance was detected in root after foliar application of PH either under low or high nitrogen regime (**Figure 1B**). Our findings could be related to the time needed by foliarly applied PH to reach the roots through phloematic system. Furthermore, *AAT1* gene was strongly up-regulated after substrate drench application of PH in root and leaf tissues in tomato plants supplied with high N supply (**Figures 2A,B**), confirming an active role of this transporter in the amino acid allocation either in leaf or root tissues. However, a different behavior was observed after foliar application where the expression level of *AAT1* was markedly suppressed in leaf (**Figure 1A**). This different behavior could be associated to the different ability of leaf and root to uptake amino acid and peptides. Obviously, root cells could uptake peptides contained in the biostimulant product through permeases and hydrolyze them in amino acids; differently leaf cells have poor ability to uptake peptides or proteins.

Transcript Levels of Key Genes Involved in Nitrogen Assimilation

In the present study, expression data highlighted a drastic reduction of *NR* transcripts in leaves of tomato plants grown under low N regime after 6 h from foliar spray with PH (**Figure 3A**). The other genes (*NiR, GS2*, *GLT*, *GLS*, and *GDH*) were not affected except for *GS1* that was up-regulated twofold (**Figure 3A**). Foliar application of PH did not modulate the expression of *NR*, *NiR*, *GS1*, *GLS*, and *GDH* in root; only the genes *GLT* and *GS2* were up-regulated in comparison with untreated plants (**Figure 3B**).

Substrate drench application of PH on tomato plants grown with low N availability had a remarkable repressive effect on the expression of *NR*, *GS1*, *GS2*, *GLT*, *GLS* in leaf and *NR*, *NiR*, *GLS* in root (**Figures 4A,B**). The remaining genes were not affected except for the *GLT* and *GS2* transcripts that were considerably increased in root (**Figure 4B**).

It was evident that both biostimulant application methods (foliar spray and substrate drench) down-regulated the key gene involved in the first steps of nitrate assimilation (*NR*) in leaves of

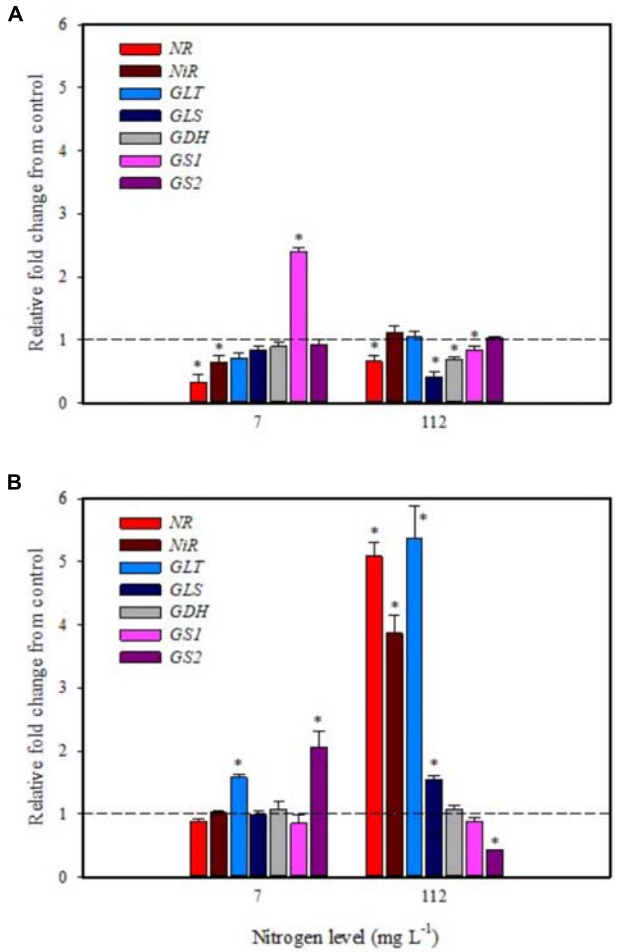


FIGURE 3 | Gene expression of nitrate reductase (*NR*), nitrite reductase (*NiR*), ferredoxin-glutamate synthases (*GLT*), NADH-dependent glutamate synthases (*GLS*), glutamate dehydrogenase (*GDH*), and glutamine synthetase isozymes [cytosolic (*GS1*), and plastidic (*GS2*)] in leaves **(A)** and roots **(B)** of tomato plants grown under low (7 mg L^{-1}) and high nitrogen supply (112 mg L^{-1}) after 6 h from foliar spray with a legume-derived protein hydrolysate. The values are reported as relative fold change from control, which was normalized to 1; values >1 represent up-regulation and <1 down-regulation. Vertical bars indicate ± standard error of means; *$P < 0.05$ compared with control.

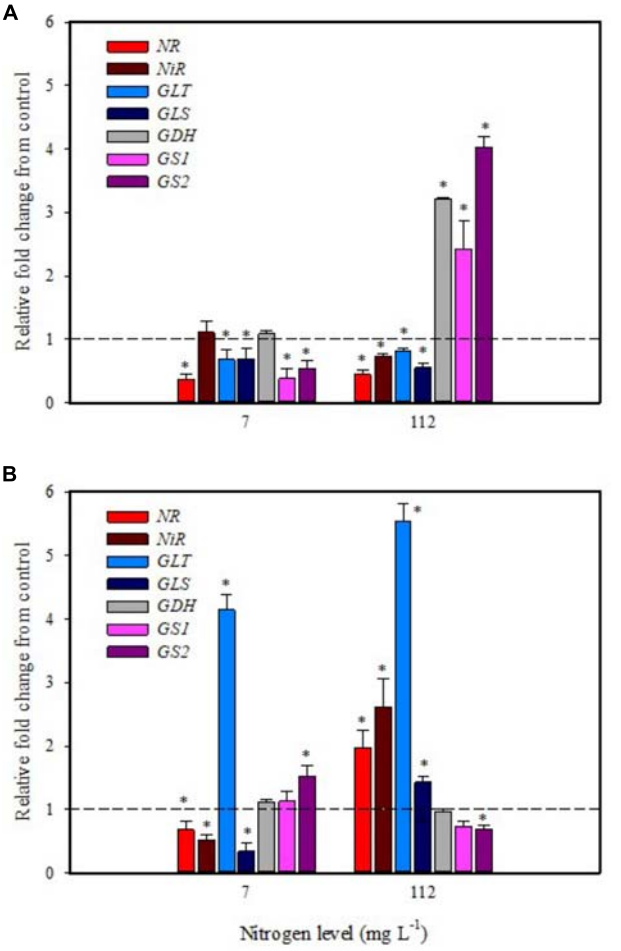


FIGURE 4 | Gene expression of nitrate reductase (*NR*), *NiR*, ferredoxin-glutamate synthases (*GLT*), NADH-dependent glutamate synthases (*GLS*), glutamate dehydrogenase (*GDH*), and glutamine synthetase isozymes [cytosolic (*GS1*), and plastidic (*GS2*)] in leaves **(A)** and roots **(B)** of tomato plants grown under low (7 mg L^{-1}) and high nitrogen supply (112 mg L^{-1}) after 6 h from substrate drench with a legume-derived protein hydrolysate. The values are reported as relative fold change from control, which was normalized to 1; values >1 represent up-regulation and <1 down-regulation. Vertical bars indicate ± standard error of means; *$P < 0.05$ compared with control.

plants grown under low N regime. These findings were associated with the reduction of transcript levels for the ammonium and nitrate transporters (**Figures 1A**, **2A**). Because of the increase in total biomass and total nitrogen content in PH treated plants (**Table 1**), we hypothesized that PH acted as N source especially when it was supplied as substrate drench treatment under low N regime. Plants can take up organic nitrogen compounds of low molecular mass, including amino acids and small peptides (di- and tripeptides), via membrane transporters (Paungfoo-Lonhienne et al., 2008). Moreover, roots exude proteolytic enzymes that digest large peptides leading to an increase of free amino acids for plant uptake (Paungfoo-Lonhienne et al., 2008). These findings may explain the better performances of Trainer® in improving total N content of leaves when it was applied as substrate drench instead of foliar spray. This hypothesis was strengthened by the enhancement of *AAT1* expression both in leaves and roots after PH applications, suggesting a rapid mobilization of amino acids in plant tissues. For instance, Miller et al. (2007) suggested that amino acids (especially glutamine) can provide a signal for the regulation of nitrate uptake. In their study the authors found a strong reduction of the transcript levels of the nitrate and ammonium transporters in roots treated with exogenous amino acids. Another possible mechanism could be that PH mediated-root growth enhancement increased the root uptake of mineral nitrogen from the substrate especially under high N availability in the rootzone.

A different effect on the transcript levels of *NR*, *NiR*, *GS,* and *GOGAT* was observed in maize plants treated with an alfalfa

protein hydrolysate (Schiavon et al., 2008) where these genes were significantly up-regulated by PH application. A plausible reason for the different behavior could be the growing conditions: Schiavon et al. (2008) provided the PH continuously for 48 h in a hydroponic system, whereas in our study plants were grown in substrate and treated twice with PH.

The application of legume-derived PH in tomato plants grown under high N concentration did not produce significant changes of transcript abundance for all genes in leaves at 6 h after foliar application (except for *NR, GDH, GLS* that were down-regulated) (**Figure 3A**); a different trend was observed in roots, where *NR*, *NiR*, *GLS*, and *GLT* were strongly induced and *GS2* was significantly repressed (**Figure 3B**). The application of legume-derived PH by substrate drench produced a similar effect on *NR*, *NiR*, *GLS*, and *GLT* in roots (**Figure 4B**). These findings are consistent with those of Liu and Lee (2012) who demonstrated that the application of mixed amino acids incurred significant increase in the enzymes activities (NR, NiR, and GS) as well as the assimilatory pathway.

Glutamine synthetase 1, *GS2*, and *GDH* transcripts were strongly up-regulated in leaf at 6 h after PH applications as substrate drench (**Figure 4A**); this behavior could reveal a signaling activity of the biostimulant on the activation of amino acid turnover and ammonium recycling. A positive correlation between GS activity and nitrogen assimilation was previously described in wheat (Kichey et al., 2007). Moreover, several studies clearly demonstrated that GS activity was also associated with improved productivity in rice, wheat, and maize (Martin et al., 2006; Kichey et al., 2007; Brauer et al., 2011).

Overall, significant findings in the current work concerning the action of PH applications include their negative impact on the gene expression of *NRT2.1* and *NRT2.3*, which in turn promoted the development of the root apparatus in PH-treated plants compared to the untreated control. Another significant effect of PH application is the stimulation of N assimilation through increased expression of the two key genes for NR and NIR in plants grown under high N supply. Additionally, both methods of biostimulant application (foliar spray and substrate drench) strongly stimulated gene expression of the amino acid transporter *AAT1*, indicating that some free amino acids may be directly absorbed by plant. Finally, the data presented in the current paper contribute significantly toward the advancement of knowledge concerning the effects of plant biostimulants on plant growth and N content.

CONCLUSION

The continuous and increasing pressure on vegetable growers and horticultural professionals to boost crop performance and at the same time to limit the use of synthetic mineral fertilizers, represents a strong motivation for the research community to seek for alternative technologies able to ensure high productivity in a sustainable manner (i.e., by enhancing nutrient use efficiency). Tomato growth as well as key genes involved in N assimilation was assessed in a multifactorial approach accounting for the influence of PH-biostimulant treatments and N regimes. At both nitrogen regimes, the application of legume-derived PH especially as a substrate drench enhanced the tomato performance parameters and N content indicating the importance of the application method. The increase in plant biomass was associated to the stimulation of the root growth, thus inducing a "*nutrient acquisition response*" that favors N uptake and translocation. Our results also demonstrated that PH application differentially regulated in a N-dependent manner the expression of genes involved in nitrate, ammonium and amino acid transporters as well as the key genes involved in N metabolism. Under low nitrogen supply, PH upregulated the expression of genes encoding for amino acid transporter and ferredoxin-glutamate synthases, and *GS* in roots whereas expression of genes encoding for nitrate and ammonium transporters, and *NR* were downregulated especially in leaves. Under high nitrogen supply, PH upregulated the expression of genes encoding for ammonium and amino acid transporter especially in roots and *NR*, *NIR*, and ferredoxin-dependent glutamate synthase in roots whereas expression of genes encoding for nitrate transporter in roots, and *NR* in leaves were downregulated. These results highlighted the potential benefits of using legume PH in tomato production to increase growth and N-nutritional status of plants grown under both high and low nitrogen regimes. Overall, the PH mediated-increase of total N content in leaves can be explained by the stimulation of root growth and the upregulation of genes involved in the N assimilation.

AUTHOR CONTRIBUTIONS

FS wrote the first draft of the manuscript and followed the molecular analysis and data interpretation. YR wrote many parts of the manuscript and followed the agronomic measurements. MC wrote many parts of the manuscripts and followed the samplings and analysis of nitrogen, nitrate, and ammonium. AP made the molecular analysis of gene expression. PB was involved in writing part of the manuscript on molecular analysis and data interpretation. RC was involved in the implementation of the manuscript. GC provided the intellectual input, set up the experiment, and corrected the manuscript.

FUNDING

This work was partially funded by the Lazio Region in the frame of FILAS-RU-2014-1108 Project "MIGLIORA" and by Italpollina S.p.A.

SUPPLEMENTARY MATERIAL

The Supplementary Material for this article can be found online at: https://www.frontiersin.org/articles/10.3389/fpls.2018.01233/full#supplementary-material

REFERENCES

Amirkhani, M., Netravali, A. N., and Huang, W. (2016). Investigation of soy protein–based biostimulant seed coating for broccoli seedling and plant growth enhancement. *HortScience* 51, 1121–1126. doi: 10.21273/hortsci10913-16

Anderson, J. M., and Ingram, J. S. I. (1989). "Colorimetric determination of ammonium," in *Tropical Soil Biology and Fertility. A Handbook of Methods*, 2nd Edn, eds J. M. Anderson and J. S. I. Ingram (Wallingford: CAB International Wallingford UK), 42–43.

Bernard, S. M., and Habash, D. Z. (2009). The importance of cytosolic glutamine synthetase in nitrogen assimilation and recycling. *New Phytol.* 182, 608–620. doi: 10.1111/j.1469-8137.2009.02823.x

Botta, A. (2013). Enhancing plant tolerance to temperature stress with amino acids: an approach to their mode of action. *Acta Hortic.* 1009, 29–35. doi: 10.17660/actahortic.2013.1009.1

Brauer, E. K., Rochon, A., Bi, Y. M., Bozzo, G. G., Rothstein, S. J., and Shelp, B. J. (2011). Reappraisal of nitrogen use efficiency in rice overexpressing glutamine synthetase1. *Physiol. Plant.* 141, 361–372. doi: 10.1111/j.1399-3054.2011.01443.x

Bremner, J. M. (1965). "Total nitrogen," in *Methods of Soil Analysis*, eds C. A. Black, D. D. Evans I, L. White, L. E. Ensminger, and F. E. Clark (Madison, WI: Agronomy Monograph 9), 1149–1178.

Calvo, P., Nelson, L., and Kloepper, J. W. (2014). Agricultural uses of plant biostimulants. *Plant Soil* 383, 3–41. doi: 10.1007/s11104-014-2131-8

Cataldo, D. A., Haroon, M., Schrader, L. E., and Youngs, V. L. (1975). Rapid colorimetric determination of nitrate in plant-tissue by nitration of salicylic-acid. *Commun. Soil Sci. Plant Anal.* 6, 71–80. doi: 10.1080/00103627509366547

Cavani, L., and Ciavatta, C. (2007). Attività biostimolante negli idrolizzati proteici. *Inf. Agrar.* 44, 46–52.

Colla, G., Cardarelli, M., Bonini, P., and Rouphael, Y. (2017a). Foliar applications of protein hydrolysate, plant and seaweed extracts increase yield but differentially modulate fruit quality of greenhouse tomato. *HortScience* 52, 1214–1220. doi: 10.21273/hortsci12200-17

Colla, G., Hoagland, L., Ruzzi, M., Cardarelli, M., Bonini, P., Canaguier, R., et al. (2017b). Biostimulant action of protein hydrolysates: unraveling their effects on plant physiology and microbiome. *Front. Plant Sci.* 8:2202. doi: 10.3389/fpls.2017.02202

Colla, G., Nardi, S., Cardarelli, M., Ertani, A., Lucini, L., Canaguier, R., et al. (2015). Protein hydrolysates as biostimulants in horticulture. *Sci. Hortic.* 196, 28–38. doi: 10.1016/j.scienta.2015.08.037

Colla, G., and Rouphael, Y. (2015). Biostimulants in horticulture. *Sci. Hortic.* 196, 1–2. doi: 10.1016/j.scienta.2015.10.044

Colla, G., Rouphael, Y., Canaguier, R., Svecova, E., and Cardarelli, M. (2014). Biostimulant action of a plant-derived protein hydrolysate produced through enzymatic hydrolysis. *Front. Plant Sci.* 5:448. doi: 10.3389/fpls.2014.00448

Colla, G., Rouphael, Y., Mirabelli, C., and Cardarelli, M. (2011). Nitrogen-use efficiency traits of mini-watermelon in response to grafting and nitrogen-fertilization doses. *J. Plant Nutr. Soil Sci.* 174, 933–941. doi: 10.1002/jpln.201000325

Colla, G., Suãrez, C. M. C., Cardarelli, M., and Rouphael, Y. (2010). Improving nitrogen use efficiency in melon by grafting. *HortScience* 45, 559–565.

Colla, G., Svecova, E., Rouphael, Y., Cardarelli, M., Reynaud, H., Canaguier, R., et al. (2013). Effectiveness of a plant –derived protein hydrolysate to improve crop performances under different growing conditions. *Acta Hortic.* 1009, 175–179. doi: 10.17660/actahortic.2013.1009.21

du Jardin, P. (2015). Plant biostimulants: definition, concept, main categories and regulation. *Sci. Hortic.* 196, 3–14. doi: 10.1016/j.scienta.2015.09.021

Ertani, A., Cavani, L., Pizzeghello, D., Brandellero, E., Altissimo, A., Ciavatta, C., et al. (2009). Biostimulant activities of two protein hydrolysates on the growth and nitrogen metabolism in maize seedlings. *J. Plant. Nutr. Soil. Sci* 172, 237–244. doi: 10.1002/jpln.200800174

Ertani, A., Pizzeghello, D., Francioso, O., Sambo, P., Sanchez-Cortes, S., and Nardi, S. (2014). Capsicum Chinensis L. growth and nutraceutical properties are enhanced by biostimulants in a long-term period: chemical and metabolomic approaches. *Front. Plant Sci.* 5:375. doi: 10.3389/fpls.2014.00375

Ertani, A., Sambo, P., Nicoletto, C., Santagata, S., Schiavon, M., and Nardi, S. (2015). The use of organic biostimulants in hot pepper plants to help low input sustainable agriculture. *Chem. Biol. Technol. Agric.* 2, 11. doi: 10.1186/s40538-015-0039-z

Ertani, A., Schiavon, M., Muscolo, A., and Nardi, S. (2013). Alfalfa plant-derived biostimulant stimulate short-term growth of salt stressed Zea mays L. plants. *Plant Soil.* 364, 145–158. doi: 10.1007/s11104-012-1335-z

Ertani, A., Schiavon, M., and Nardi, S. (2017). Transcriptome-wide identification of differentially expressed genes in *Solanum lycopersicon* L. in response to an alfalfa-protein hydrolysate using microarrays. *Front. Plant Sci.* 8:1159. doi: 10.3389/fpls.2017.01159

Forde, B. G. (2000). Nitrate transporters in plants: structure, function and regulation. *Biochim. Biophys. Acta* 1465, 219–235. doi: 10.1016/S0005-2736(00)00140-1

Fu, Y., Yi, H., Bao, J., and Gong, J. (2015). LeNRT2.3 functions in nitrate acquisition and long-distance transport in tomato. *FEBS Lett.* 589, 1072–1079. doi: 10.1016/j.febslet.2015.03.016

Halpern, M., Bar-Tal, A., Ofek, M., Minz, D., Muller, T., and Yermiyahu, U. (2015). The use of biostimulants for enhancing nutrient uptake. *Adv. Agron.* 130, 141–174. doi: 10.1016/bs.agron.2014.10.001

Hildebrandt, U., Schmelzer, E., and Bothe, H. (2002). Expression of nitrate transporter genes in tomato colonized by an arbuscular mycorrhizal fungus. *Physiol. Plant.* 115, 125–136. doi: 10.1034/j.1399-3054.2002.1150115.x

Hirel, B., Le Gouis, J., Ney, B., and Gallais, A. (2007). The challenge of improving nitrogen use efficiency in crop plants: towards a more central role for genetic variability and quantitative genetics within integrated approaches. *J. Exp. Bot.* 58, 2369–2387. doi: 10.1093/jxb/erm097

Husted, S., Mattsson, M., Möllers, C., Wallbraun, M., and Schjoerring, J. K. (2002). Photorespiratory $NH4^+$ production in leaves of wild-type and glutamine synthetase 2 antisense oilseed rape. *Plant Physiol.* 130, 989–998. doi: 10.1104/pp.006759

Jannin, L., Arkoun, M., Etienne, P., Laîné, P., Goux, D., Garnica, M., et al. (2013). *Brassica napus* growth is promoted by *Ascophyllum nodosum* (L.) Le Jol. seaweed extract: microarray analysis and physiological characterization of N. C, and S metabolisms. *J. Plant Regul.* 32, 31–52. doi: 10.1007/s00344-012-9273-9

Kichey, T., Hirel, B., Heumez, E., Dubois, F., and Le Gouis, J. (2007). In winter wheat (*Triticum aestivum* L.), post-anthesis nitrogen uptake and remobilisation to the grain correlates with agronomic traits and nitrogen physiological markers. *Field Crops Res.* 102, 22–32. doi: 10.1016/j.fcr.2007.01.002

Lancien, M., Lea, P. J., and Azevedo, R. A. (2007). "Amino acid synthesis in plastids," in *The Structure and Function of Plastids. Advances in Photosynthesis and Respiration*, Vol. 23, eds R. R. Wise and J. K. Hoober (Dordrecht: Springer).

Little, D. Y., Rao, H., Oliva, S., Daniel-Vedele, F., Krapp, A., and Malamy, J. E. (2005). The putative high-affinity nitrate transporter NRT2. 1 represses lateral root initiation in response to nutritional cues. *Proc. Natl. Ac. Sci. U.S.A.* 102, 13693–13698. doi: 10.1073/pnas.0504219102

Liu, X. Q., and Lee, K. S. (2012). "Effect of mixed amino acids on crop growth," in *Agricultural Science*, ed. G. Aflakpui (Rijeka: InTech Europe Publisher), 119–158. doi: 10.5772/37461

Loqué, D., and von Wirén, N. (2004). Regulatory levels for the transport of ammonium in plant roots. *J. Exp. Bot.* 55, 1293–1305. doi: 10.1093/jxb/erh147

Lucini, L., Rouphael, Y., Cardarelli, M., Canaguier, R., Kumar, P., and Colla, G. (2015). The effect of a plant-derived protein hydrolysate on metabolic profiling and crop performance of lettuce grown under saline conditions. *Sci. Hortic.* 182, 124–133. doi: 10.1016/j.scienta.2014.11.022

Malamy, J. E., and Ryan, K. S. (2001). Environmental regulation of lateral root initiation in Arabidopsis. *Plant Physiol.* 127, 899–909. doi: 10.1104/pp.010406

Martin, A., Lee, J., Kichey, T., Gerentes, D., Zivy, M., Tatout, F., et al. (2006). Two cytosolic glutamine synthetase isoforms of maize are specifically involved in the control of grain production. *Plant Cell* 18, 3252–3274. doi: 10.1105/tpc.106.042689

Matsumiya, Y., and Kubo, M. (2011). "Soybean peptide: novel plant growth promoting peptide from soybean," in *Soybean and Nutrition*, ed. H. El-Shemy (Rijeka: In Tech Europe Publisher), 215–230.

Miller, A. J., Fan, X., Shen, Q., and Smith, S. J. (2007). Amino acids and nitrate as signals for the regulation of nitrogen acquisition. *J. Exp. Bot.* 59, 111–119. doi: 10.1093/jxb/erm208

Nardi, S., Pizzeghello, D., Schiavon, M., and Ertani, A. (2016). Plant biostimulants: physiological responses induced by protein hydrolyzed-based products and

humic substances in plant metabolism. *Sci. Agric.* 73, 18–23. doi: 10.1590/0103-9016-2015-0006

Ono, F., Frommer, W. B., and Von Wiren, N. (2000). Coordinated diurnal regulation of low-and high-affinity nitrate transporters in tomato. *Plant Biol.* 2, 17–23. doi: 10.1055/s-2000-297

Paungfoo-Lonhienne, C., Lonhienne, T. G. A., Rentsch, D., Robinson, N., Christie, M., Webb, R. I., et al. (2008). Plants can use protein as a nitrogen source without assistance from other organisms. *Proc. Natl. Acad. Sci. U.S.A.* 105, 4524–4529. doi: 10.1073/pnas.0712078105

Pecha, J., Fürst, T., Kolomaznik, K., Friebrova, V., and Svoboda, P. (2012). Protein biostimulant foliar uptake modeling: the impact of climatic conditions. *AIChE J.* 58, 2010–2019. doi: 10.1002/aic.12739

Remans, T., Nacry, P., Pervent, M., Girin, T., Tillard, P., Lepetit, M., et al. (2006). A central role for the nitrate transporter NRT2.1 in the integrated morphological and physiological responses of the root system to nitrogen limitation in Arabidopsis. *Plant Physiol.* 140, 909–921. doi: 10.1104/pp.105.075721

Rouphael, Y., Cardarelli, M., Bonini, P., and Colla, G. (2017a). Synergistic action of a microbial-based biostimulant and a plant derived-protein hydrolysate enhances lettuce tolerance to alkalinity and salinity. *Front. Plant Sci.* 8:131. doi: 10.3389/fpls.2017.00131

Rouphael, Y., Colla, G., Giordano, M., El-Nakhel, C., Kyriacou, M. C., and De Pascale, S. (2017b). Foliar applications of a legume-derived protein hydrolysate elicit dose-dependent increases of growth, leaf mineral composition, yield and fruit quality in two greenhouse tomato cultivars. *Sci. Hortic.* 226, 353–360. doi: 10.1016/j.scienta.2017.09.007

Rouphael, Y., De Micco, V., Arena, C., Raimondi, G., Colla, G., and De Pascale, S. (2017c). Effect of Ecklonia maxima seaweed extract on yield, mineral composition, gas exchange and leaf anatomy of zucchini squash grown under saline conditions. *J. Appl. Phycol.* 29, 459–470. doi: 10.1007/s10811-016-0937-x

Rouphael, Y., Giordano, M., Cardarelli, M., Cozzolino, E., Mori, M., Kyriacou, M. C., et al. (2018). Plant- and seaweed-based extracts increase yield but differentially modulate nutritional quality of greenhouse spinach through biostimulant action. *Agronomy* 8:126. doi: 10.3390/agronomy8070126

Rouphael, Y., Franken, P., Schneider, C., Schwarz, D., Giovannetti, M., Agnolucci, M., et al. (2015). Arbuscular mycorrhizal fungi act as biostimulants in horticultural crops. *Sci. Hortic.* 196, 91–108. doi: 10.1016/j.scienta.2015.09.002

Ryan, C. A., Pearce, G., Scheer, J., and Moura, D. S. (2002). Polypeptide hormones. *Plant Cell* 14(Suppl.), S251–S264. doi: 10.1105/tpc.010484

Santi, C., Zamboni, A., Varanini, Z., and Pandolfini, T. (2017). Growth stimulatory effects and genome-wide transcriptional changes produced by protein hydrolysates in maize seedlings. *Front. Plant Sci.* 8:433. doi: 10.3389/fpls.2017.00433

Schiavon, M., Ertani, A., and Nardi, S. (2008). Effects of an alfalfa protein hydrolysate on the gene expression and activity of enzymes of TCA cycle and N metabolism in Zea mays L. *J. Agric. Food Chem.* 56, 11800–11808. doi: 10.1021/jf802362g

Sestili, F., Janni, M., Doherty, A., Botticella, E., D'Ovidio, R., Masci, S., et al. (2010). Increasing the amylose content of durum wheat through silencing of the SBEIIa genes. *BMC Plant Biol.* 10:144. doi: 10.1186/1471-2229-10-144

Snowden, C. J., Thomas, B., Baxter, C. J., Smith, J. A. C., and Sweetlove, L. J. (2015). A tonoplast Glu/Asp/GABA exchanger that affects tomato fruit amino acid composition. *Plant J.* 81, 651–660. doi: 10.1111/tpj.12766

Tercé-Laforgue, T., Bedu, M., Dargel-Graffin, C., Dubois, F., Gibon, Y., Restivo, F. M., et al. (2013). Resolving the role of plant glutamate dehydrogenase: II. Physiological Characterization of plants overexpressing individually or simultaneously the two enzyme subunits. *Plant. Cell. Physiol.* 54, 1634–1647. doi: 10.1093/pcp/pct108

Tilman, D., Cassman, K. G., Matson, P. A., Naylor, R., and Polasky, S. (2002). Agricultural sustainability and intensive production practices. *Nature* 418, 671–677. doi: 10.1038/nature01014

Ugolini, L., Cinti, S., Righetti, L., Stefan, A., Matteo, R., D'Avino, L., et al. (2015). Production of an enzymatic protein hydrolyzate from defatted sunflower seed meal for potential application as a plant biostimulant. *Ind. Crops Prod.* 75, 15–23. doi: 10.1016/j.indcrop.2014.11.026

von Wirén, N., Lauter, F. R., Ninnemann, O., Gillissen, B., Walch-Liu, P., Engels, C., et al. (2000). Differential regulation of three functional ammonium transporter genes by nitrogen in root hairs and by light in leaves of tomato. *Plant J.* 21, 167–175. doi: 10.1046/j.1365-313x.2000.00665.x

Williams, L. E., and Miller, A. J. (2001). Transporters responsible for the uptake and partitioning of nitrogenous solutes. *Annu. Rev. Plant Physiol. Plant Mol. Biol.* 52, 659–688. doi: 10.1146/annurev.arplant.52.1.659

Zhang, Z., Xiong, S., Wei, Y., Meng, X., Wang, X., and Ma, X. (2017). The role of glutamine synthetase isozymes in enhancing nitrogen use efficiency of N-efficient winter wheat. *Sci. Rep.* 7:1000. doi: 10.1038/s41598-017-01071-1

Conflict of Interest Statement: The authors declare that the research was conducted in the absence of any commercial or financial relationships that could be construed as a potential conflict of interest.

frontiers in Microbiology

OPINION
published: 03 September 2018
doi: 10.3389/fmicb.2018.02054

Is Phosphate Solubilization the Forgotten Child of Plant Growth-Promoting Rhizobacteria?

Camille E. Granada[1], Luciane M. P. Passaglia[2], Eduardo M. de Souza[1] and Raul A. Sperotto[1]*

[1] Graduate Program in Biotechnology, University of Taquari Valley - Univates, Lajeado, Brazil, [2] Department of Genetics, Institute of Biosciences, Federal University of Rio Grande do Sul (UFRGS), Porto Alegre, Brazil

Keywords: P-fertilizers, inoculation, P-solubilization, rhizobacteria, sustainable agriculture

OPEN ACCESS

Edited by:
Youssef Rouphael, Università degli Studi di Napoli Federico II, Italy

Reviewed by:
Claudio Valverde, Universidad Nacional de Quilmes (UNQ), Argentina
Gyöngyvér Mara, Sapientia Hungarian University of Transylvania, Romania

****Correspondence:***
Camille E. Granada
cegranada@univates.br

Specialty section:
This article was submitted to Plant Microbe Interactions, a section of the journal Frontiers in Microbiology

Received: *09 July 2018*
Accepted: *13 August 2018*
Published: *03 September 2018*

Citation:
Granada CE, Passaglia LMP, de Souza EM and Sperotto RA (2018) Is Phosphate Solubilization the Forgotten Child of Plant Growth-Promoting Rhizobacteria? Front. Microbiol. 9:2054. doi: 10.3389/fmicb.2018.02054

CHEMICAL FERTILIZERS × PLANT GROWTH-PROMOTING RHIZOBACTERIA

Plant growth-promoting rhizobacteria (PGPR) is a well-known group of microorganisms able to promote plant growth through enhanced biological nitrogen fixation (BNF), synthesis of plant hormones, soil nutrient solubilization (as phosphorus [P] and potassium [K]; Gupta et al., 2015), besides preventing deleterious effects of soil-borne phytopathogens (Compant et al., 2005). Due to the high importance of nitrogen (N) for plant development and the low persistence time that synthetic N fertilizer presents in the soil (Galloway et al., 2003), most of the studies are focused on microorganisms able to biologically fix atmospheric N. BNF is performed by symbiotic PGPR, which are restricted to association of leguminous plants and rhizobial isolates (e.g., *Rhizobium* spp., *Bradyrhizobium* spp., *Mesorhizobium* spp., and *Allorhizobium* spp.), or by free-living bacterial isolates (e.g., *Azospirillum* spp., *Pseudomonas* spp., *Burkholderia* spp., *Gluconacetobacter* spp., and *Herbaspirillum* spp.; Remigi et al., 2016). However, the research focused only in BNF neglects the high biotechnological potential of PGPR to agriculture.

Overuse of synthetic fertilizers and agrochemical pesticides has sustained the high crop yield and, consequently, the population growth in the last century (Stewart et al., 2005). However, environment does not sustain these practices any more. The consequences are already observed as high eutrophication of rivers, groundwater contamination, atmospheric pollution, and losses of soil quality (Stewart et al., 2005; Mondal et al., 2017). These scenarios have stimulated several agricultural researches. Replacement of synthetic N inputs by PGPR inoculation has been possible only due to the deep knowledge about BNF. It is interesting to farmers, since it reduces production costs besides being an environmental-friendly technique. However, PGPR inoculation can go further, since it presents a potential to reduce the amount of the most important synthetic inputs applied on crops, which is of paramount importance regarding fertilizers obtained from finite sources.

SOIL PHOSPHORUS (P) AND P-FERTILIZATION

Phosphorus (P) is a good example of an essential nutrient for plant development derived from finite resources. P fertilizer is extracted from P-rich rock in the form of phosphate. Morocco, China, South Africa and the U.S. account for approximately 83% of the world's reserves of exploitable phosphate rock (Vaccari, 2009). Therefore, P deficiency is one of the major limitations to crop production and it is estimated that 5.7 billion hectares of land worldwide are deficient in P (Mouazen and Kuang, 2016). These numbers highlight the high importance of P fertilizers for achieving optimal

crop production. Bouwman et al. (2013) estimated that annual P consumption in agriculture will increase around 2.5% per year. Considering the finite sources of P, this data and other studies indicated that a global P crisis is near (Abelson, 1999; Vaccari, 2009; Jones et al., 2015). However, none of these studies have considered the residual P in the soil (Sattari et al., 2012).

Some tropical agricultural soils are P-fixing, and the vast majority of P fertilizer added to them are adsorbed onto soil minerals [metal oxides (mainly iron and aluminum) and clay minerals], precipitated as P minerals (predominantly apatite-like minerals), and immobilized as organic P compounds (soil organic matter and phytate), making its residual P less available to crops (Martinez-Viveros et al., 2010; Hinsinger et al., 2011). Due to such P immobilization and environmental losses, producers need to apply twice or more P fertilizers than are actually needed for optimal yield production (Roy et al., 2016). It is estimated that 2–8 million tons of P fertilizer are applied to the soils every year, and ∼1–4 million tons remain in the soil as a residual part. In a future scenario (2050), 4–14 million tons will be applied, and 2–7 million tons will remain in the soils (Roy et al., 2016). Considering that P fertilizer costs approximately US$ 400 per ton, around US$ 400 million to US$ 1.6 billion are lost with P fertilizers in crops around the world every year. It certainly means a substantial increase on the food prices for consumers.

IS PHOSPHORUS SOLUBILIZATION THE FORGOTTEN CHILD OF PGPR?

Recently, Roy et al. (2016) made a tricky question: is it possible that the increasing amount of immobilized P in the tropical agricultural soils eventually become available to plants and support crop productivity? In the case we keep using the same fertilization strategies used for many years, the answer is certainly no. However, we do believe that using adequate biotechnological approaches, the immobilized P could return to the plants in a soluble and available form. Screening of new PGP isolates for inoculant production aiming to optimize plant growth and BNF comprise an essential stage of *in vitro* phosphate solubilization analysis (Collavino et al., 2010; Souza et al., 2013, 2015; Walitang et al., 2017; Marag et al., 2018). These studies identified several bacterial isolates able to promote plant growth, improve rhizosphere area and solubilize different sources of immobilized P. Given the low mobility of P in soils, the enlargement of volume and geometry of the rhizosphere provided by PGPR inoculation determines the amount of P available to plants (Richardson et al., 2009). Therefore, inoculation of PGPR seems to be a reasonable tool to maximize such approach. Microorganisms increase the availability of inorganic P through the production of protons, organic acids, and ligands, which are ubiquitous among rhizosphere P-solubilizing microorganisms (Hinsinger et al., 2011), and also mobilize phytate (organic P) probably by phytase production (Jorquera et al., 2008). However, in greenhouse and/or field conditions, most of the studies do not evaluate different P-fertilization levels, phosphate solubilization in the soil and P uptake by the plants. The majority of the studies considers only plant agronomic parameters and plant N content in conditions with or without N fertilization.

REDUCTION OF P-FERTILIZATION THROUGH PGPR INOCULATION

Increasing P efficiency in crops without increasing or even decreasing P inputs requires a more efficient exploitation of soil microbial resources in agroecosystems. Some studies clearly

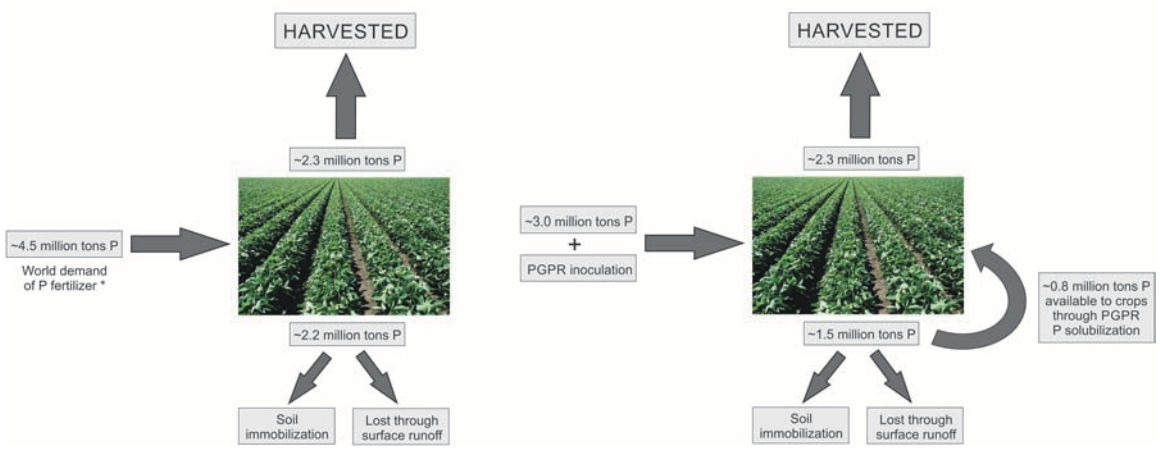


FIGURE 1 | Schematic model of current/traditional approach **(A)** and proposed/biotechnological approach **(B)**. *On the current agricultural approach, world demand of P fertilizer is approximately 4.5 million tons, according to FAO (http://www.fao.org/3/a-i6895e.pdf). From these, 2.2 million tons are unavailable to crops (soil immobilization or surface runoff), and 2.3 million tons are harvested with the crops. On the proposed biotechnological approach, we suggest the reduction of up to 33% on the P fertilizer dose applied on the soil, along with PGPR inoculation. Such reduction on P fertilizer together with PGPR inoculation would result in less P unavailable to the crops. Nearly half of such unavailable P can be further solubilized by PGPR and uptaked by the crops, resulting in the same 2.3 million tons of harvested P (adapted from Roy et al., 2016).

report that plant inoculation with new PGPR can improve P uptake. Rudresh et al. (2005) showed that chickpea plants inoculated with *Rhizobium* sp. and *Bacillus* sp. present higher yield (two-fold) and higher P content (four-fold) in the grain. Vyas and Gulati (2009) and Granada et al. (2013) demonstrated that inoculation of maize (*Zea mays*) with *Pseudomonas* spp., and *Lupinus albescens* plants with free-living *Sphingomonas* sp. results in almost three-fold increases in their shoot P contents, respectively. Studying wheat (*Triticum aestivum* L.) plants, Kumar et al. (2014) showed that inoculation of *Bacillus megaterium*, *Arthrobacter chlorophenolicus,* and *Enterobacter* improves grain yield and the amount of P in the straw and grain up to two-fold in greenhouse and field experiments. Thus, it is already known that inoculation of efficient P-solubilizer bacteria significantly improve P absorption by plants, even though most of the experiments use the recommended P fertilizer dose, and reduction of the P-fertilization has not been evaluated.

Khalafallah et al. (1982) developed an important work inoculating *Vicia faba* plants with P-solubilizing bacteria. This work showed the possibility of reducing the P-fertilization up to 50%, once plants that received half of the recommended P-fertilizer dose presented similar plant dry weight and P-uptake when compared to plants that received usual P-fertilizer dose. More recently, Lavakush et al. (2014) observed the same potential in rice plants inoculated with the P-solubilizing bacteria *Azotobacter chroococcum*, *Azospirillum brasilense*, and combined *Pseudomonas* spp. culture. Inoculated rice plants presented similar performance in plant height, panicle length, grain number per panicle and grain yield when fertilized with 30 and 60 kg P ha^{-1} in a greenhouse experiment. Dutta and Bandyopadhyay (2009) showed that reduction of up to one-third in P-fertilization of chickpea plants (inoculated with P-solubilizing *Pseudomonas* sp.) did not cause any decrease in plant development parameters.

Therefore, PGPR inoculation can probably be used to reduce P-fertilization, being an excellent biotechnological tool. However, this research area is neglected by researches and certainly needs more investigation. All plant species are able to establish a relationship with some PGPR, and the selection of new bacterial isolates, able to solubilize different forms of P *in vitro*, is an important and necessary first step. We hope the results obtained in greenhouse and field inoculation experiments with selected P-solubilizing bacterial isolates and plant species subjected to reduced amounts of P-fertilizer could serve as an alert to producers about the high costs of normally used fertilization strategies, the concerns about finite P sources, and the environmentally friend biotechnological option of using PGPR. Based on previous works which addressed P-solubilization potential by PGPR inoculation in plants (mainly Khalafallah et al., 1982; Dutta and Bandyopadhyay, 2009; Kumar et al., 2014; Lavakush et al., 2014; Anzuay et al., 2015; Kaur and Reddy, 2015), we consider that an average reduction of 33% in P-fertilization could be achieved with the use of high efficient P-solubilizing bacterial isolates as crop inoculants, as indicated on the proposed biotechnological approach in **Figure 1**. Therefore, future experiments need to be specifically designed for such purposes. Considering the complexity of these mechanisms, an interdisciplinary approach taking into account molecular, biochemical, physiological, and agronomic parameters has a good probability to generate positive results. We have a long way to cross until reaching similar knowledge and applicability achieved by bacterial inoculants regarding the reduction of N-fertilizers. However, reasonable use of environmental resources should be the basis for modern and sustainable agriculture development.

AUTHOR CONTRIBUTIONS

All authors listed have made a substantial, direct and intellectual contribution to the work, and approved it for publication.

REFERENCES

Abelson, P. H. (1999). A potential phosphate crisis. *Science* 283:2015. doi: 10.1126/science.283.5410.2015

Anzuay, M. S., Ludueña, L. M., Angelini, J. G., Fabra, A., and Taurian, T. (2015). Beneficial effects of native phosphate solubilizing bacteria on peanut (*Arachis hypogaea* L) growth and phosphorus acquisition. *Symbiosis* 66, 89–97. doi: 10.1007/s13199-015-0337-z

Bouwman, L., Goldewijk, K. K., Van Der Hoek, K. W., Beusen, A. H. W., Van Vuuren, D. P., Willems, J., et al. (2013). Exploring global changes in nitrogen and phosphorus cycles in agriculture induced by livestock production over the 1900-2050 period. *Proc. Natl. Acad. Sci. U.S.A.* 110, 20882–20887. doi: 10.1073/pnas.1012878108

Collavino, M. M., Sansberro, P. A., Mroginski, L. A., and Aguilar, O. M. (2010). Comparison of *in vitro* solubilization activity of diverse phosphate-solubilizing bacteria native to acid soil and their ability to promote *Phaseolus vulgaris* growth. *Biol. Fertil. Soils* 46, 727–738. doi: 10.1007/s00374-010-0480-x

Compant, S., Duffy, B., Nowak, J., Cle, C., and Barka, E. A. (2005). Use of plant growth-promoting bacteria for biocontrol of plant diseases: principles, mechanisms of action, and future prospects. *Appl. Environ. Microbiol.* 71, 4951–4959. doi: 10.1128/AEM.71.9.4951-4959.2005

Dutta, D., and Bandyopadhyay, P. (2009). Performance of chickpea (*Cicer arietinum* L.) to application of phosphorus and bio-fertilizer in laterite soil. *Arch. Agron. Soil Sci.* 55, 147–155. doi: 10.1080/03650340802398864

Galloway, J. N., Aber, J. D., Erisman, J. W., Seitzinger, S. P., Howarth, R. W., Cowling, E. B., et al. (2003). The nitrogen cascade. *Bioscience* 53, 341–356. doi: 10.1641/0006-3568(2003)053[0341:TNC]2.0.CO;2

Granada, C., da Costa, P. B., Lisboa, B. B., Vargas, L. K., and Passaglia, L. M. P. (2013). Comparison among bacterial communities present in arenized and adjacent areas subjected to different soil management regimes. *Plant Soil* 373, 339–358. doi: 10.1007/s11104-013-1796-8

Gupta, G., Parihar, S. S., Ahirwar, N. K., Snehi, S. K., and Singh, V. (2015). Plant growth promoting rhizobacteria (PGPR): current and future prospects for development of sustainable agriculture. *J. Microb. Biochem. Technol.* 7, 96–102. doi: 10.4172/1948-5948.1000188

Hinsinger, P., Betencourt, E., Bernard, L., Brauman, A., Plassard, C., Shen, J., et al. (2011). P for two, sharing a scarce resource: soil phosphorus acquisition in the rhizosphere of intercropped species. *Plant Physiol.* 156, 1078–1086. doi: 10.1104/pp.111.175331

Jones, C., Nomosatryo, S., Crowe, S. A., Bjerrum, C. J., and Canfield, D. E. (2015). Iron oxides, divalent cations, silica, and the early earth phosphorus crisis. *Geology* 43, 135–138. doi: 10.1130/G36044.1

Jorquera, M. A., Hernández, M. T., Rengel, Z., Marschner, P., and De La Luz Mora, M. (2008). Isolation of culturable phosphobacteria with both phytate-mineralization and phosphate-solubilization activity from the rhizosphere of plants grown in a volcanic soil. *Biol. Fertil. Soils* 44, 1025–1034. doi: 10.1007/s00374-008-0288-0

Kaur, G., and Reddy, M. S. (2015). Effects of phosphate-solubilizing bacteria, rock phosphate and chemical fertilizers on maize-wheat cropping cycle and economics. *Pedosphere* 25, 428–437. doi: 10.1016/S1002-0160(15)30010-2

Khalafallah, M. A., Saber, M. S. M., and Abd-El-Maksoun, H. K. (1982). Influence of phosphate dissolving bacteria on the efficiency of superphosphate in a calcareous soil cultivated with *Vicia faba*. *Z. Pflanzenernaehr. Bodenk*. 145, 455–459. doi: 10.1002/jpln.19821450505

Kumar, A., Maurya, B. R., and Raghuwanshi, R. (2014). Isolation and characterization of PGPR and their effect on growth, yield and nutrient content in wheat (*Triticum aestivum* L.). *Biocatal. Agric. Biotechnol.* 3, 121–128. doi: 10.1016/j.bcab.2014.08.003

Lavakush, Y. J., Verma, J. P., Jaiswal, D. K., and Kumar, A. (2014). Evaluation of PGPR and different concentration of phosphorus level on plant growth, yield and nutrient content of rice (*Oryza sativa*). *Ecol. Engin.* 62, 123–128. doi: 10.1016/j.ecoleng.2013.10.013

Marag, P. S., Suman, A., and Gond, S. (2018). Prospecting endophytic bacterial colonization and their potential plant growth promoting attributes in hybrid maize (*Zea mays* L.). *Int. J. Curr. Microbiol. Appl. Sci.* 7, 1292–1304. doi: 10.20546/ijcmas.2018.703.154

Martinez-Viveros, O., Jorquera, M. A., Crowley, D. E., Gajardo, G., and Mora, M. L. (2010). Mechanisms and practical considerations involved in plant growth promotion by Rhizobacteria. *J. Soil Sci. Plant Nutr.* 10, 293–319. doi: 10.4067/S0718-95162010000100006

Mondal, T., Datta, J. K., and Mondal, N. K. (2017). Chemical fertilizer in conjunction with biofertilizer and vermicompost induced changes in morpho-physiological and bio-chemical traits of mustard crop. *J. Saudi Soc. Agric. Sci.* 16, 135–144. doi: 10.1016/j.jssas.2015.05.001

Mouazen, A. M., and Kuang, B. (2016). On-line visible and near infrared spectroscopy for in-field phosphorous management. *Soil Tillage Res.* 155, 471–477. doi: 10.1016/j.still.2015.04.003

Remigi, P., Zhu, J., Young, J. P., and Masson-Boivin, C. (2016). Symbiosis within symbiosis: evolving nitrogen-fixing legume symbionts. *Trends Microbiol.* 24, 63–75. doi: 10.1016/j.tim.2015.10.007

Richardson, A. E., Baréa, J. M., McNeill, A. M., and Prigent-Combaret, C. (2009). Acquisition of phosphorus and nitrogen in the rhizosphere and plant growth promotion by microorganisms. *Plant Soil* 321, 305–339. doi: 10.1007/s11104-009-9895-2

Roy, E. D., Richards, P. D., Martinelli, L. A., Coletta, L. D., Lins, S. R. M., Vazquez, F. F., et al. (2016). The phosphorus cost of agricultural intensification in the tropics. *Nat. Plants* 43, 1–6.

Rudresh, D. L., Shivaprakash, M. K., and Prasad, R. D. (2005). Effect of combined application of *Rhizobium*, phosphate solubilizing bacterium and *Trichoderma* spp. on growth, nutrient uptake and yield of chickpea *(Cicer aritenium L.). Appl. Soil Ecol.* 28, 139–146. doi: 10.1016/j.apsoil.2004.07.005

Sattari, S. Z., Bouwman, A. F., Giller, K. E., and van Ittersum, M. K. (2012). Residual soil phosphorus as the missing piece in the global phosphorus crisis puzzle. *Proc. Natl. Acad. Sci. U.S.A.* 109, 6348–6353. doi: 10.1073/pnas.1113675109

Souza, R., Beneduzi, A., Ambrosini, A., Costa, P. B., Meyer, J., Vargas, L. K., et al. (2013). The effect of plant growth-promoting rhizobacteria on the growth of rice (*Oryza sativa* L.) cropped in southern Brazilian fields. *Plant Soil* 366, 585–603. doi: 10.1038/nplants.2016.43

Souza, R., Meyer, J., Schoenfeld, R., Costa, P. B., and Passaglia, L. M. P. (2015). Characterization of plant growth-promoting bacteria associated with rice cropped in iron-stressed soils. *Ann. Microbiol.* 65, 951–964. doi: 10.1007/s13213-014-0939-3

Stewart, W. M., Dibb, D. W., Johnston, A. E., and Smyth, T. J. (2005). The contribution of commercial fertilizer nutrients to food production. *Agron. J.* 97, 1–6. doi: 10.2134/agronj2005.0001

Vaccari, D. A. (2009). Phosphorus: a looming crisis. *Scientific American* 300, 54–59. doi: 10.1038/scientificamerican0609-54

Vyas, P., and Gulati, A. (2009). Organic acid production *in vitro* and plant growth promotion in maize under controlled environment by phosphate-solubilizing fluorescent *Pseudomonas*. *BMC Microbiol.* 9:174. doi: 10.1186/1471-2180-9-174

Walitang, D. I., Kim, K., Madhaiyan, M., Kim, Y. K., Kang, Y., and Sa, T. (2017). Characterizing endophytic competence and plant growth promotion of bacterial endophytes inhabiting the seed endosphere of rice. *BMC Microbiol.* 17:209. doi: 10.1186/s12866-017-1117-0

Conflict of Interest Statement: The authors declare that the research was conducted in the absence of any commercial or financial relationships that could be construed as a potential conflict of interest.

frontiers in Plant Science

MINI REVIEW
published: 10 September 2018
doi: 10.3389/fpls.2018.01329

Arbuscular Mycorrhizas: A Promising Component of Plant Production Systems Provided Favorable Conditions for Their Growth

Michael Bitterlich[1], Youssef Rouphael[2], Jan Graefe[1] and Philipp Franken[1,3]*

[1] Department of Plant-Microbe Interactions, Leibniz-Institute of Vegetable and Ornamental Crops e.V., Großbeeren, Germany, [2] Department of Agricultural Sciences, University of Naples Federico II, Naples, Italy, [3] Department of Plant Physiology, Institute of Biology, Humboldt-Universität zu Berlin, Berlin, Germany

Arbuscular mycorrhizal (AM) fungi have become an attractive target as biostimulants in agriculture due to their known contributions to plant nutrient uptake and abiotic stress tolerance. However, inoculation with AM fungi can result in depressed, unchanged, or stimulated plant growth, which limits security of application in crop production systems. Crop production comprises high diversity and variability in atmospheric conditions, substrates, plant species, and more. In this review, we emphasize that we need integrative approaches for studying mycorrhizal symbioses in order to increase the predictability of growth outcomes and security of implementation of AM fungi into crop production. We briefly review known mechanisms of AM on nutrient uptake and drought tolerance of plants, on soil structure and soil hydraulic properties. We carve out that an important factor for both nutrient availability and drought tolerance is yet not well understood; the AM effects on soil hydraulic properties. We gave special emphasis to circular references between atmospheric conditions, soil hydraulic properties and plant nutrient and water uptake. We stress that interdisciplinary approaches are needed that account for a variability of atmospheric conditions and, how this would match to mycorrhizal functions and demands in a way that increased plant nutrient and water uptake can be effectively used for physiological processes and ultimately growth. Only with integrated analyses under a wide range of growing conditions, we will be able to make profound decisions whether or not to use AM in particular crop production systems or can adjust culture conditions in ways that AM plants thrive.

Keywords: arbuscular mycorrhiza, soil hydraulic properties, plant production, environment, atmospheric conditions

OPEN ACCESS

Edited by:
Angeles Calatayud, Instituto Valenciano de Investigaciones Agrarias, Spain

Reviewed by:
Consuelo Penella, Instituto Valenciano de Investigaciones Agrarias, Spain
Vincenzo Candido, University of Basilicata, Italy

****Correspondence:***
Michael Bitterlich
Bitterlich@igzev.de

Specialty section:
This article was submitted to Crop and Product Physiology, a section of the journal Frontiers in Plant Science

Received: *22 February 2018*
Accepted: *23 August 2018*
Published: *10 September 2018*

Citation:
Bitterlich M, Rouphael Y, Graefe J and Franken P (2018) Arbuscular Mycorrhizas: A Promising Component of Plant Production Systems Provided Favorable Conditions for Their Growth. Front. Plant Sci. 9:1329. doi: 10.3389/fpls.2018.01329

VARIABLE PLANT GROWTH RESPONSES TO THE APPLICATION OF MYCORRHIZA IN COMMERCIAL PRODUCTION SYSTEMS

In search for improvements in sustainability and resource use efficiency in agriculture, application of arbuscular mycorrhizal (AM) fungi has become an attractive tool given its known contribution to nutrient acquisition, pathogen resistance, and abiotic stress tolerance of plants (Rouphael et al., 2015). This ubiquitous symbiosis is characterized by low host specificity, but also by variable plant responses in experimental systems and plant production (Smith and Read, 2010).

Inoculation with AMF under nursery conditions, in pots and in fields can increase plant growth and marketable yield of many horticultural crops (Regvar et al., 2003; Mena-Violante et al., 2006; Sorensen et al., 2008; Candido et al., 2013, 2015; Conversa et al., 2013; Colla et al., 2015), but this is not always the case (Bosco et al., 2007). Research studies revealed a mutualism-parasitism continuum in terms of symbiotic outcomes (Johnson et al., 1997). From an ecological point of view, the whole symbiotic continuum can underpin the resilience of symbiotic plants under particular environmental conditions. Reduced growth may allow for prolonged survival periods under resource-limited conditions, while enhanced growth may drive advantages for resource acquisition. In contrast to natural systems, growers seek to grow plants efficiently and maximize yields under given resources and economic inputs; hence, positive mycorrhizal growth responses are desired by growers. At the same time, growers are tasked with growing plants artificially and in protected environments. Likewise, plant scientists grow plants under engineered nutrient and water conditions, in restricted volumes, on constructed substrates, and at times under suboptimal climatic conditions. Those man-made conditions may be on the boundaries of evolutionary spectra or beyond, which can drive variability and unpredictability of growth outcomes under random application of AM fungi (Johnson et al., 1997) and hence, promote uncertainty rather than desired benefits for plant productivity. To confront that, one needs awareness that the application of AM fungi adds complexity to the cultivation system and advanced understanding is required of the growing conditions under which proper mycorrhizal function mitigates plant growth limitations.

Plant growth in particular environments is mostly determined by climatic conditions and the properties of the growing medium. As sessile organisms, plants have developed mechanisms for adjusting to variable environments in order to match resource supply and demand for survival and growth. AM fungi contribute to nutrient and water supply, but as obligate biotrophs, they also increase symbiotic carbon demands to develop hyphae and to complete their life cycle (Smith and Read, 2010). Hence, AM fungi application to production systems in particular, which are characterized by certain constraints to resource supply and demand, adds costs and benefits for plants. Since AM fungi change both resource supply and demand, it is likely that optimal growth conditions for symbiotic plants will differ from those required for non-symbiotic plants. All AM fungi have in common that they grow beyond the ambit of roots and can directly deliver nutrients to hosts. Besides direct nutrient delivery, they also change the properties of the growing medium (Rillig and Mummey, 2006; Rillig et al., 2010; Leifheit et al., 2014), which in turn alters constraints to nutrient and water extractability from this medium. The extent of AM effects in changing soil properties and their relevance to plant physiology and growth expectably differs with the underground habitat, i.e., physical and chemical properties and volume of soils and substrates (Querejeta, 2017), and the accompanied climatic conditions (Bitterlich et al., 2018b). This either allows effective usage of changes in supply or creates costs by superfluity of additional supply under low demand scenarios. Thus, integrative approaches that account for climatic conditions and edaphic properties are required to maximize and stabilize mycorrhizal growth outcomes.

NUTRITIONAL BENEFITS AND DROUGHT TOLERANCE OF MYCORRHIZAL PLANTS: TWO EFFECTS OF THE SAME ORIGIN?

The best understood characteristic of AM symbioses is the delivery of phosphorus (P) to plants from areas beyond root P depletion zones. Phosphorus is taken up by extraradical hyphae, transported toward the root and delivered to the plant via intracellular highly branched organs called arbuscules (Smith and Read, 2010). Since plant P uptake depends on diffusion and P has a low mobility in many soils, plant P uptake causes the formation of P depletion zones around roots, which are bypassed by fungal hyphae (Schachtman et al., 1998). Thus, AM plants become less dependent on the P solute concentration around roots. When soil moisture declines, water filled pores are emptied, reducing the diffusional cross sections and the extent of P depletion zones around roots (Gahoonia et al., 1994). Hyphae may cross P depletion zones and air-filled pore spaces, hence providing less tortuous pathways for P, making plant P uptake less dependent on soil moisture, and allow maintenance of higher P flow toward roots under drought.

For extraradical growth, AM fungi receive carbon from plant photosynthesis. Hence, with hyphal proliferation, carbon is redistributed to hyphae beyond root areas and into soils (Jakobsen and Rosendahl, 1990). The addition of organic matter and/or AM fungi into soils is known to contribute to soil structure, e.g., by entangling and enmeshing particles to aggregates, bridging of large voids, or by releasing organic matter during turnover (Miller and Jastrow, 2000; Rillig and Mummey, 2006). This in turn will modulate water retention (Rawls et al., 2003) and water and solute mobility, i.e., hydraulic conductivity (Durner, 1994), and thereby, water extractability for plants. Hitherto, the importance of AM effects on soil hydraulic properties for plant water relations is not well understood or characterized and will vary considerably with species identity, substrate properties, and rooting density. Substrate hydraulic properties influence the moisture stress that plants experience (Tardieu and Simonneau, 1998). The soil water status is sensed by roots via hydraulic and non-hydraulic signals (Tardieu and Davies, 1993); hence, apparent changes in soil hydraulic properties induced by AM fungi imply differential sensing of drought intensities by plants growing in AM and NM substrates given equal irrigation and/or during different time points in drying episodes. AM plants often grow better under moisture stress and AM-colonized substrates often have to dry more before host plants achieve a comparable physiological drought response (Augé, 2001). That may delay inhibition of transpiration and

subsequently water flow in AM substrates (Bitterlich et al., 2018a,b).

In addition to putatively altered plant stress responses upon water retention and hydraulic conductivity modulations in AM substrates, AM colonized substrates may possess different water and solute flow resistances. If mycorrhizal effects on substrate hydraulic properties cause a reduction of flow resistance and/or an enhancement of water availability, two scenarios could be proposed: (i) AM plants either require fewer physiological adjustments to scavenge water and nutrients or, (ii) increased acquisition of water and nutrients facilitates resource deployment for growth while expending equal investments in physiological stress responses.

Both putative scenarios have the same prerequisites: AM fungi must colonize additional substrate volumes and, the plant must deliver the necessary resources for that. A feasible point of view would be that observed alterations of plant physiological responses in AM plants upon particular moisture levels are at least partially corollary effects of changes in substrate properties.

UNDERSTANDING FAVORABLE GROWTH CONDITIONS CAN UNDERPIN STABILITY OF GROWTH OUTCOMES: AN INTEGRATED VIEW

In nature and in plant production systems, continuous crop cycles face periodical transitions in growth limiting factors deriving from aerial and underground conditions. Conceptually, conditions under which the application of AM fungi leads to a mitigation of growth limiting factors are desired by growers. To elucidate those conditions, the fact that AM fungi are biotrophic soil microbes should be the basis. It is necessary to determine the underground constraints for nutrient and water availability, the variation of mycorrhizal impact on these constraints across different soils or substrates, and the corresponding atmospheric conditions that create a scenario where growth is limited by underground supply.

The increased independency of AM plants on P availability around roots can mitigate growth limitations under low P availability (Smith and Read, 2010). However, alleviation of P starvation in AM plants may not necessarily promote growth if analogous demands for nutrients other than P, especially nitrogen (N), cannot be satisfied. N and P have been shown to be the main nutrients regulating symbiotic intensity and growth responses and, insufficient N supply can lead to marked decreases in N/P ratios and relative N starvation in aerial tissues (Nouri et al., 2014). Although N may also be delivered by hyphae in non-negligible amounts (Govindarajulu et al., 2005), significant plant N acquisition (nitrate under most scenarios) is driven by plant transpiration, which induces mass flow toward the root and/or affects N diffusion indirectly (Oyewole et al., 2014). Vice versa, N availability determines mass flow driven acquisition of other nutrients (Matimati et al., 2013). This constitutes a circular reference to constraints set by substrate hydraulic characteristics and alterations in physiological moisture stress response (Matimati et al., 2013). Hence, direct (hyphal delivery) and indirect (substrate transport) AM effects in concert may be decisive factors in growth-limiting environments. A grower's desire would then be growing conditions, where both ways of delivery are stimulated by AM fungi.

Frequently observed AM fungi-mediated changes to plant nutrient levels and drought stress responses (stomatal conductance) alter the capacity for leaf C assimilation (Augé, 2001; Augé et al., 2015). It is imperative that growth of AM plants should not be carbon limited, since photosynthesis also feeds the fungus. Indeed, it is known that low light intensities can be detrimental to positive growth responses in AM plants (Konvalinková and Jansa, 2016). This is logical, because apparent improvements in leaf nutrient status and CO_2 availability (stomatal conductance) can become superfluous if the photosynthetic process is energy limited. Under such conditions, photosynthetic nutrient and water use efficiency would decline in AM plants. Then, AM fungi can constitute a carbon costly scenario at the expense of fungal demands (Konvalinková et al., 2015). Assuming photosynthesis is not energy restrained and leaf nutrient contents are high in AM plants, stomatal conductance should not limit photosynthesis. Vice versa, if nutrient contents are not affected and sufficient light is available, higher stomatal conductance in AM plants could alleviate CO_2-imposed limitation to photosynthesis. Both scenarios can constitute a carbon gain scenario, which should be targeted by the grower. To close the circle, stomatal conductance will respond to substrate water potentials sensed by plant roots (Tardieu and Simonneau, 1998), which is determined partly by the water holding and transport capacity of the substrate. Such AM-induced alterations exhibit particularity for the combination of substrate, fungus, and plant.

MYCORRHIZAL EFFECTS ON SUBSTRATE HYDRAULIC PROPERTIES MAY BE CRITICAL FOR SYMBIOTIC PLANT GROWTH AND NUTRIENT-WATER UPTAKE, BUT THEIR ACTUAL CONTRIBUTION REMAINS UNQUANTIFIABLE

The effects of AM fungi on soil structure have been studied since decades and were reviewed extensively (Miller and Jastrow, 2000; Rillig and Mummey, 2006; Leifheit et al., 2014). These effects are specific to substrate and fungus–plant interaction and are mostly considered to originate from stabilization, formation (Leifheit et al., 2014), or even breakdown of aggregates, which induce changes to the secondary structure of soils. Besides AM effects on aggregation, AM fungi can have surfactant effects, i.e., they may change surface wettability and profiles of soil particles (Hallett, 2008; Rillig et al., 2010). AM fungi could also form micro-channels or contribute to the interconnectivity of pore spaces, by serving as a solid pathway for water across air spaces with direct contact to roots (Miller and Jastrow,

2000). The development of secondary structure and the other mentioned effects on soils contribute to soil water retention and hydraulic conductivity (Durner, 1992, 1994; Hallett, 2008), which are quantitative measures for underground water availability and transport. Water retention in rooted substrates has been shown to be affected by AM inoculation in different directions in soils and constructed substrates (Augé et al., 2001; Bearden, 2001; Augé, 2004; Daynes et al., 2013; Bitterlich et al., 2018a,b). Hitherto, only one study used root exclusion compartments to test whether hyphal ingrowth alone is sufficient to induce substrate water retention changes (Bitterlich et al., 2018a). Comparative studies that analyze AM effects in different substrates are missing, although physical reasoning implies that AM fungi-induced modifications of soil hydraulic properties will be substrate specific (Querejeta, 2017), depending on which soil or substrate matrix with its given properties is "offered" to AM fungi. Only recently, unsaturated hydraulic conductivity was observed to be enhanced in equally rooted substrates (Bitterlich et al., 2018b) and under root growth exclusion (Bitterlich et al., 2018a). To the best of our knowledge, those are the only studies that report AM influence on unsaturated hydraulic conductivity of substrates as a strict physical property independent of plant or fungal activity.

Water retention and hydraulic conductivity set constraints to plant water uptake and induce particular plant physiological responses to water shortage (Tardieu and Simonneau, 1998). In addition, water retention and hydraulic conductivity limit water and solute movement in the soil (Vogel et al., 2000). Feasibly, changes to soil hydraulic properties upon AM colonization could induce alterations in the physiological stress response of plants and, hence, induce subsequent changes to aerial plant gas exchange (Augé et al., 2001; Augé, 2004) and underground water and nutrient uptake.

Commonly observed is that AM plants require prolonged drying or more thoroughly dried substrates, before plants achieve a comparable physiological drought response (reviewed in Augé, 2001; Khalvati et al., 2005). In addition, non-hosts growing in a mycorrhizal soil showed improved stomatal conductance (Augé, 2004). And, whole plant transpiration of AM plants of equal size and root length densities was quantitatively limited by soil water flux, at higher drought intensity, i.e., substrate water potentials (Bitterlich et al., 2018b). The study showed that AM alleviated soil water flux limitations by enhancing hydraulic conductivity under higher drought intensities and by mitigating the decline in substrate water potential with water loss. Especially the latter cited studies imply that plants growing in a mycorrhizal substrate experience an underground environment that differs from non-colonized substrates in its hydraulic constraints (Augé et al., 2001; Augé, 2004; Bitterlich et al., 2018a,b). The result is that AM plants will respond with physiological adjustments to drought at varying soil water contents and/or consume resources in different time frames, which at least partially does not require direct alterations of their physiological state (Bitterlich et al., 2018b).

The vast majority of AM studies dealing with plant reactions to drought use distinct drought treatments, e.g., maintaining particular degrees of water contents/potentials during cultivation (e.g., Ruiz-Lozano et al., 1995; Porcel and Ruiz-Lozano, 2004), which are determined in advance (e.g., Subramanian et al., 1995; Porcel and Ruiz-Lozano, 2004), deliver a particular volume of water per irrigation cycle or withhold water for a certain time (e.g., Allen et al., 1981; Nelsen and Safir, 1982; Duan et al., 1996; Khalvati et al., 2005; Aroca et al., 2007; Ruth et al., 2011). However, indication mounts that soil water contents/potentials evoked by particular amounts of irrigation can diverge in AM and NM substrates after a certain time of cultivation (Bitterlich et al., 2018b). In particular, water contents do not need to correspond to equal water potentials or hydraulic conductivities in colonized and non-colonized substrates (Augé et al., 2001; Bitterlich et al., 2018a,b). This may change the physiological drought reaction in the plant. Moreover, a different physiological stress response in AM plants may induce shifts to mass flows in the substrate driven by altered transpiration (Bitterlich et al., 2018b), which in turn would affect mass flow driven nutrient transport (Matimati et al., 2013). Consequently, altered substrate drying rates induced by changes in substrate hydraulic conductivity or changed plant activity will result in different soil water contents. Subsequently, the resulting changes in soil moisture can lead to changes in water dependent nutrient diffusion coefficients, e.g., for P (Bhadoria et al., 1991; Gahoonia et al., 1994).

The effects of AM fungi on soil hydraulic properties will be substrate specific but are far from elucidated (Querejeta, 2017). At any rate, changes to hydraulic properties will partly determine the optimal choice for irrigation and fertilization practices that stimulate mycorrhizal benefits and avoid detrimental conditions for AM plants under given climatic factors. A mechanistic understanding of AM fungi-induced changes in hydraulic properties is therefore required and should be targeted in future research.

CHALLENGES AHEAD

A positive AM-induced plant growth response will depend on the sum of events that constitute nutrient and water benefits by AM fungi, which can be efficiently used for carbon assimilation. Cultural practices for intensive plant production, normally absent in nature, impose risks to the safe use of AM fungi. Constraints to soil volumes, for example, hinder subsequent delivery of nutrients and water from the periphery and limit the explored volume. This may salvage economic risks caused by superfluity of application when AM effects become marginal, e.g., under high rooting densities and unfavorable growth conditions. Additionally, a more exhaustive behavior of AM plants can be induced when uptake is higher under particular growing conditions, which is less buffered by limited substrate volumes or not timely met by irrigation and fertilization (Bitterlich et al., 2018b). These shifts to time frames of resource exploitations in AM plants are dependent on atmospheric conditions and have to be known if growers want to adjust growing conditions to those in which AM plants thrive. Moreover, integrated approaches should be used that account for atmospheric conditions, plant and rhizosphere sizes and soil hydraulic properties. Mechanistic photosynthesis-based

(e.g., Farquhar et al., 1980; Farquhar and Von Caemmerer, 1982) and quantitative plant-based uptake models (e.g., van Lier et al., 2013) are available, but have yet been scarcely applied to AM-studies (e.g., Boldt et al., 2011; Romero-Munar et al., 2017; Bitterlich et al., 2018b). Such models enable to quantify limitations of carbon assimilation and of water and nutrient uptake. Plant-based models also enable to analyze AM and NM plants of different sizes, because this is accounted for by implicit inputs. Knowing how substrate-borne limitations are mitigated by AM fungi and, under which conditions those mitigations become effective, will guide modulation of growing conditions to produce enlarged AM plants or enable decision making on whether or not to use AM fungi in a particular occasion.

AUTHOR CONTRIBUTIONS

MB wrote the manuscript. YR, JG, and PF critically revised the manuscript and contributed to writing.

FUNDING

The project received funding from the Ministry of Consumer Protection, Food and Agriculture of the Federal Republic of Germany, from the Ministry for Science, Research and Culture of the State of Brandenburg, and from the Thuringian Ministry of Infrastructure and Agriculture.

REFERENCES

Allen, M. F., Smith, W. K., Moore, T. S., and Christensen, M. (1981). Comparative water relations and photosynthesis of mycorrhizal and non-mycorrhizal *Bouteloua Gracilis* HBK Lag Ex Steud. *New Phytol.* 88, 683–693. doi: 10.1111/j.1469-8137.1981.tb01745.x

Aroca, R., Porcel, R., and Ruiz Lozano, J. M. (2007). How does arbuscular mycorrhizal symbiosis regulate root hydraulic properties and plasma membrane aquaporins in *Phaseolus vulgaris* under drought, cold or salinity stresses? *New Phytol.* 173, 808–816. doi: 10.1111/j.1469-8137.2006.01961.x

Augé, R. M. (2001). Water relations, drought and vesicular-arbuscular mycorrhizal symbiosis. *Mycorrhiza* 11, 3–42. doi: 10.1007/s005720100097

Augé, R. M. (2004). Arbuscular mycorrhizae and soil/plant water relations. *Can. J. Soil Sci.* 84, 373–381. doi: 10.4141/s04-002

Augé, R. M., Stodola, A. J. W., Tims, J. E., and Saxton, A. M. (2001). Moisture retention properties of a mycorrhizal soil. *Plant Soil* 230, 87–97. doi: 10.3389/fpls.2018.00154

Augé, R. M., Toler, H. D., and Saxton, A. M. (2015). Arbuscular mycorrhizal symbiosis alters stomatal conductance of host plants more under drought than under amply watered conditions: a meta-analysis. *Mycorrhiza* 25, 13–24. doi: 10.1007/s00572-014-0585-4

Bearden, B. N. (2001). Influence of arbuscular mycorrhizal fungi on soil structure and soil water characteristics of vertisols. *Plant Soil* 229, 245–258. doi: 10.1023/A:1004835328943

Bhadoria, P. B. S., Kaselowsky, J., Claassen, N., and Jungk, A. (1991). Phosphate diffusion coefficients in soil as affected by bulk density and water content. *J. Plant Nutr. Soil Sci.* 154, 53–57. doi: 10.1002/jpln.19911540111

Bitterlich, M., Franken, P., and Graefe, J. (2018a). Arbuscular Mycorrhiza improves substrate hydraulic conductivity in the plant available moisture range under root growth exclusion. *Front. Plant Sci.* 9:301. doi: 10.3389/fpls.2018.00301

Bitterlich, M., Sandmann, M., and Graefe, J. (2018b). Arbuscular mycorrhiza alleviates restrictions to substrate water flow and delays transpiration limitation to stronger drought in tomato. *Front. Plant Sci.* 9:154. doi: 10.3389/fpls.2018.00154

Boldt, K., Pors, Y., Haupt, B., Bitterlich, M., Kuhn, C., Grimm, B., et al. (2011). Photochemical processes, carbon assimilation and RNA accumulation of sucrose transporter genes in tomato arbuscular mycorrhiza. *J. Plant Physiol.* 168, 1256–1263. doi: 10.1016/j.jplph.2011.01.026

Bosco, M., Giovannetti, G., Picard, C., Baruffa, E., Brondolo, A., and Sabbioni, F. (2007). "Commercial plant-probiotic microorganisms for sustainable organic tomato production systems," in *Proceedings of the 3rd QLIF Congress: Improving Sustainability in Organic and Low Input Food Production Systems,* (Stuttgart: University of Hohenheim), 468–471.

Candido, V., Campanelli, G., D'Addabbo, T., Castronuovo, D., Renco, M., and Camele, I. (2013). Growth and yield promoting effect of artificial mycorrhization combined with different fertiliser rates on field-grown tomato. *Ital. J. Agron.* 8:22. doi: 10.4081/ija.2013.e22

Candido, V., Campanelli, G. D., Addabbo, T., Castronuovo, D., Perniola, M., and Camele, I. (2015). Growth and yield promoting effect of artificial mycorrhization on field tomato at different irrigation regimes. *Sci. Hortic.* 187, 35–43. doi: 10.1016/j.scienta.2015.02.033

Colla, G., Rouphael, Y., Di Mattia, E., El-Nakhel, C., and Cardarelli, M. (2015). Co inoculation of *Glomus intraradices* and *Trichoderma atroviride* acts as a biostimulant to promote growth, yield and nutrient uptake of vegetable crops. *J. Sci. Food Agric.* 95, 1706–1715. doi: 10.1002/jsfa.6875

Conversa, G., Lazzizera, C., Bonasia, A., and Elia, A. (2013). Yield and phosphorus uptake of a processing tomato crop grown at different phosphorus levels in a calcareous soil as affected by mycorrhizal inoculation under field conditions. *Biol. Fertil. Soils* 49, 691–703. doi: 10.1007/s00374-012-0757-3

Daynes, C. N., Field, D. J., Saleeba, J. A., Cole, M. A., and McGee, P. A. (2013). Development and stabilisation of soil structure via interactions between organic matter, arbuscular mycorrhizal fungi and plant roots. *Soil Biol. Biochem.* 57, 683–694. doi: 10.1016/j.soilbio.2012.09.020

Duan, X. G., Neuman, D. S., Reiber, J. M., Green, C. D., Saxton, A. M., and Augé, R. M. (1996). Mycorrhizal influence on hydraulic and hormonal factors implicated in the control of stomatal conductance during drought. *J. Exp. Bot.* 47, 1541–1550. doi: 10.1093/jxb/47.10.1541

Durner, W. (1992). "Predicting the unsaturated hydraulic conductivity using multi-porosity water retention curves," in *Indirect Methods for Estimating the Hydraulic Properties of Unsaturated Soils*, ed. L. J. Lund (Berkeley: University of Californoia), 185–202.

Durner, W. (1994). Hydraulic conductivity estimation for soils with heterogeneous pore structure. *Water Resour. Res.* 30, 211–223. doi: 10.1029/93WR02676

Farquhar, G., and Von Caemmerer, S. (1982). "Modelling of photosynthetic response to environmental conditions," in . *Physiological Plant Ecology II*, ed. O. L. Lange (Berlin: Springer), 549–587. doi: 10.1007/978-3-642-68150-9_17

Farquhar, G., Von Caemmerer, S., and Berry, J. (1980). A biochemical model of photosynthetic CO2 assimilation in leaves of C3 species. *Planta* 149, 78–90. doi: 10.1007/BF00386231

Gahoonia, T. S., Raza, S., and Nielsen, N. E. (1994). Phosphorus depletion in the rhizosphere as influenced by soil moisture. *Plant Soil* 159, 213–218. doi: 10.1007/BF00009283

Govindarajulu, M., Pfeffer, P. E., Jin, H., Abubaker, J., Douds, D. D., Allen, J. W., et al. (2005). Nitrogen transfer in the arbuscular mycorrhizal symbiosis. *Nature* 435, 819–823. doi: 10.1038/nature03610

Hallett, P. D. (2008). A brief overview of the causes, impacts and amelioration of soil water repellency-a review. *Soil Water Res.* 3, 521–528. doi: 10.17221/1198-swr

Jakobsen, I., and Rosendahl, L. (1990). Carbon flow into soil and external hyphae from roots of mycorrhizal cucumber plants. *New Phytol.* 115, 77–83. doi: 10.1111/j.1469-8137.1990.tb00924.x

Johnson, N. C., Graham, J. H., and Smith, F. A. (1997). Functioning of mycorrhizal associations along the mutualism-parasitism continuum. *New Phytol.* 135, 575–586. doi: 10.1046/j.1469-8137.1997.00729.x

Khalvati, M. A., Hu, Y., Mozafar, A., and Schmidhalter, U. (2005). Quantification of water uptake by arbuscular mycorrhizal hyphae and its significance for leaf growth, water relations, and gas exchange of barley subjected to drought stress. *Plant Biol.* 7, 706–712. doi: 10.1055/s-2005-872893

Konvalinková, T., and Jansa, J. (2016). Lights off for arbuscular mycorrhiza: on its symbiotic functioning under light deprivation. *Front. Plant Sci.* 7:782. doi: 10.3389/fpls.2016.00782

Konvalinková, T., Püschel, D., Janoušková, M., Gryndler, M., and Jansa, J. (2015). Duration and intensity of shade differentially affects mycorrhizal growth-and phosphorus uptake responses of *Medicago truncatula*. *Front. Plant Sci.* 6:65. doi: 10.3389/fpls.2015.00065

Leifheit, E. F., Veresoglou, S. D., Lehmann, A., Morris, E. K., and Rillig, M. C. (2014). Multiple factors influence the role of arbuscular mycorrhizal fungi in soil aggregation-a meta-analysis. *Plant Soil* 374, 523–537. doi: 10.1007/s11104-013-1899-2

Matimati, I., Verboom, G. A., and Cramer, M. D. (2013). Nitrogen regulation of transpiration controls mass-flow acquisition of nutrients. *J. Exp. Bot.* 65, 159–168. doi: 10.1093/jxb/ert367

Mena-Violante, H. G., Ocampo-Jiménez, O., Dendooven, L., Martínez-Soto, G., González-Castaneda, J., Davies, F. T., et al. (2006). Arbuscular mycorrhizal fungi enhance fruit growth and quality of chile ancho (*Capsicum annuum* L. cv San Luis) plants exposed to drought. *Mycorrhiza* 16, 261–267. doi: 10.1007/s00572-006-0043-z

Miller, R., and Jastrow, J. (2000). "Mycorrhizal fungi influence soil structure," in *Arbuscular mycorrhizas: Physiology and Function*, ed. Y. Kapulnik (Berlin: Springer), 3–18. doi: 10.1007/978-94-017-0776-3_1

Nelsen, C. E., and Safir, G. (1982). Increased drought tolerance of mycorrhizal onion plants caused by improved phosphorus nutrition. *Planta* 154, 407–413. doi: 10.1007/BF01267807

Nouri, E., Breuillin-Sessoms, F., Feller, U., and Reinhardt, D. (2014). Phosphorus and nitrogen regulate arbuscular mycorrhizal symbiosis in *Petunia hybrida*. *PLoS One* 9:e90841. doi: 10.1371/journal.pone.0090841

Oyewole, O. A., Inselsbacher, E., and Näsholm, T. (2014). Direct estimation of mass flow and diffusion of nitrogen compounds in solution and soil. *New Phytol.* 201, 1056–1064. doi: 10.1111/nph.12553

Porcel, R., and Ruiz-Lozano, J. M. (2004). Arbuscular mycorrhizal influence on leaf water potential, solute accumulation, and oxidative stress in soybean plants subjected to drought stress. *J. Exp. Bot.* 55, 1743–1750. doi: 10.1093/jxb/erh188

Querejeta, J. (2017). "Soil water retention and availability as influenced by mycorrhizal symbiosis: consequences for individual plants, communities, and ecosystems," in *Mycorrhizal Mediation of Soil*, ed. J. Jansa (New York, NY: Elsevier), 299–317. doi: 10.1016/B978-0-12-804312-7.00017-6

Rawls, W., Pachepsky, Y. A., Ritchie, J., Sobecki, T., and Bloodworth, H. (2003). Effect of soil organic carbon on soil water retention. *Geoderma* 116, 61–76. doi: 10.1016/S0016-7061(03)00094-6

Regvar, M., Vogel-Mikus, K., and Severkar, T. (2003). Effect of AMF inoculum from field isolates on the yield of green pepper, parsley, carrot, and tomato. *Folia Geobot.* 38, 223–234. doi: 10.1007/BF02803154

Rillig, M. C., Mardatin, N. F., Leifheit, E. F., and Antunes, P. M. (2010). Mycelium of arbuscular mycorrhizal fungi increases soil water repellency and is sufficient to maintain water-stable soil aggregates. *Soil Biol. Biochem.* 42, 1189–1191. doi: 10.1016/j.soilbio.2010.03.027

Rillig, M. C., and Mummey, D. L. (2006). Mycorrhizas and soil structure. *New Phytol.* 171, 41–53. doi: 10.1111/j.1469-8137.2006.01750.x

Romero-Munar, A., Del-Saz, N. F., Ribas-Carbó, M., Flexas, J., Baraza, E., Florez-Sarasa, I., et al. (2017). Arbuscular mycorrhizal symbiosis with *Arundo donax* decreases root respiration and increases both photosynthesis and plant biomass accumulation. *Plant Cell Environ.* 40, 1115–1126. doi: 10.1111/pce.12902

Rouphael, Y., Franken, P., Schneider, C., Schwarz, D., Giovannetti, M., Agnolucci, M., et al. (2015). Arbuscular mycorrhizal fungi act as biostimulants in horticultural crops. *Sci. Hortic.* 196, 91–108. doi: 10.1016/j.scienta.2015.09.002

Ruiz-Lozano, J. M., Azcon, R., and Gomez, M. C. (1995). Effects of Arbuscular-Mycorrhizal *Glomus* species on drought tolerance - physiological and nutritional plant-responses. *Appl. Environ. Microbiol.* 61, 456–460.

Ruth, B., Khalvati, M., and Schmidhalter, U. (2011). Quantification of mycorrhizal water uptake via high-resolution on-line water content sensors. *Plant Soil* 342, 459–468. doi: 10.1007/s11104-010-0709-3

Schachtman, D. P., Reid, R. J., and Ayling, S. M. (1998). Phosphorus uptake by plants: from soil to cell. *Plant Physiol.* 116, 447–453. doi: 10.1104/pp.116.2.447

Smith, S. E., and Read, D. J. (2010). *Mycorrhizal Symbiosis*. London: Academic press.

Sorensen, J. N., Larsen, J., and Jakobsen, I. (2008). Pre-inoculation with arbuscular mycorrhizal fungi increases early nutrient concentration and growth of field-grown leeks under high productivity conditions. *Plant Soil* 307, 135–147. doi: 10.1007/s11104-008-9591-7

Subramanian, K. S., Charest, C., Dwyer, L. M., and Hamilton, R. I. (1995). Arbuscular mycorrhizas and water relations in maize under drought stress at tasselling. *New Phytol.* 129, 643–650. doi: 10.1111/j.1469-8137.1995.tb03033.x

Tardieu, F., and Davies, W. (1993). Integration of hydraulic and chemical signalling in the control of stomatal conductance and water status of droughted plants. *Plant Cell Environ.* 16, 341–349. doi: 10.1111/j.1365-3040.1993.tb00880.x

Tardieu, F., and Simonneau, T. (1998). Variability among species of stomatal control under fluctuating soil water status and evaporative demand: modelling isohydric and anisohydric behaviours. *J. Exp. Bot.* 49, 419–432. doi: 10.1093/jxb/49.special_issue.419

van Lier, Q., de van Dam, J. C., Durigon, A., Dos Santos, M. A., and Metselaar, K. (2013). Modeling water potentials and flows in the soil–plant system comparing hydraulic resistances and transpiration reduction functions. *Vadose Zone J.* 12:vzj2013.02.0039. doi: 10.2136/vzj2013.02.0039

Vogel, T., Gerke, H. H., Zhang, R., and Van Genuchten, M. T. (2000). Modeling flow and transport in a two-dimensional dual-permeability system with spatially variable hydraulic properties. *J. Hydrol.* 238, 78–89. doi: 10.1016/S0022-1694(00)00327-9

Conflict of Interest Statement: The authors declare that the research was conducted in the absence of any commercial or financial relationships that could be construed as a potential conflict of interest.

The reviewer CP and handling Editor declared their shared affiliation.

frontiers in Plant Science

ORIGINAL RESEARCH
published: 13 September 2018
doi: 10.3389/fpls.2018.01327

Characterization of Biostimulant Mode of Action Using Novel Multi-Trait High-Throughput Screening of *Arabidopsis* Germination and Rosette Growth

Lydia Ugena[1†], Adéla Hýlová[1†], Kateřina Podlešáková[1], Jan F. Humplík[1,2], Karel Doležal[1], Nuria De Diego[1] and Lukáš Spíchal[1]*

[1] Department of Chemical Biology and Genetics, Centre of the Region Haná for Biotechnological and Agricultural Research, Faculty of Science, Palacký University, Olomouc, Czechia, [2] Laboratory of Growth Regulators, Centre of the Region Haná for Biotechnological and Agricultural Research, Institute of Experimental Botany, Czech Academy of Sciences, Olomouc, Czechia

OPEN ACCESS

Edited by:
Giuseppe Colla,
Università degli Studi della Tuscia, Italy

Reviewed by:
Magdalena Maria Julkowska,
King Abdullah University of Science and Technology, Saudi Arabia
Antonio Ferrante,
Università degli Studi di Milano, Italy
Barbara De Lucia,
Università degli Studi di Bari Aldo Moro, Italy

***Correspondence:**
Nuria De Diego
nuria.de@upol.cz

[†] These authors have contributed equally to this work

Specialty section:
This article was submitted to Crop and Product Physiology, a section of the journal Frontiers in Plant Science

Received: 31 May 2018
Accepted: 23 August 2018
Published: 13 September 2018

Citation:
Ugena L, Hýlová A, Podlešáková K, Humplík JF, Doležal K, De Diego N and Spíchal L (2018) Characterization of Biostimulant Mode of Action Using Novel Multi-Trait High-Throughput Screening of Arabidopsis Germination and Rosette Growth. Front. Plant Sci. 9:1327. doi: 10.3389/fpls.2018.01327

Environmental stresses have a significant effect on agricultural crop productivity worldwide. Exposure of seeds to abiotic stresses, such as salinity among others, results in lower seed viability, reduced germination, and poor seedling establishment. Alternative agronomic practices, e.g., the use of plant biostimulants, have attracted considerable interest from the scientific community and commercial enterprises. Biostimulants, i.e., products of biological origin (including bacteria, fungi, seaweeds, higher plants, or animals) have significant potential for (i) improving physiological processes in plants and (ii) stimulating germination, growth and stress tolerance. However, biostimulants are diverse, and can range from single compounds to complex matrices with different groups of bioactive components that have only been partly characterized. Due to the complex mixtures of biologically active compounds present in biostimulants, efficient methods for characterizing their potential mode of action are needed. In this study, we report the development of a novel complex approach to biological activity testing, based on multi-trait high-throughput screening (MTHTS) of *Arabidopsis* characteristics. These include the *in vitro* germination rate, early seedling establishment capacity, growth capacity under stress and stress response. The method is suitable for identifying new biostimulants and characterizing their mode of action. Representatives of compatible solutes such as amino acids and polyamines known to be present in many of the biostimulant irrespective of their origin, i.e., well-established biostimulants that enhance stress tolerance and crop productivity, were used for the assay optimization and validation. The selected compounds were applied through seed priming over a broad concentration range and the effect was investigated simultaneously under control, moderate stress and severe salt stress conditions. The new MTHTS approach represents a powerful tool in the field of biostimulant research and development and offers direct classification of the biostimulants mode of action into three categories: (1) plant growth promotors/inhibitors, (2) stress alleviators, and (3) combined action.

Keywords: biostimulants, multi-trait high-throughput screening assay, proline, polyamines, plant biostimulant characterization index, salinity

INTRODUCTION

Agricultural crop production will be extremely challenging in the coming decades. Due to the increase in population, a 50% (maximum) increase in the demand for food is expected by 2030. During the growing season, crops around the world are subjected to environmental stresses that affect plant germination, metabolism, growth and yield. Breeders worldwide have therefore focused on quantitative analyses of plant traits in order to accelerate the development of appropriate strategies for improving lines or varieties which are adaptable to resource-limited environments (Rahaman et al., 2017). Soil salinity is an important environmental factor that results in decreased crop productivity on a global scale. In fact, owing to this factor, an estimated 1.5 million hectares of land is taken out of production each year and by 2050 a 50% loss of cultivable lands is expected (Ibrahim, 2016).

The application of biostimulants represents one of the most innovative and promising strategies for minimizing stress impact, including salinity. A plant biostimulant is defined as a material of biological origin which includes bacteria, fungi, seaweeds, higher plants, animals and humate-containing raw materials (Sharma et al., 2014; Yakhin et al., 2016; Cristiano et al., 2018). This material induces beneficial plant processes (including nutrient uptake, nutrient use efficiency, tolerance to abiotic stress and crop quality), independently of its nutrient content (Calvo et al., 2014; Yakhin et al., 2016). Exposure of seeds to abiotic stresses, such as salinity among others, results in lower seed viability, reduced germination, and poor seedling establishment (Savvides et al., 2016). Increasing the salt concentration of the soil leads to a decrease in the germination percentage and delays the germination starting point (Kaveh et al., 2011; Thiam et al., 2013; Ibrahim, 2016). Seed-priming might improve seed stress-tolerance through 'priming memory,' which is established during priming and can be recruited later when seeds are exposed to stresses during germination (Chen and Arora, 2013). Seeds primed with biostimulants from varied origins trigger fast seed germination (Zeng et al., 2012; Colla et al., 2014; Garcia-Gonzalez and Sommerfeld, 2016). Besides, priming seeds with certain biostimulants can promote tolerance to adverse environmental conditions during the imbibition and germination stages (Mahdavi, 2013; Sharma et al., 2014; Pichyangkura and Chadchawan, 2015; Van Oosten et al., 2017).

Recently, the global biostimulant market has grown rapidly and, to satisfy crop requirements, many companies are actively introducing various innovative products and ingredients (Calvo et al., 2014; Sharma et al., 2014). However, in general, the raw materials used by the biostimulant manufacturers exhibit considerable compositional variations which may impact on the composition and concentration of major components (Povero et al., 2016; Sharma et al., 2016). The origin of biostimulants is diverse, and can range from single compounds to complex matrices with different groups of bioactive components that have only been partly characterized (du Jardin, 2015). Irrespective of their complexity, biostimulants are known to contain different groups of plant signaling compounds such as plant hormones, amino acids, and polyamines among others (Craigie, 2011; du Jardin, 2015). The exogenous application of these signaling molecules has been reported to ameliorate the adverse effect of stress through a sophisticated crosstalk among them leading to the activation of conserved pathways [reviewed in Podlešáková et al. (2018)].

In this work we present a novel approach for biostimulant mode of action characterization based on multi-trait high-throughput screening (MTHTS) of *Arabidopsis* germination and rosette growth under salinity. The analyzed traits included the germination rate, rosette growth rate and color. The potential of the approach was demonstrated by applying (via seed priming) representatives of the most common compounds present in biostimulants (i.e., polyamines and amino acids). In addition, we optimized the principles of two previously described protocols for implementation into the MTHTS approach. These included (i) the fast scoring of the germination rate based on a standardized 96-well plate test coupled with spectrophotometric reading of tetrazolium salt reduction (Pouvreau et al., 2013) and (ii) an automated method for high-throughput screening of *Arabidopsis* rosette growth in multi-well plates (De Diego et al., 2017). A highly efficient and reliable method for characterizing biostimulant efficacy at various salt stress levels was realized by developing and combining a high-throughput seed germination assay in *Arabidopsis* with the improved *Arabidopsis* rosette growth assay.

MATERIALS AND METHODS

HTS of *Arabidopsis in vitro* Seed Germination

Arabidopsis thaliana (L.) Heynh seeds (accession Col-0) were surface-sterilized by soaking in 70% Ethanol plus 0.01% Triton X-100 for 10 min. After that, the seeds were washed with sterilized water and then resuspended at a density of 10 g L^{-1} in 1 mM HEPES [4-(2-hydroxyethyl)-1-piperazineethanesulfonic acid] buffer (Carl Roth GmbH + Co. KG., Germany) (pH 7.5). Seeds were stratified at 4°C in the dark for 72 h. To investigate the effect of biostimulants on *Arabidopsis in vitro* seed germination, four single active compounds commonly present in many commercial biostimulant products were selected for seed priming; three polyamines: putrescine (Put) (1,4-butanediamine dihydrochloride), spermidine (Spd) (*N*-(3-aminopropyl)-1,4-butanediamine trihydrochloride), spermine (Spm) [N-(3-Aminopropyl)-1,4-butanediamine trihydrochloride] and the amino acid *L*-proline (Pro) [(*S*)-Pyrrolidine-2-carboxylic acid], all purchased from Sigma-Aldrich, Inc., (Germany). These compounds were added before the stratification, reaching final concentrations of 0.001, 0.01, 0.1, or 1 mM. After the cold stratification, seed suspension was washed three times with 20 mL sterile water to remove the biostimulants. In the last wash half of the water volume was removed and an additional 10 mL solution of sterilized 0.1% agarose with 1 mM of HEPES

Abbreviations: GLI, green leaf index; MTHTS, multi-trait high-throughput screening; NGRDI, normalized green red difference index; Pro, *L*-proline; Put, putrescine; Spd, spermidine; Spm, Spermine; VARI, visible atmospherically resistant index.

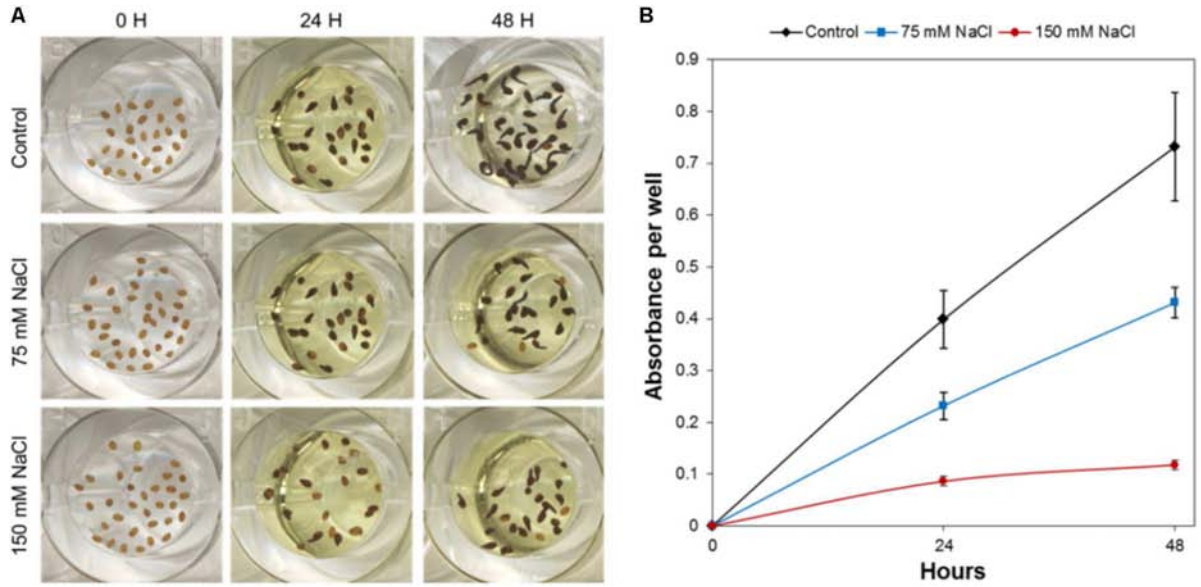


FIGURE 1 | HTS of *Arabidopsis* germination under control, moderate (75 mM NaCl), and severe (150 mM NaCl) stress conditions. **(A)** Characteristics of *Arabidopsis* seeds in one well of the 96-well plate before germination (0 h), and after 24 and 48 h, respectively, of germination with subsequent MTT treatment. **(B)** Absorbance of MTT after solubilization of formazan from *Arabidopsis* seeds germinated under control, moderate (75 mM NaCl), and severe (150 mM NaCl) stress conditions. The values represent *Mean* ± *SE*.

buffer was added. This is because seeds do not sediment in 0.05% agarose and are suspended in an adequate homogeneous solution for pipetting. The 96-well plate was filled with the seed suspension, 50 μL per well, representing ∼20–30 seeds per well. The final volume was adjusted to 100 μL per well with demineralized water or, in the case of the salt stress treatments, a NaCl solution that yields a final concentration of 75 or 150 mM NaCl in the well. Plates were sealed and incubated for seed germination at 21°C in darkness.

For the quantification of the *Arabidopsis* germination rate, the methylthiazolyldiphenyl-tetrazolium bromide (MTT; Sigma-Aldrich, Inc.) assay was performed in accordance with Pouvreau et al. (2013). In this process, 10 μL of 0.5% MTT solution per well was added after 24 or 48 h under germination conditions. Plates were placed in the culture chamber for an additional 24 h in darkness and a redox reaction, which is a reduction of MTT to formazan, lasted for 24 h (**Figure 1**). After MTT addition, the formazan salt deposit was solubilized by adding 100 μL of lysis buffer (10% Triton X-100, 0.04 mol L^{-1} HCl in isopropanol) to each well, and holding at 21°C in darkness for another 24 h. Subsequently, the absorbance was read with a BioTek™ Synergy™ H4 Hybrid Microplate Reader (BioTek Instruments, Inc., United States). For each well, the final absorbance was calculated by subtracting the absorbance at a reference wavelength of 690 nm from a test absorbance of 570 nm (A570–690 nm).

Image Acquisition and Data Analysis

Images were acquired by scanning each plate twice (HP ScanJet 5300c; resolution 1200 DPI; HP Development Company, L.P., United States), immediately after placing the seeds in the 96 multi-well plates (0 h) and after 24 or 48 h under seed-germination conditions with the subsequent 24 h MTT treatment. The images were saved as TIFF format. For seed counting, the images of *Arabidopsis* seeds at 0 h (immediately after cold stratification) were used and the number of seeds per well was estimated using an in-house software routine implemented in MATLAB R2015. The free of charge access to the software application for academical purposes is described in the next section.

HTS of *Arabidopsis* Rosette Growth

Experimental Setup and Assay Conditions

The protocol for analysis of *Arabidopsis* rosette growth described by De Diego et al. (2017) was modified as follows. Seeds of *A. thaliana* (ecotype Col-0) were surface- sterilized and sown on 12 cm × 12 cm square plates containing a 0.5× MS medium (Murashige and Skoog, 1962) (pH 5.7) supplemented with a gelling agent (0.6% Phytagel; Sigma-Aldrich, Germany). The seeds were kept for 4 days at 4°C in the dark (in the case of primed variants, the growth medium contained the tested biostimulant described below). The plates were then positioned vertically in a growth chamber under controlled conditions (22°C, 16/8 h light/dark cycle with the light cycle starting at 5 a.m., photon irradiance: 120 μmol photons of PAR m^{-2} s^{-1}). Three days after germination, seedlings of similar size were transferred under sterile conditions into 48-well plates (Jetbiofil, Guangzhou, China). One seedling was transferred to each well filled with 850 μL 1× MS medium (pH 5.7; supplemented with 0.6% Phytagel), with NaCl added for different salt stress

intensities (75 and 150 mM NaCl) and the plates were sealed with perforated transparent foil allowing gas and water exchange. The 48-well plates containing the transferred *Arabidopsis* seedlings were placed the OloPhen platform[1] that uses the PlantScreen^{TM} XYZ system installed in a growth chamber with a controlled environment and cool-white LED and far-red LED lighting (Photon Systems Instruments, Brno, Czechia). The conditions were set to simulate a long day with a regime of at 22°C/20°C in a 16/8 h light/dark cycle, an irradiance of 120 mmol photons of PAR m^{-2} s^{-1} and a relative humidity of 60%. The PlantScreen^{TM} XYZ system consists of a robotically driven arm holding an RGB camera with customized lighting panel and growing tables with a total area of approximately 7 m^2. To increase the throughput of the assay, the capacity of the growing area was improved to accommodate in total 572 multi-well. The XYZ robotic arm was automatically moved above the plates to take RGB images of single plates from the top. The imaging of each 48 well plate was performed twice per day (at 10 a.m. and 4 p.m.) for 7 days. RGB images (resolution 2500 × 2000 pixels) of a single plate with a file size of approximately 10 MB in the PNG compression format were stored in a database on a server, using a filename containing information about the acquisition time and the (x, y) coordinates of the camera. The data were automatically stored in PlantScreen XYZ database, exported by PlantScreen Data Analyzer software and analyzed using an in-house software routine implemented in MATLAB R2015.

The software application for *Arabidopsis* rosette growth analysis (same as for above described *Arabidopsis* seed counting) can be used without any charge upon obtaining a license from the author. The license can be obtained by e-mail to Palacky University upon agreeing not to use the application for commercial purpose. After obtaining the license, the enduser will be provided (free of charge) with the MCRInstaller.exe. MCRInstaller simulates the MATLAB environment on computers where MATLAB is not installed and enables to execute the applications. To obtain the application executable files, please contact the author Tomas Furst by email tomas.furst@upol.cz. The email must contain the following statement: "Neither the application nor the MCRInstaller will be used for any commercial purpose."

Seed Priming With Biostimulants

The biostimulant effect was determined using Put, Spd, Spm, and Pro for seed priming. After sterilization, the aforementioned seeds were placed on 12 cm × 12 cm square plates containing a 0.5× MS medium (pH 5.7) supplemented with the tested compounds at four concentrations (0.001, 0.01, 0.1, or 1 mM). After 4 days in the dark and 3 days of germination, seedlings were transferred into 48 multi-well plates filled with a 1× MS with/without salt (75 or 150 mM NaCl solution) addition. Two plates per growth condition, compound and concentration (96 seedlings) were used as replicates for the control and 75 mM NaCl. Due to the high mortality of seedlings under severe salt stress conditions, three plates for the seedling in 150 mM NaCl were used to obtain sufficient reproducible data and an adequate number of measurable individuals.

[1]http://www.plant-phenotyping.org/db_infrastructure#/tool/57

Biometrical Parameters

The changes in green area (Pixels) were measured twice per day in each *Arabidopsis* seedling using the aforementioned automatic system. From the obtained data, the relative growth rate (RGR) per hour or day was estimated for each replicate and variant as follows:

$$\mathrm{RGR} = [\ln(\text{green area})_{ti} - \ln(\text{green area})_{ti-1}]/(ti - ti - 1) \quad (1)$$

Where t*i* is the *i* time (h or days).

Determination of the Leaf Color in *Arabidopsis* Rosette Under Control and Salt Stress Conditions

For non-invasive estimation of the changes in leaf color, we calculated three vegetative indices (NGRDI, GLI, and VARI) which have exhibited correlation with the plant biomass, nutrient status or tolerance to abiotic stress (Gitelson et al., 2002; Perry and Roberts, 2008; Hunt et al., 2013). The images captured on the seventh day of an *Arabidopsis* rosette growth assay subjected to HTS were segmented for the extraction of leaf rosettes using software described in our previous report (De Diego et al., 2017). Afterward, the values corresponding to particular color channels (red = R, green = G, and blue = B) were extracted for each pixel within the plant mask, and the vegetative indices were calculated as follows:

Normalized green red difference index

$$\mathrm{NGRDI} = (\mathrm{G\text{-}R})/(\mathrm{G} + \mathrm{R}) \quad (2)$$

Green leaf index

$$\mathrm{GLI} = (2\mathrm{G\text{-}R\text{-}B})/(2\mathrm{G} + \mathrm{R} + \mathrm{B}) \quad (3)$$

Visible atmospherically resistant index

$$\mathrm{VARI} = (\mathrm{G\text{-}R})/(\mathrm{G} + \mathrm{R} - \mathrm{B}) \quad (4)$$

Subsequently, indices representing particular seedlings were determined by calculating the mean values for each plant mask. The mean value for each 48-well plate was then calculated.

Statistical Analysis

The one-way analysis of variance (ANOVA) was used to assess the differences between the projected areas (Pixels) or seed germination (absorbance) of two or more plant groups at a particular time-point. The test compares the variance (or variation) between the data samples to variation within each particular sample. When ANOVA was significant the differences among groups was determined using Dunn & Sidák's approach.

The relationship among traits was analyzed via Pearson's correlation. Furthermore, the significance of the regression was determined by applying a Student's *t*-test to the linear curves and after linearization of non-linear curves.

RESULTS

Development of HTS of *Arabidopsis* Seed Germination Under Control and Salt Stress Conditions

To efficiently determine the effect of biostimulant priming on the seed germination rate, we developed a HTS assay for seed germination using the MTT method proposed by Pouvreau et al. (2013). In this method, the MTT is used as a marker of metabolic activity in the embryo and its reduction to purple formazan can be quantified spectrophotometrically in a microtiter plate. We optimized this assay for *Arabidopsis* seeds and validated the assay for determining the effect of salinity at two time points (i.e., 24 and 48 h; see **Figure 1**). The severity of the salinity was expected to exert a dose-dependent negative effect on seed germination (seed staining and radicle length decrease; **Figure 1A**), leading to a decrease in the absorbance values measured (**Figure 1B**). During optimization of the assay, we observed a strong correlation between the absorbance values and the number of seeds per well under all three growth conditions (**Figure 2A**). Thus, a stable number of seeds per well was critical to reducing the variability in the experiment. This is, however, technically difficult under HTS conditions when a high number of wells/plates must be rapidly filled. To solve this problem we used 0.05% agarose solution allowing homogeneous suspension of seeds through vortexing. This way using multi-step pipette the average number of 21 ± 5.4 (mean ± SD) seeds per well was achieved. Besides, we handled the relatively high variability (∼25%) by developing an automatic simple software that counts the exact number of seeds per well (rather than finding a technical solution that allows precise and repeated filling of the plate wells with the same number of seeds). Using this software routine, the measured absorbance per well can be recounted to the absorbance per seed. In the first step of this process, the software identifies the wells in the plate. The seeds are then identified via simple thresholding in the R, G, and B channels and single seeds or clusters of seeds are subsequently separated from the background. Afterward, single seeds are distinguished from clusters by computing the solidity (i.e., the ratio of the area of the convex hull of an object to the area of an object) of each object. Single seeds have a high solidity (usually >0.9), whereas clusters of seeds are larger and have lower solidity. The number of seeds in a cluster is estimated by dividing the area of the cluster by the average seed area which is determined from previous runs of the software. The accuracy of the software was determined by manually counting the seeds on several plates, and a high correlation was obtained between the real number and the software-estimated number of seeds (**Figure 2B**). As shown in **Table 1**, the counting of the seeds allowed us to reduce the dispersion of the absorbance per variant, with an at least three times lower standard deviation (28 vs. 9%) in the two analyzed points at 24 and 48 h. Thus, we observed a significant correlation ($p < 0001$) between the absorbance per seed and the percentage of *Arabidopsis* seeds germinated under control and salt stress conditions (**Figure 2C**).

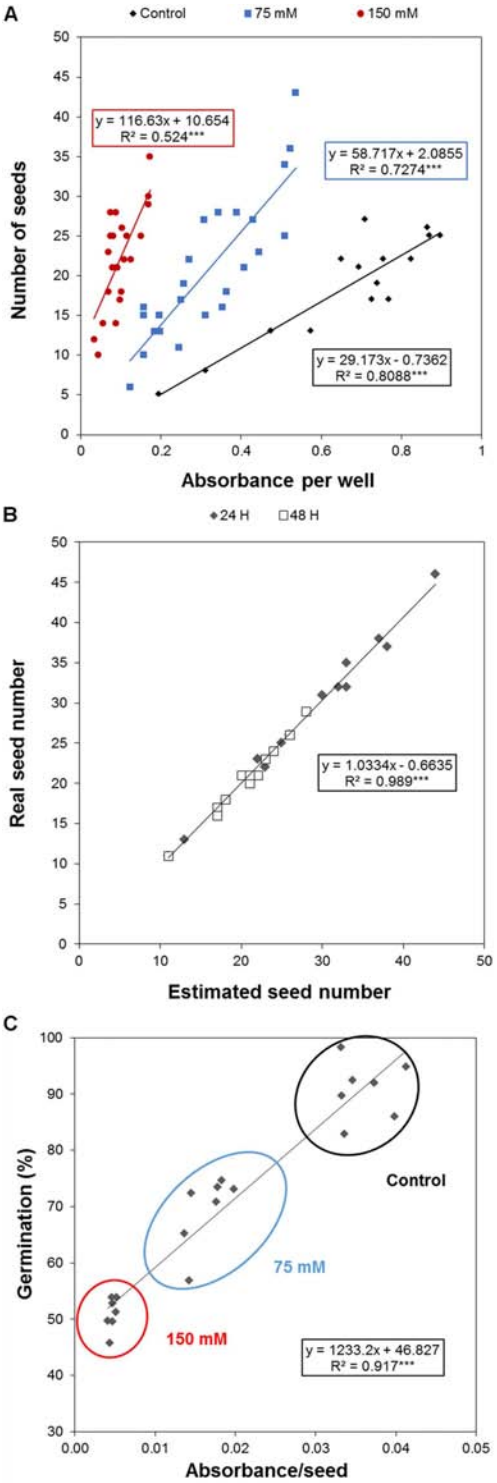


FIGURE 2 | Optimization of *Arabidopsis* germination HTS. Correlation between the **(A)** number of seeds per well and the absorbance under control, moderate (75 mM NaCl), and severe (150 mM NaCl) stress conditions, **(B)** manually counted number of seeds and the software-estimated number of seeds per well, and **(C)** germination rate (%) and the estimated number of seeds. The equation of the curve and the Pearson's correlation coefficient with significance according to ANOVA were calculated. ***$p < 0.001$.

TABLE 1 | Comparison of measured overall formazan absorbance and the absorbance recounted per seed after 24 or 48 h of germination.

	24 h		48 h	
	Absorbance	**Absorbance/seed**	**Absorbance**	**Absorbance/seed**
Mean	0.399	0.019	0.73	0.041
SD	0.112	0.002	0.21	0.003
%	28.08	9.14	28.52	8.28

Mean ± SD and contribution of the SD to each mean.

Effect of Biostimulant Seed Priming on *Arabidopsis* Seed Germination

We used the above-described optimized protocol to evaluate the effect of biostimulant (Put, Spd, Spm, and Pro) seed priming on seed germination under salt stress conditions. After 24 h, the tested variants differed only slightly (**Figure 3**). However, 1 mM Spd inhibited seed germination under control conditions and after 48 h of exposure to 75 mM NaCl, but exerted no effect under severe salt conditions (**Figure 3**). The same holds true for 1 mM Spm which also inhibited seed germination in 75 mM NaCl. The most visible effect was obtained for seeds primed with 0.01 and 0.1 mM Put and (to a lesser extent) 1 mM Pro, which yielded a significant increase in the germination in 150 mM NaCl (**Figure 3**).

Seed Size Conditions Associated With *Arabidopsis* Rosette Growth

To determine the effect of biostimulants on the early seedling development of *Arabidopsis* plants under salt stress conditions, we further optimized our previously published protocol (De Diego et al., 2017) for HTS of the rosette growth. For rapid characterization of the plant biostimulants, the protocol was improved as follows: the response of 4-day-old *Arabidopsis* seedlings grown in 1× MS was evaluated using 48 well plates with four biological replicates randomly distributed in the platform. Due to the rapid image acquisition of our system (∼250 plates per hour) the seedlings were imaged twice per day (at 10:00 and at 16:00) for seven consecutive days (**Supplementary Figure S1**). The time-dependent increase in the rosette area (represented by the green region) and RGR were determined for each replicate. The green area differed negligibly among the replicates according to ANOVA (**Figure 4A**), which also exhibited similar RGR. Using this approach, we could record fluctuations in the RGR (per hour) between the 2 days sessions, thereby increasing the sensitivity and applicability of the assay to analysis of circadian rhythms. Higher RGR occurred in the period from 10:00 a.m. to 4:00 p.m. (**Figure 1B**) than in other sessions.

The effect of seed size on the variability of early seedling development via rosette growth was evaluated to further increase the technical precision of the assay. Using sieves, the seed batch was separated into three different size categories: 250–280, 280–300, and >300 μm. Seeds larger than 280 μm produced seedlings with similar rosette area (see **Figure 4C**), whereas seeds with sizes of 250–280 μm yielded significantly smaller rosettes (**Supplementary Table S1**). Although seeds with sizes of 280–300 μm were quite abundant, seeds larger than 300 μm were rare. Thus, due to their abundance and good growth performance, we selected the 280–300 μm seeds as the standard for subsequent experiments.

HTS of *Arabidopsis* Rosette Growth as a Suitable Assay for the Characterization of Biostimulants Under Control and Salt Stress Conditions

Our OloPhen platform has sufficient capacity for the simultaneous testing of numerous variants (De Diego et al., 2017). To demonstrate the capacity for large-scale stress-response studies, we performed an experiment analogous to the germination assay using a 1× MS medium supplemented with two concentrations of NaCl (75 or 150 mM). The seeds were primed with Put, Spd, Spm, and Pro over the same concentration range (0.001, 0.01, 0.1, and 1 mM) described in the Methods section. The 4-day old seedlings were transferred for continued growth under three different conditions: control, moderate salt (75 mM NaCl) and severe salt (150 mM NaCl). In this experimental design, 119 units of 48 well plates containing a total of 5,712 plants were analyzed in a single run. As shown in **Figure 5**, seed priming with biostimulants induced significant differences in the rosette growth of individual variants (**Supplementary Table S2**). All concentrations of Put and Spd improved rosette growth and RGR, in both control and salt stress conditions, acting as plant growth promotors and stress alleviators (**Figure 5**). The best results were obtained with Put and Spd (**Figures 5**, **6**), especially under the severe salt condition (150 mM NaCl). In this case, exponential growth of the plants was maintained (**Figure 5**) through more efficient RGR per day (**Figure 7**) than that associated with other conditions. Spm priming promoted concentration-dependent growth under control and moderate salt stress conditions, although this growth stimulation was less than that induced by Put or Spd (**Figure 5**). Although Spm application improved rosette growth under severe stress conditions, maximum growth of the Spm-primed seedlings occurred earlier than that of seedlings grown only with 150 mM NaCl (**Figure 5**). Spm can therefore be classified as a plant growth promotor rather than a stress alleviator. In the case of stress-related amino acid Pro, we observed that low concentrations of Pro inhibited plant growth, whereas the highest concentrations stimulated growth in control and 75 mM NaCl conditions (**Figure 5** and **Supplementary Table S2**). Under the moderate stress induced by 75 mM NaCl, high concentrations of Pro exerted a stress-alleviating effect, but had

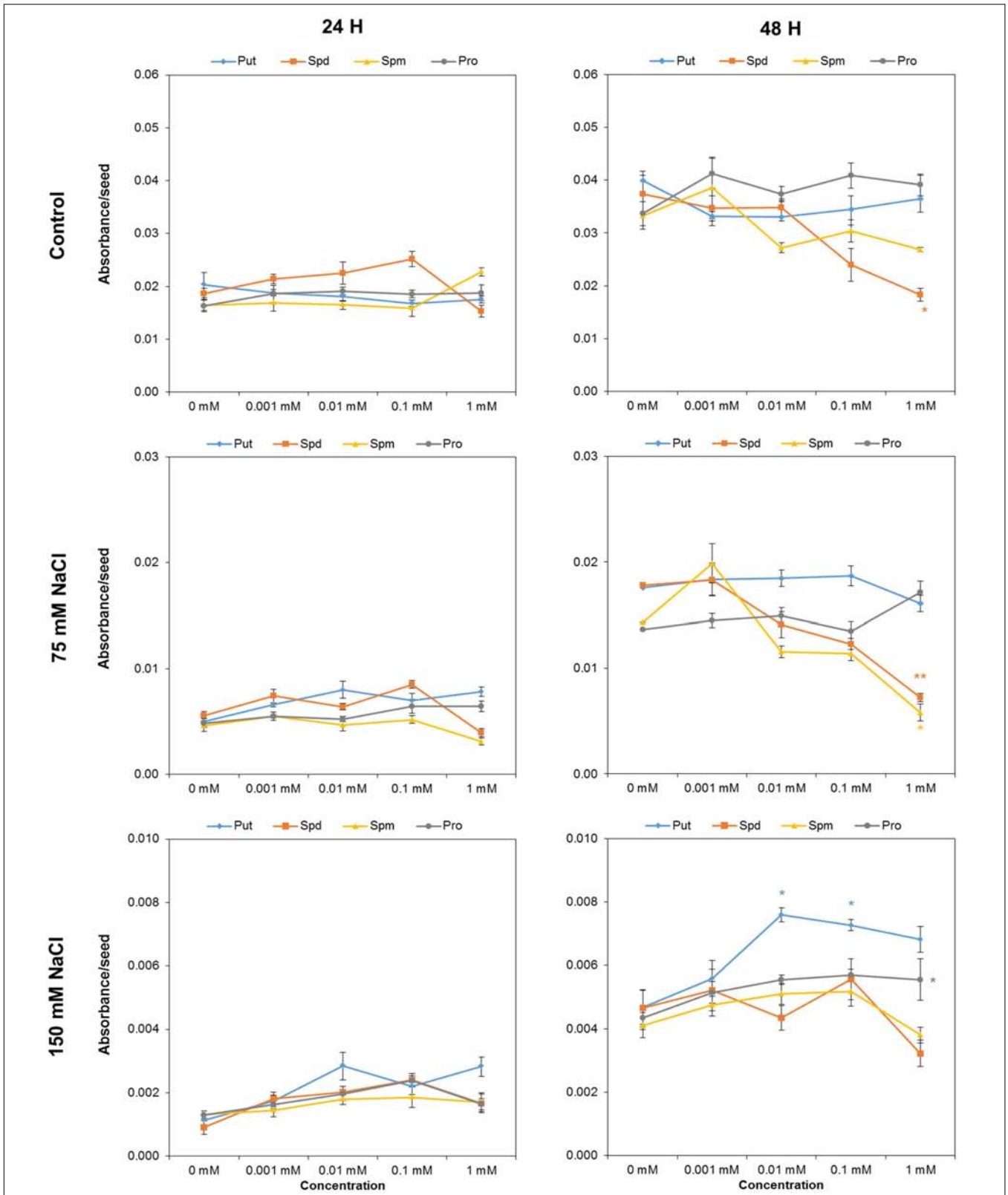


FIGURE 3 | Absorbance per seed of *Arabidopsis* seeds primed with Put, Spd, Spm, and Pro at four concentrations (0.001, 0.01, 0.1, and 1 mM), after 24 or 48 h of germination. *Mean* ± *SE*. Statistical analysis was performed via the Kruskal-Wallis test. Asterisks indicate differences relative to the non-treated variant $^{**}p < 0.01$; $^{*}p < 0.05$.

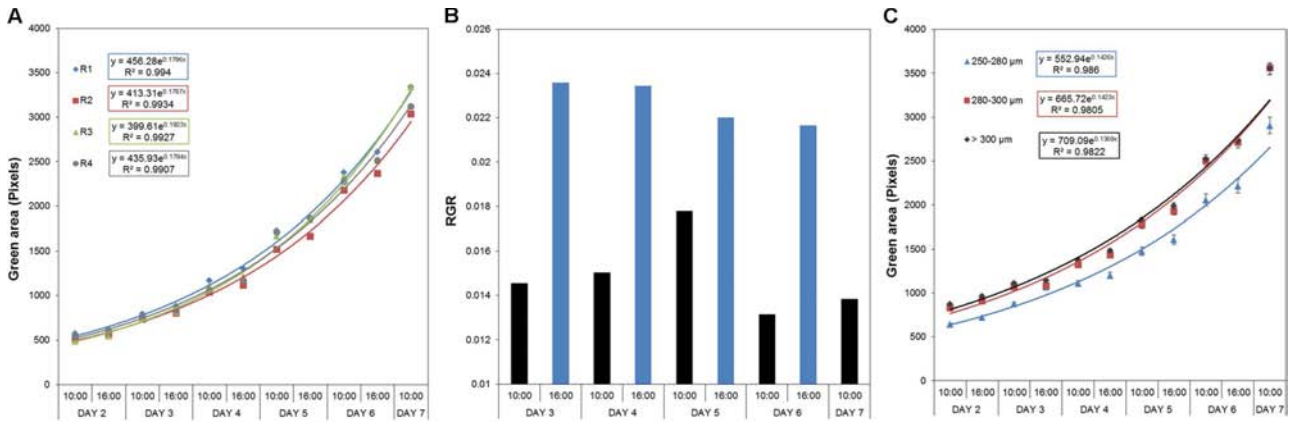


FIGURE 4 | Natural variation in *Arabidopsis* rosette growth in 48 multi-well plates under control conditions. **(A)** Green area (pixels) associated with the growth of four DAG *Arabidopsis* seedlings in independent 48-well plates (replicates; R1–R4) for 7 days. *Mean* ± *SE.* **(B)** Relative growth ratio (RGR, pixel pixel^{-1} hour^{-1}) of four DAG *Arabidopsis* seedlings grown in 48-well plates (n = 192). **(C)** Effect of the seed size on the green area (pixels) associated with the growth of four DAG *Arabidopsis* seedlings in independent 48-well plates. Three different size categories of seeds were considered: 250–280, 280–300, and >300 µm. The equation of the curve and the Pearson's correlation coefficient were calculated. 250–280 µm seeds were significantly smaller than 280–300 and >300 µm ones, according to the multiple comparisons after ANOVA.

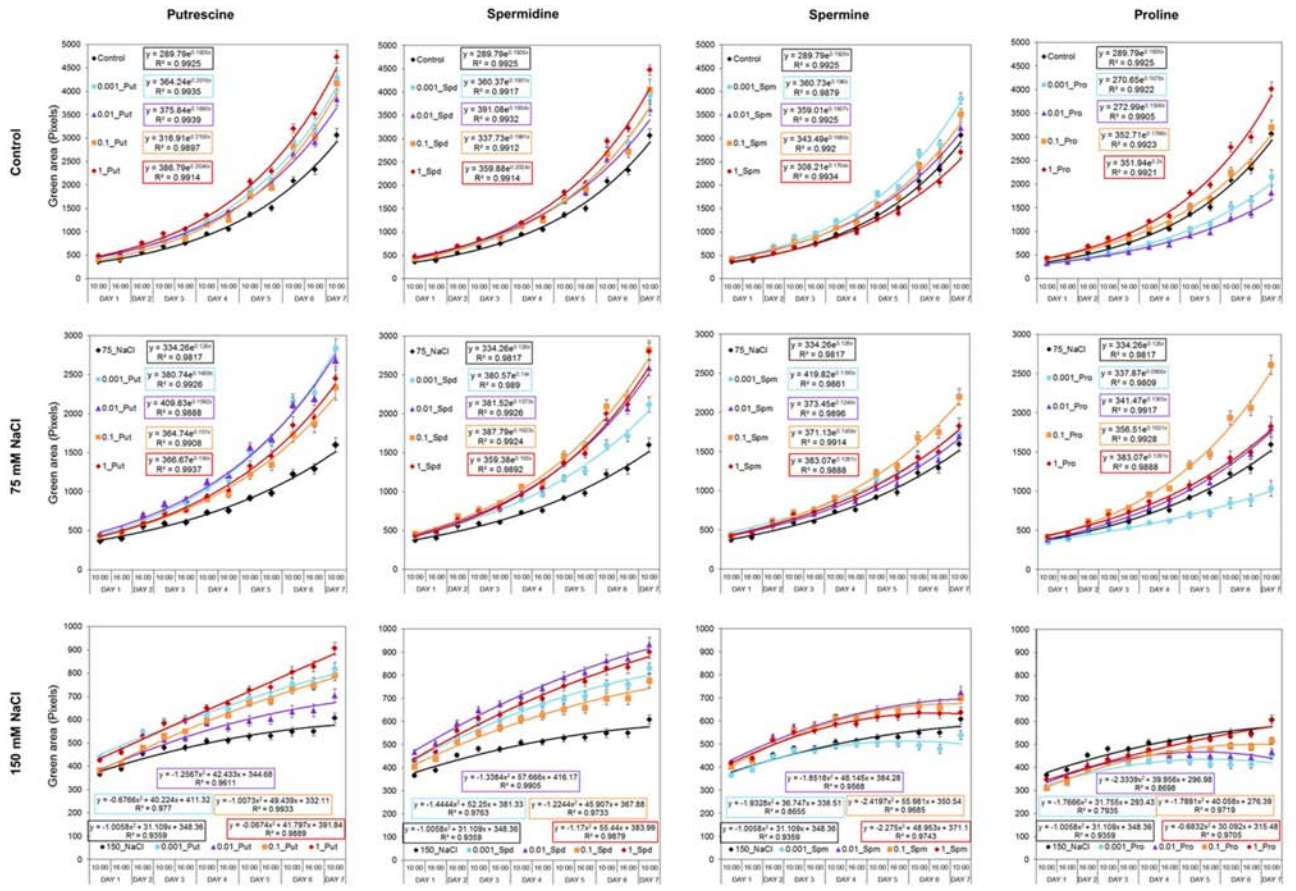


FIGURE 5 | Variation in *Arabidopsis* rosette growth from primed seeds with moderate and severe salt stress. Green area (pixels) of four DAG *Arabidopsis* seedlings primed with Put, Spd, Spm, and Pro at four concentrations (0.001, 0.01, 0.1, and 1 mM) and grown for 7 days in 48-well plates under control, moderate (75 mM NaCl), and severe (150 mM NaCl) salt stress conditions. *Mean* ± *SE.* The equation of the curve and the Pearson's correlation coefficient were calculated.

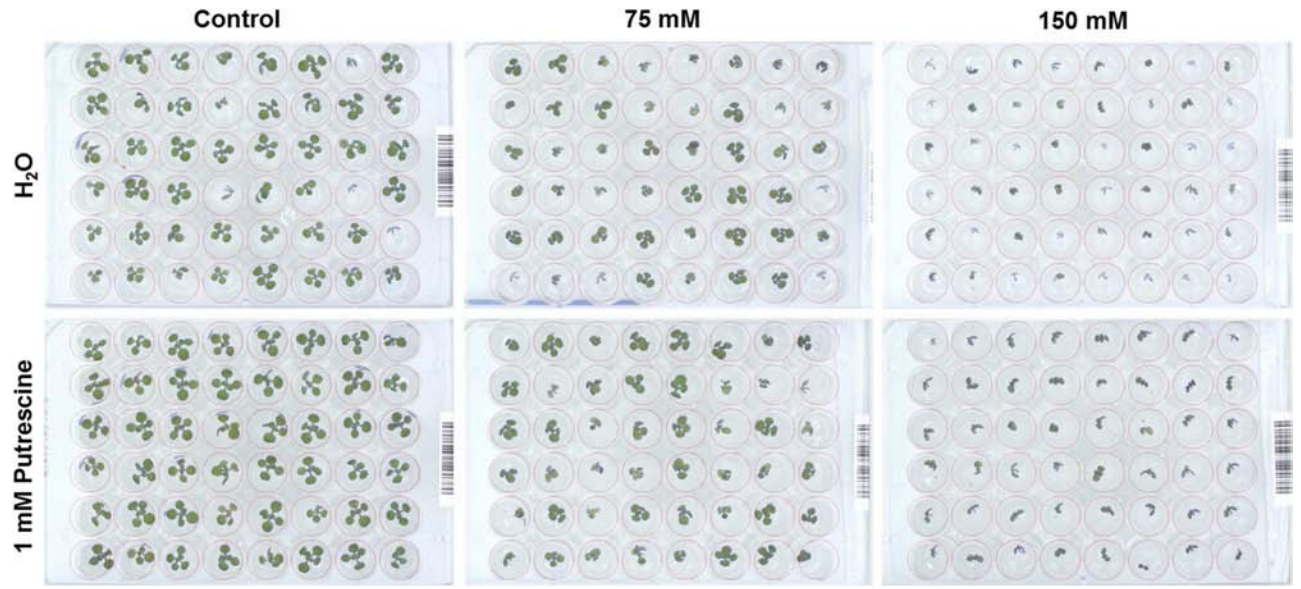


FIGURE 6 | RGB image of an individual 48-well plate containing four DAG *Arabidopsis* seedlings primed with 1 mM Put grown for 7 days under control, moderate (75 mM NaCl), and severe (150 mM NaCl) salt stress conditions.

a rather negative effect under the severe salt stress condition (**Figure 5** and **Supplementary Table S2**).

Effect of Biostimulant on *Arabidopsis* Seedling Establishment

Analysis of the dataset recorded from the above-described HTS of rosette growth revealed the effect of seed priming on early-seedling establishment. In this case, we analyzed the green area of the *Arabidopsis* seedlings immediately after the transfer to 48 well plates, corresponding to time zero of the HTS focused on *Arabidopsis* rosette growth as a suitable assay. Without salt stress, the sizes of seedlings established from primed seeds differed significantly from the sizes of seedlings resulting from non-primed seeds (**Figure 8**). For the entire range of concentrations, the priming by Put and Spd resulted in significantly larger rosettes compared to those seedlings from non-primed seeds. Except for the highest (1 mM) concentration, all Spm concentrations lead to a significant increase in the green area of the seedlings, whereas for Pro a considerable increase was observed only at the highest concentration (**Figure 8**). These results showed that our method can record traits in a complex manner that describes the effect of priming on all important stages of early development (e.g., germination, early seedling establishment and rosette growth).

Effect of Biostimulants on the Leaf Color of *Arabidopsis* Rosettes Under Control and Salt Stress Conditions

The degradation of chlorophyll, manifested as a change in leaf color, represents one of the most important symptoms of stresses in plants. This change in color may serve as an important marker in stress-related plant studies, especially in those employing salinity. To obtain this information, we introduced another trait into our method describing the effect of seed priming on the plant stress response. As described in the Methods section, the leaf color of the *Arabidopsis* rosettes was determined. We also evaluated the potential of three vegetation indices (VI) calculated using all three mixed visible bands (i.e., R, G, and B bands) which included the NGRDI, GLI, and VARI as indicators of leaf color change. These indices were strongly correlated with changes in the rosette area of the *Arabidopsis* seedlings and the values thereof depended on the seed priming treatment and salt intensity (**Figures 9A,B**). Of the three indices, GLI exhibited the highest sensitivity to salt stress (R^2: 0.97; R^2 for NGRDI and VARI: 0.95). However, when the three VI were separately evaluated for the seedling with 150 mM NaCl, a significant positive correlation with the green area of the *Arabidopsis* rosette (**Figures 9D–F**) was obtained only for GLI. The seed priming with Put and Spd generated *Arabidopsis* rosettes with the highest greenness under control and salt stress conditions. The highest values were observed for GLI where 1 mM Put and Spd yielded 22 and 31%, respectively, higher levels of greenness than that of the non-treated seeds (**Figure 9E**).

PBC Index for Estimating the Biostimulant Mode of Action

We developed a Plant Biostimulant Characterization (PBC) index aimed at integrating both HTS methods into a pipeline that yields straight-forward information allowing simple selection of the best treatment under each condition. The index can represent up to four analyzed traits: seed germination rate (%), seedling establishment (green pixels after transfer to 48 well plates), growth capacity (Pixels) and the leaf color

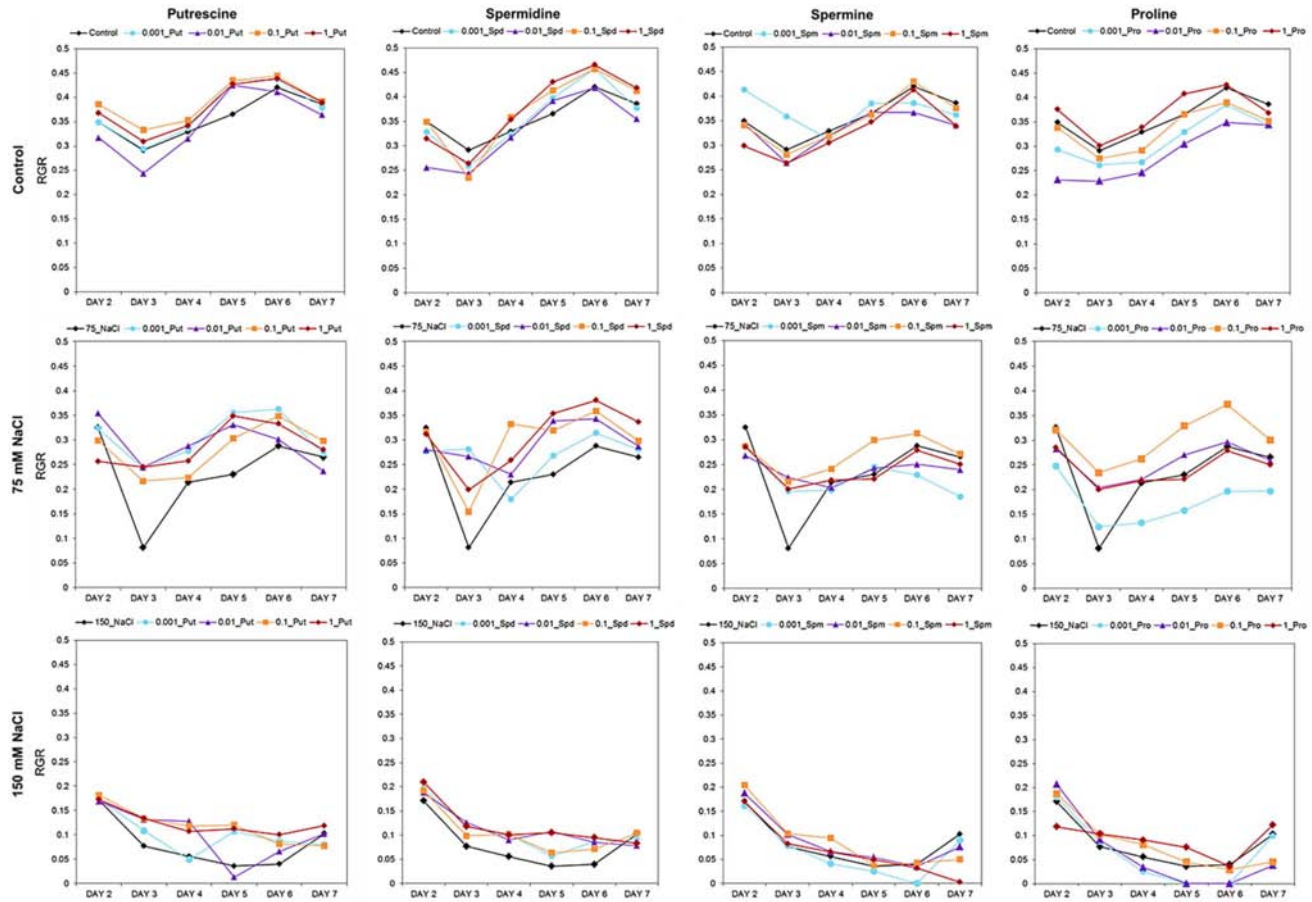


FIGURE 7 | Relative growth ratio (RGR, pixel pixel^{-1} day^{-1}) of four DAG *Arabidopsis* seedlings primed with Put, Spd, Spm, and Pro at four concentrations (0.001, 0.01, 0.1, and 1 mM) and grown for 7 days in 48-well plates under control, moderate (75 mM NaCl), and severe (150 mM NaCl) salt stress conditions.

index (GLI) for the primed and non-primed seeds. For the index calculation first the differences between the controls of the different growth conditions and variants (compound and concentration) under the same conditions were calculated as the log2 of the ratio. The number represented by the independent traits and treatment constituting the PBC index can be then represented in a parallel coordinate plot (**Figures 10**, **11**). This type of representation allows a better visualization (than that provided by other representations) of the variant-induced changes in each trait. In addition, the connection between the traits can be quickly identified. For example, under control conditions, it is easier to visualize that the seed priming with Put and Spd mainly improved *Arabidopsis* growth capacity, and to less extend the early seedling establishment and leaf color index, whereas the germination remained unchanged or was even inhibited by these agents (**Figure 10**). Under salt stress condition, seed priming with polyamines improved *Arabidopsis* growth capacity and leaf color index under both intensities tested (75 and 150 mM) (**Figures 11A,B**). Nevertheless, only under severe conditions, the priming with polyamines improved seed germination in almost all cases compared with their respective control (**Figure 11A**).

The concentration effect of the tested compound under three different growth conditions (control, 75 mM NaCl or 150 mM NaCl) was then determined by summing the relative changes (log2) obtained for the parallel coordinate plot ending with a single number as shown in **Figure 12**. This sum yielded a total that can reach a positive (biostimulant- blue) or negative (inhibitor-red) value. The resulting numbers were then plotted in a multidimensional graphic "radar chart" using the concentrations as quantitative variables (**Figure 12**). From these results we confirmed that Put was the most efficient plant growth promotor and stress alleviator with higher values in each concentration and growth condition, compared with the controls. The remaining compounds exhibited a concentration- and growth-condition-dependent response. For example, Spd and Spm yielded the highest index values at low concentrations, whereas Pro acted as plant biostimulants at high concentrations only, and its effectiveness increased with increasing salt stress intensity (**Figures 12B,C**). These results confirm that the presented MTHTS approach is an adequate tool for a fast and simultaneous analysis of various concentrations and growth conditions for identification and, especially, characterization of the operation mode associated with new biostimulants.

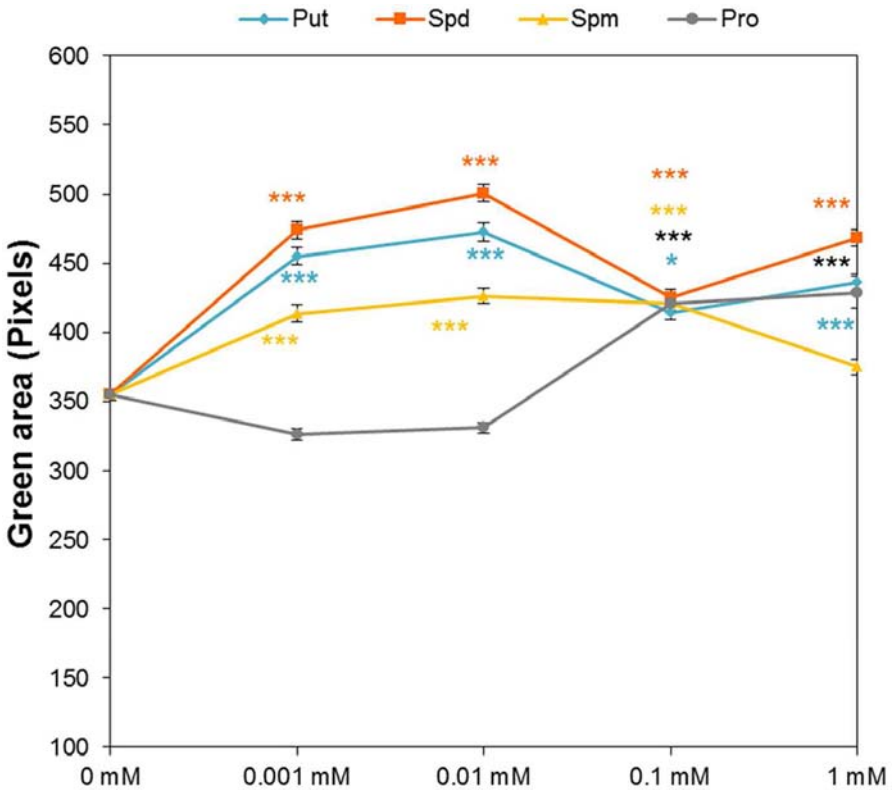


FIGURE 8 | Effect of biostimulant seed priming on the seedling establishment of *Arabidopsis*. Green area (pixels) of four DAG *Arabidopsis* seedlings primed with Put, Spd, Spm, and Pro at four concentrations (0.001, 0.01, 0.1, and 1 mM) and grown under control conditions after the transfer to 48-well plates. Statistical analysis was performed via the Kruskal-Wallis test. Asterisks indicate differences relative to the non-treated variant. ***$p < 0.001$; *$p < 0.05$.

DISCUSSION

Uniform and efficient seed germination and establishment of early seedlings are crucial for agricultural crop production under stress conditions, especially drought and/or salinity (Savvides et al., 2016). Seed priming, where seeds are pre-sown with certain compounds with the aim of increasing the uniformity and vigor of seedlings, represents an innovative alternative to coping with the negative stress effects. In addition, the use of natural compounds or biostimulants as priming agents can improve the efficiency of crop production and yield under suboptimal conditions. The use of these substances is more sustainable and environmentally friendly compared with the use of other materials. The priming with single compounds such as polyamines and amino acids can be a good technology against different abiotic stresses (Savvides et al., 2016). However, despite the fact that most of the complex biostimulants of several origins (i.e., protein hydrolysis from agroindustrial by-product from both plant sources and animal waste, and seaweed extracts) contain these types of compounds (du Jardin, 2015), their biostimulant activity potential hasn't been fully evaluated. For this reason, we used in this study the stress related amino acid Pro and polyamines' representatives as priming agents to bring additional information about their possible biostimulant mode of action. Therefore, biostimulant manufacturers require tools for identifying new biostimulants, characterizing and quantifying their biological effects and describing the corresponding mode of action. Moreover, during biostimulant preparation, the tools for rapid control of the quality during the extraction processes and production of different batches are needed. Taking into account the mentioned facts, we suggest that Put, Spd, Spm, and Pro have potential to be used as positive controls in the biostimulant research and manufacturing.

Screening platforms based on the semi-automated or automated bioassaying of simple traits based on *in vitro Arabidopsis* assays might be useful to accelerate the process for preliminary screening of stability, composition and effect of raw material. This testing allows for a rapid first-step screening on plants, eliminating the influence of soil and other environmental parameters (Povero et al., 2016). The testing of biostimulants using bioassays has been traditionally performed with Petri dishes, thus having low-throughput requiring posterior manual quantification (Durand et al., 2003; Colla et al., 2014; Povero et al., 2016). Recently, Rodriguez-Furlán et al. (2016) published an *in vitro* bioassay using *Arabidopsis* for the testing of several compounds. However, the use of scanners for image analysis yields an analysis rate of 20 min per plate and the analysis is performed only at one time-point (Rodriguez-Furlán et al., 2016). Several other manual and semi-automated HTS protocols using RGB imaging for phenotyping of *Arabidopsis*

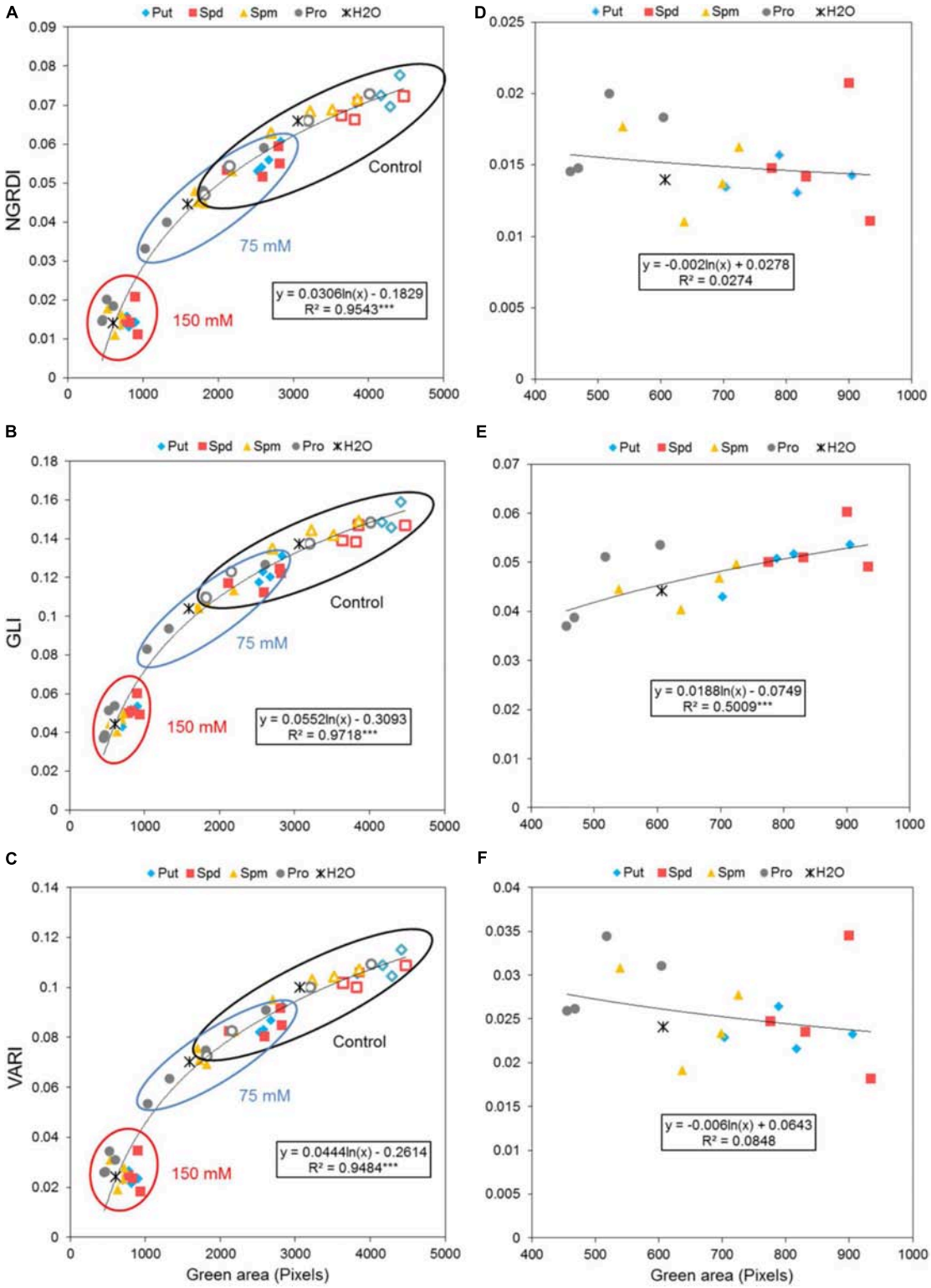


FIGURE 9 | Effect of biostimulant seed priming on the stress response of *Arabidopsis*. **(A)** Correlation between the color index; NGRDI **(A)**, GLI **(B)**, or VARI **(C)** and the green area (Pixels) of four DAG *Arabidopsis* seedlings primed with Put, Spd, Spm, and Pro at four concentrations (0.001, 0.01, 0.1, and 1 mM) and grown under control (empty symbols), moderate (75 mM NaCl), and severe (150 mM NaCl) (filled symbols) salt stress conditions for 7 days. **(B)** Correlation between NGRDI **(D)**, GLI **(E)**, or VARI **(F)** and the green area of the *Arabidopsis* seedlings grown only under severe stress conditions. The equation of the curve and the Pearson's correlation coefficient with significance, according to ANOVA, were calculated after linearization. ***$p < 0.001$.

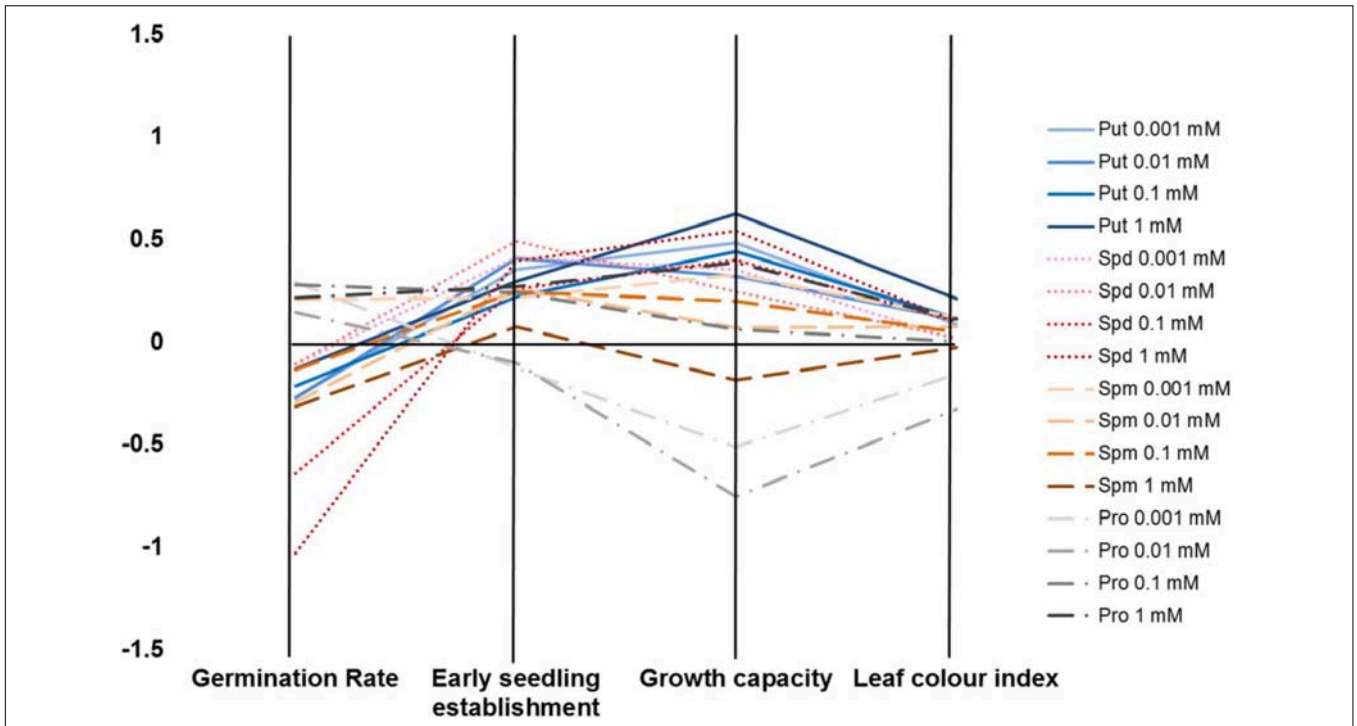


FIGURE 10 | Characterization of plant biostimulants under control conditions. Parallel coordinate plot of the traits (germination, seedling establishment, plant growth capacity, and leaf color index) obtained from the Multi-trait HTS of *Arabidopsis* seeds primed with Put, Spd, Spm, and Pro at four concentrations (0.001, 0.01, 0.1, and 1 mM) and grown under control, moderate (75 mM NaCl), and severe (150 mM NaCl) salt stress conditions.

in the controlled conditions have been published with different throughputs and (dis)advantages. The method of Granier et al. (2006) showed possibilities solving potential complications and methodological difficulties with the spatial and temporal variability of micrometeorological conditions within a growth chamber, reaching throughput of 500 plants per hour. Recently, simple HTS protocol based on *in vitro* growth of *Arabidopsis* using square plates with 16 seedlings and manual image acquisition followed by analysis of plant size and color was published by Faragó et al. (2018). The protocol presented by us is based on our previous report of an automated method for HTS of *Arabidopsis* rosette growth in multi-well plates accessible at OloPhen facility (De Diego et al., 2017). The potential of this method was in our recent protocol improved in several ways through (1) increase of the number of plates per run from 480 to 572; (2) significant increase of the total number of plants analyzed by use of 48-well plates, instead of 24-well plates that increased the number of analyzed plants to more than 27.000 in less than 3 h; and (3) through increase of the resolution of the growth analysis by automated measurement twice a day within 1 week. As presented here, our new method allows a simultaneous study of different growth conditions without compromising the number of variants, replicates and plants per treatment. Moreover, compared to Faragó et al. (2018), the growth analysis of each plant is done for the whole cycle by imaging of the same plant individual. Further, the use of independent wells per plant permits an easier detection of the single plant so they are located in a concrete XY position. Thus, there is no requirement of any manual adjustment to separate individual plants. As clear example illustrating the potential of our method, in this work we automatically recorded the rosette growth of 5,712 *Arabidopsis* (119 plates × 48 seedling). The imaging of each well-plate was performed twice per day (at 10 a.m. and 4 p.m.) for 7 days, ending with 14 data points per plant in very short time. Altogether, we developed a very fast *in vitro* bioassay to analyze simultaneously a huge amount of treatments and plants.

The improved HTS of rosette growth under control and stress salinity was integrated in a pipe-line for the screening of biostimulants together with the HTS of *Arabidopsis* seed germination. For that, we developed a simple and fast bioassay for *Arabidopsis* seed germination based on (Pouvreau et al., 2013) using spectrophotometric analysis of MTT reduction in microtiter plates. With the classical method using a microscope, the distinction between non-germinated seeds and germinated seeds with a very short protruded radicle is very difficult, increasing the risk of germination rate underestimation (Pouvreau et al., 2013). However, the MTT method is simple and accurate and can be easily adapted for high-throughput germination bioassays. The HTS method is performed in 96 well plates. These plates allow many variants per plate (compounds, concentrations, and/or germination conditions) using a spectrophotometric MTT method with a simple read out of the germination rate per variant (**Figure 1**). In addition, we developed a simple in-house software routine to automatically count the seed number per well. This reduced the time consuming counting of the seed number per well necessary

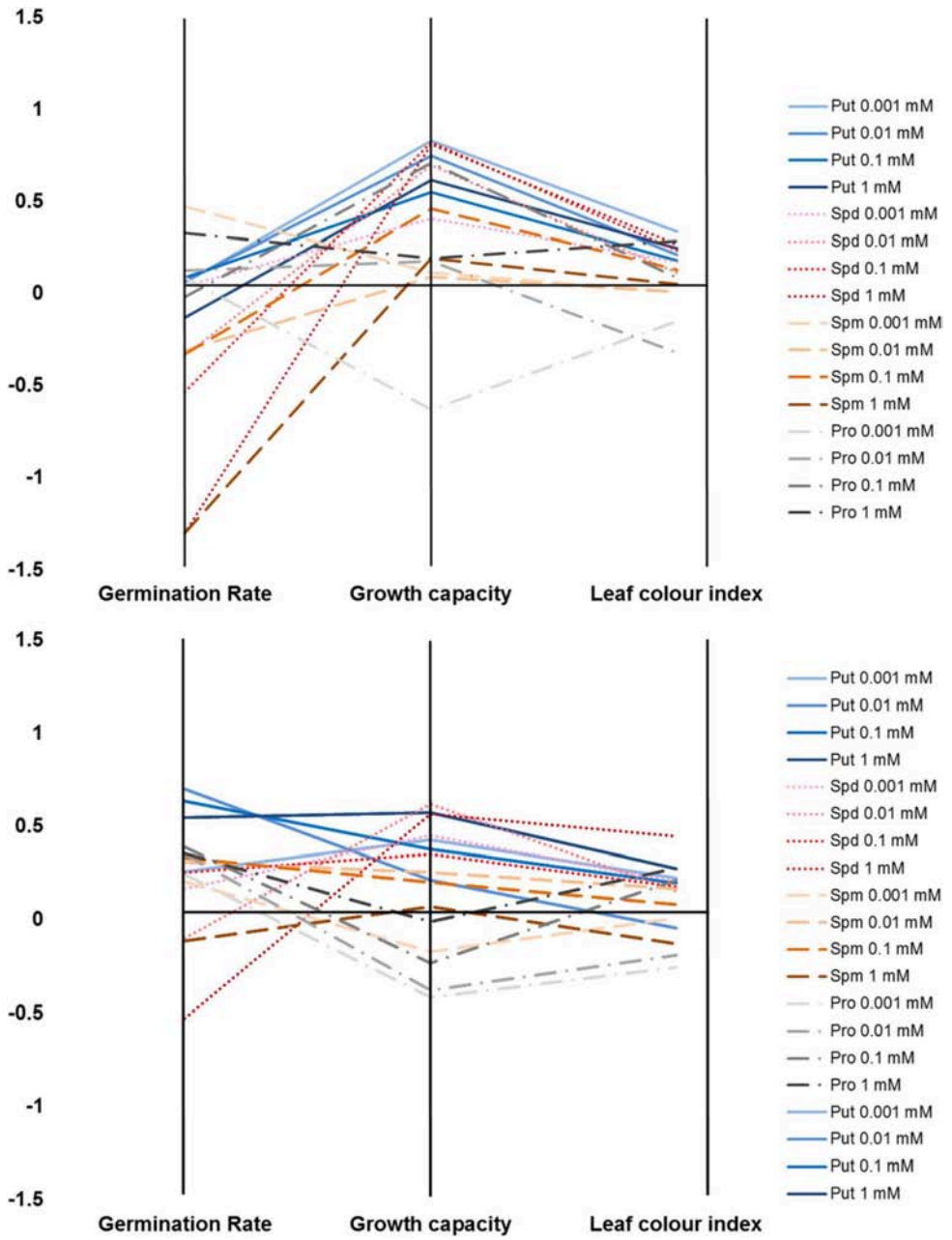
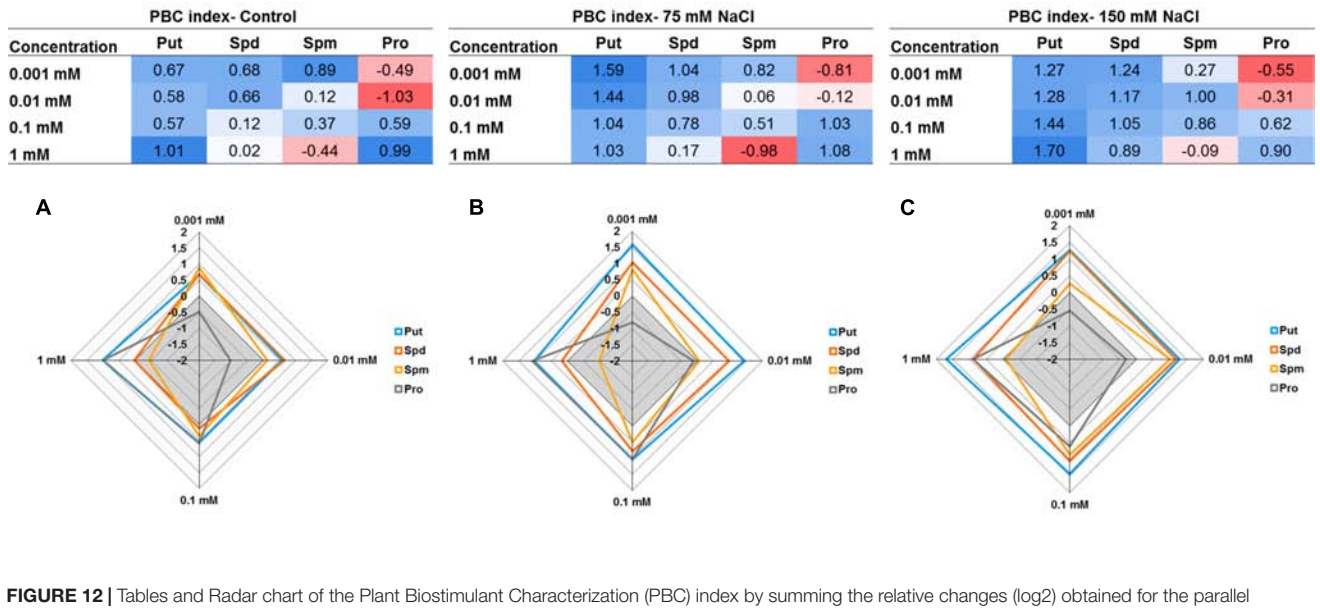


FIGURE 11 | Characterization of plant biostimulants under moderate **(A)** or severe **(B)** salt stress conditions. Parallel coordinate plot of the traits (germination, plant growth capacity, and leaf color index) obtained from the Multi-trait HTS of *Arabidopsis* seeds primed with Put, Spd, Spm, and Pro at four concentrations (0.001, 0.01, 0.1, and 1 mM) and grown under moderate (75 mM NaCl) or severe (150 mM NaCl) salt stress conditions.

for increasing the accuracy of the method by reducing the variability within treatments (**Figure 2A**). Although free software applications exist for image-based analysis of seeds allowing automated definition of the seed shape and size (Tanabata et al., 2012), for our purpose we created a very simple software routine in MATLAB suitable for detecting and counting objects (seeds) in multi-well plates at 0 h (immediately after cold stratification and before seed germination). The obtained number is then used to recalculate the total absorbance of the well recorded by spectrophotometer to the absorbance per seed that represents the germination rate. This trait together with those obtained from the HTS of Arabidopsis rosette growth (plant establishment, plant growth capacity under different conditions and leaf color index), constitute the MTHTS for biostimulant characterization achievable within 1 week.

Many biostimulants contain various groups of components including complex mixtures of biologically active compounds



PBC index- Control

Concentration	Put	Spd	Spm	Pro
0.001 mM	0.67	0.68	0.89	-0.49
0.01 mM	0.58	0.66	0.12	-1.03
0.1 mM	0.57	0.12	0.37	0.59
1 mM	1.01	0.02	-0.44	0.99

PBC index- 75 mM NaCl

Concentration	Put	Spd	Spm	Pro
0.001 mM	1.59	1.04	0.82	-0.81
0.01 mM	1.44	0.98	0.06	-0.12
0.1 mM	1.04	0.78	0.51	1.03
1 mM	1.03	0.17	-0.98	1.08

PBC index- 150 mM NaCl

Concentration	Put	Spd	Spm	Pro
0.001 mM	1.27	1.24	0.27	-0.55
0.01 mM	1.28	1.17	1.00	-0.31
0.1 mM	1.44	1.05	0.86	0.62
1 mM	1.70	0.89	-0.09	0.90

FIGURE 12 | Tables and Radar chart of the Plant Biostimulant Characterization (PBC) index by summing the relative changes (log2) obtained for the parallel coordinate plot for each compound (Put, Spd, Spm, or Pro) concentration (0.001, 0.01, 0.1, and 1 mM) and growth condition; control **(A)**, 75 mM NaCl **(B)**, or 150 mM NaCl **(C)**.

and, hence, the testing should be performed over a broad concentration range allowing evaluation of concentration-dependent effects. We selected individual molecules as a first step in optimizing our bioassays for biostimulant characterization. The polyamines Put, Spd, and Spm, and the amino acid Pro, which also have been identified in the raw material of complex formulations from different natural origins, were selected (Colla et al., 2014; du Jardin, 2015). Moreover, we selected salinity as a stressor, owing to its negative impact on seed germination and plant growth. Using our approach, each compound can be simultaneously tested at different concentrations and plant growth conditions in both HTS methods. The results revealed differences in the mode of action for the four compounds applied to *Arabidopsis* seed germination and rosette growth (**Figures 3**–**9**). Put and Spd were identified as plant growth promotors and stress alleviators, whereas Spm and Pro were less efficient and their positive effect was concentration dependent (**Figures 5**, **10**, and **11**). The exogenous application of polyamines yields improved salt tolerance in many crops via enhanced germination and/or plant productivity (Roychoudhury et al., 2011; Li et al., 2015; Shekari et al., 2015). For example, exogenous application of Spd in *Cucumis sativus* L. induces accumulation of endogenous polyamines that act as free radical scavengers, thereby stabilizing cellular membranes and maintaining cellular ionic balance under salinity (Shu et al., 2012). This was attributed to a relatively high Put/(Spd+Spd) ratio that rendered seed priming with Put the most efficient treatment. As confirmation, Shu et al. (2015) demonstrated that Put application regulates protein expression at transcriptional and translational levels by increasing endogenous polyamine levels in thylakoid membranes which may stabilize the photosynthetic apparatus under a salt stress. In addition, changes in polyamines biosynthesis and catabolism influence plant tolerance and recovery capacity though a sophisticated crosstalk with plant hormones, which induces changes in primary metabolism such as the synthesis of amino acids, and improves photosynthesis and nutrient uptake under stress conditions (review in Podlešáková et al., 2018). Therefore, priming with polyamines could be a cheap, healthy, and easy solution for mitigating adverse salinity-induced stresses occurring during the initial developmental phases of crops.

The priming with Pro was less effective than with polyamines, and the most positive effect was in the germination rate under a severe salt stress. This may have resulted from the fact that enhanced Pro levels in plants occur in the first phases of seed germination and the seed-to-seedling transition (Silva et al., 2017). Similar results were obtained in rice, where the seeds pre-treated with Pro provided significant evidence for assessing the salt tolerance at the germination stage (Deivanai et al., 2011). However, the effect was variety dependent. In sugar cane grown *in vitro*, the anti-stress effect was also genotype dependent (Medeiros et al., 2015), but both dependences increased the stress tolerance by activating the plant antioxidative response. Other studies consider the Pro mode of action to be long-term, when the plant accumulated high levels of Pro, and attributed this action to plant recovery and hardening (De Diego et al., 2015; Sabagh et al., 2015). This could be explained by the fact that stress-tolerance improvement in many other crops required relatively high concentrations (Talat et al., 2013; Dawood et al., 2014). However, contradictory results regarding the Pro effect have been obtained for the same crop under the same stress conditions. For example, Teh et al. (2016) reported that 5 or 10 mM Pro improved salt stress tolerance of rice, but Deivanai et al. (2011) considered

the 10 mM concentration toxic. This contradiction resulted mainly from the different intensities of salinity considered. Therefore, integrating a wide range of concentrations in the same bioassay combined with different stress levels for the testing of biostimulants constitutes a viable strategy for biostimulant mode of action characterization.

CONCLUSION

In this work we present a complex pipe-line for a fast characterization of plant biostimulants suitable for seed-priming application giving straight-forward information for simple selection of the best treatments under control, moderate and severe salt stress conditions, using treatment evaluation through newly introduced index. The MTHTS approach based on the semi-automated analysis of *Arabidopsis* germination and rosette growth analyses four traits: *in vitro* germination rate, early seedling establishment capacity, growth capacity under stress and stress response based on plant greenness. The approach allows the acceleration of the biostimulant characterization through a simultaneous spanning of a broad number of biostimulants in a wide range of concentrations and stress conditions. Further, the method helps to define a biostimulant made of action based on its contribution to the plant development and stress tolerance such as plant growth promotor/inhibitor and/or stress alleviator. The presented approach (i) represents a useful tool for biostimulant research and development, and (ii) when combined with chemical-composition analysis and biological-activity measurements can help to identify the specific mode of action characterizing the biostimulants and their main bioactive ingredients.

AUTHOR CONTRIBUTIONS

LU, AH, JH, KD, LS, and NDD designed the experiments. LU, AH, JH, and KP performed the experiments. NDD and LS supervised the study and formulated the concept of the project. LU, AH, and NDD performed the data analysis. All authors discussed the results. LU, AH, JH, NDD, and LS wrote the manuscript.

FUNDING

This work was funded by the Ministry of Education, Youth and Sports of the Czechia (Grant LO1204 from the National Program of Sustainability).

SUPPLEMENTARY MATERIAL

The Supplementary Material for this article can be found online at: https://www.frontiersin.org/articles/10.3389/fpls.2018.01327/full#supplementary-material

FIGURE S1 | *Arabidopsis* rosette growth in 48 multi-well plates for 7 days under control conditions.

TABLE S1 | Statistical differences in the green area (pixels) of 4 DAG *Arabidopsis* seedlings from three different size categories of seeds (250–280, 280–300, and >300 μm) grown in 48-well plates (three biological replicates per treatment) for 7 days. Different letters indicate significant differences according to multiple comparisons performed after ANOVA.

TABLE S2 | Statistical differences in the green area (pixels) of 4 DAG *Arabidopsis* seedlings primed with Put, Spd, Spm, and Pro at four concentrations (0.001, 0.01, 0.1, and 1 mM) and grown under control, moderate (75 mM NaCl) and severe (150 mM NaCl) salt stress conditions for 7 days. Different letters indicate significant differences according to multiple comparisons performed after ANOVA.

REFERENCES

Calvo, P., Nelson, L., and Kloepper, J. W. (2014). Agricultural uses of plant biostimulants. *Plant Soil* 383, 3–41. doi: 10.1007/s11104-014-2131-8

Chen, K., and Arora, R. (2013). Priming memory invokes seed stress-tolerance. *Environ. Exp. Bot.* 94, 33–45. doi: 10.1016/j.envexpbot.2012.03.005

Colla, G., Rouphael, Y., Canaguier, R., Svecova, E., and Cardarelli, M. (2014). Biostimulant action of a plant-derived protein hydrolysate produced through enzymatic hydrolysis. *Front. Microbiol.* 5:448. doi: 10.3389/fpls.2014.00448

Craigie, J. S. (2011). Seaweed extract stimuli in plant science and agriculture. *J. Appl. Phycol.* 23, 371–393. doi: 10.1007/s10811-010-9560-4

Cristiano, G., Pallozzi, E., Conversa, G., Tufarelli, V., and De Lucia, B. (2018). Effects of an animal-derived biostimulant on the growth and physiological parameters of potted snapdragon (*Antirrhinum majus* L.). *Front. Plant Sci.* 9:861. doi: 10.3389/fpls.2018.00861

Dawood, M. G., Taie, H. A. A., Nassar, R. M. A., Abdelhamid, M. T., and Schmidhalter, U. (2014). The changes induced in the physiological, biochemical and anatomical characteristics of *Vicia faba* by the exogenous application of proline under seawater stress. *South African J. Bot.* 93, 54–63. doi: 10.1016/j.sajb.2014.03.002

De Diego, N., Fürst, T., Humplík, J. F., Ugena, L., Podlešáková, K., and Spíchal, L. (2017). An automated method for high-throughput screening of *Arabidopsis* rosette growth in multi-well plates and its validation in stress conditions. *Front. Plant Sci.* 8:1702. doi: 10.3389/fpls.2017.01702

De Diego, N., Saiz-Fernandez, I., Rodriguez, J. L., Perez-Alfocea, F., Sampedro, M. C., Barrio, R. J., et al. (2015). Metabolites and hormones are involved in the intraspecific variability of drought hardening in radiata pine. *J. Plant Physiol.* 188, 64–71. doi: 10.1016/j.jplph.2015.08.006

Deivanai, S., Xavier, R., Vinod, V., Timalata, K., and Lim, O. F. (2011). Role of exogenous proline in ameliorating salt stress at early stage in two rice cultivars. *J. Stress Physiol. Biochem.* 7, 157–174.

du Jardin, P. (2015). Plant biostimulants: definition, concept, main categories and regulation. *Sci. Hortic.* 196, 3–14. doi: 10.1016/j.scienta.2015.09.021

Durand, N., Briand, X., and Meyer, C. (2003). The effect of marine bioactive substances (N PRO) and exogenous cytokinins on nitrate reductase activity in *Arabidopsis thaliana*. *Physiol. Plant.* 119, 489–493. doi: 10.1046/j.1399-3054.2003.00207.x

Faragó, D., Sass, L., Valkai, I., Andrási, N., and Szabados, L. (2018). PlantSize offers an affordable, non-destructive method to measure plant size and color in vitro. *Front. Plant Sci.* 9:219. doi: 10.3389/fpls.2018.00219

Garcia-Gonzalez, J., and Sommerfeld, M. (2016). Biofertilizer and biostimulant properties of the microalga *Acutodesmus dimorphus*. *J. Appl. Phycol.* 28, 1051–1061. doi: 10.1007/s10811-015-0625-2

Gitelson, A. A., Kaufman, Y. J., Stark, R., and Rundquist, D. (2002). Novel algorithms for remote estimation of vegetation fraction. *Remote Sens. Environ.* 80, 76–87. doi: 10.1016/S0034-4257(01)00289-9

Granier, C., Granier, C., Aguirrezabal, L., Aguirrezabal, L., Chenu, K., Chenu, K., et al. (2006). PHENOPSIS, an automated platform for reproducible

phenotyping of plant responses to soil water de cit in. *New Phytol.* 169, 623–635. doi: 10.1111/j.1469-8137.2005.01609.x

Hunt, E. R., Doraiswamy, P. C., McMurtrey, J. E., Daughtry, C. S. T., Perry, E. M., and Akhmedov, B. (2013). A visible band index for remote sensing leaf chlorophyll content at the canopy scale. *Int. J. Appl. Earth Obs. Geoinf.* 21, 103–112. doi: 10.1016/J.JAG.2012.07.020

Ibrahim, E. A. (2016). Seed priming to alleviate salinity stress in germinating seeds. *J. Plant Physiol.* 192, 38–46. doi: 10.1016/j.jplph.2015.12.011

Kaveh, H., Nemati, H., Farsi, I. M., and Vatandoost Jartoodeh, S. (2011). How salinity affect germination and emergence of tomato lines. *J. Biol. Environ. Sci.* 5, 159–163.

Li, J., Hu, L., Zhang, L., Pan, X., and Hu, X. (2015). Exogenous spermidine is enhancing tomato tolerance to salinity-alkalinity stress by regulating chloroplast antioxidant system and chlorophyll metabolism. *BMC Plant Biol.* 15:303. doi: 10.1186/s12870-015-0699-7

Mahdavi, B. (2013). Seed germination and growth responses of Isabgol (*Plantago ovata* Forsk) to chitosan and salinity. *Int. J. Agric. Crop Sci.* 5, 1084–1088.

Medeiros M. J. L., Silva M. M. A., Granja, M. M. C., Souza e Silva Júnior, G., Camara, T. R., Willadino, L. et al. (2015). Effect of exogenous proline in two sugarcane genotypes grown *in vitro* under salt stress. *Acta biol. Colomb.* 20, 57–63. doi: 10.15446/abc.v20n2.42830

Murashige, T., and Skoog, F. (1962). A revised medium for rapid growth and bio assays with tobacco tissue cultures. *Physiol. Plant.* 15, 473–497. doi: 10.1111/j.1399-3054.1962.tb08052.x

Perry, E. M., and Roberts, D. A. (2008). Sensitivity of narrow-band and broad-band indices for assessing nitrogen availability and water stress in an annual crop. *Agron. J.* 100:1211. doi: 10.2134/agronj2007.0306

Pichyangkura, R., and Chadchawan, S. (2015). Biostimulant activity of chitosan in horticulture. *Sci. Hortic.* 196, 49–65. doi: 10.1016/j.scienta.2015.09.031

Podlešáková, K., Ugena, L., Spíchal, L., Doležal, K., and De Diego, N. (2018). Phytohormones and polyamines regulate plant stress responses by altering GABA pathway. *N. Biotechnol.* doi: 10.1016/j.nbt.2018.07.003 [Epub ahead of print].

Pouvreau, J.-B., Gaudin, Z., Auger, B., Lechat, M.-M., Gauthier, M., Delavault, P., et al. (2013). A high-throughput seed germination assay for root parasitic plants. *Plant Methods* 9:32. doi: 10.1186/1746-4811-9-32

Povero, G., Mejia, J. F., Di Tommaso, D., Piaggesi, A., and Warrior, P. (2016). A systematic approach to discover and characterize natural plant biostimulants. *Front. Plant Sci.* 7:435. doi: 10.3389/fpls.2016.00435

Rahaman, M. M., Ahsan, M. A., Gillani, Z., and Chen, M. (2017). Digital biomass accumulation using high-throughput plant phenotype data analysis. *J. Integr. Bioinform.* 14, 1–13. doi: 10.1515/jib-2017-0028

Rodriguez-Furlán, C., Miranda, G., Reggiardo, M., Hicks, G. R., and Norambuena, L. (2016). High throughput selection of novel plant growth regulators: assessing the translatability of small bioactive molecules from *Arabidopsis* to crops. *Plant Sci.* 245, 50–60. doi: 10.1016/j.plantsci.2016.01.001

Roychoudhury, A., Basu, S., and Sengupta, D. N. (2011). Amelioration of salinity stress by exogenously applied spermidine or spermine in three varieties of indica rice differing in their level of salt tolerance. *J. Plant Physiol.* 168, 317–328. doi: 10.1016/j.jplph.2010.07.009

Sabagh, A., El Islam, M. S., Ueda, A., Saneoka, H., and Barutçular, C. (2015). Increasing reproductive stage tolerance to salinity stress in soybean. *Int. J. Agric. Crop Sci.* 8, 738–745.

Savvides, A., Ali, S., Tester, M., and Fotopoulos, V. (2016). Chemical priming of plants against multiple abiotic stresses: mission possible? *Trends Plant Sci.* 21, 329–340. doi: 10.1016/j.tplants.2015.11.003

Sharma, H. S. S., Fleming, C., Selby, C., Rao, J. R., and Martin, T. (2014). Plant biostimulants: a review on the processing of macroalgae and use of extracts for crop management to reduce abiotic and biotic stresses. *J. Appl. Phycol.* 26, 465–490. doi: 10.1007/s10811-013-0101-9

Sharma, H. S. S., Selby, C., Carmichael, E., McRoberts, C., Rao, J. R., Ambrosino, P., et al. (2016). Physicochemical analyses of plant biostimulant formulations and characterisation of commercial products by instrumental techniques. *Chem. Biol. Technol. Agric.* 3:13. doi: 10.1186/s40538-016-0064-6

Shekari, F., Danalo, A. A., and Mustafavi, S. H. (2015). Exogenous polyamines improve seed germination of borage under salt stress via involvement in antioxidant defenses. *WALIA J.* 31, 57–63.

Shu, S., Yuan, L. Y., Guo, S. R., Sun, J., and Liu, C. J. (2012). Effects of exogenous spermidine on photosynthesis, xanthophyll cycle and endogenous polyamines in cucumber seedlings exposed to salinity. *Afr. J. Biotechnol.* 11, 6064–6074. doi: 10.5897/AJB11.1354

Shu, S., Yuan, Y., Chen, J., Sun, J., Zhang, W., Tang, Y., et al. (2015). The role of putrescine in the regulation of proteins and fatty acids of thylakoid membranes under salt stress. *Sci. Rep.* 5:14390. doi: 10.1038/srep14390

Silva, A. T., Ligterink, W., and Hilhorst, H. W. M. (2017). Metabolite profiling and associated gene expression reveal two metabolic shifts during the seed-to-seedling transition in *Arabidopsis thaliana*. *Plant Mol. Biol.* 95, 481–496. doi: 10.1007/s11103-017-0665-x

Talat, A., Nawaz, K., Hussian, K., Bhatti, K. H., Siddiqi, E. H., Khalid, A., et al. (2013). Foliar application of proline for salt tolerance of two wheat (*Triticum aestivum* L.) cultivars. *World Appl. Sci. J.* 22, 547–554. doi: 10.5829/idosi.wasj.2013.22.04.19570

Tanabata, T., Shibaya, T., Hori, K., Ebana, K., and Yano, M. (2012). SmartGrain: high-throughput phenotyping software for measuring seed shape through image analysis. *Plant Physiol.* 160, 1871–1880. doi: 10.1104/pp.112.205120

Teh, C. Y., Shaharuddin, N. A., Ho, C. L., and Mahmood, M. (2016). Exogenous proline significantly affects the plant growth and nitrogen assimilation enzymes activities in rice (Oryza sativa) under salt stress. *Acta Physiol. Plant* 38:151. doi: 10.1007/s11738-016-2163-1

Thiam, M., Champion, A., Diouf, D., and Mame Ourèye, S. Y. (2013). NaCl effects on in vitro germination and growth of some senegalese cowpea (*Vigna unguiculata* (L.) *Walp.)* Cultivars. *ISRN Biotechnol.* 2013:382417. doi: 10.5402/2013/382417

Van Oosten, M. J., Pepe, O., De Pascale, S., Silletti, S., and Maggio, A. (2017). The role of biostimulants and bioeffectors as alleviators of abiotic stress in crop plants. *Chem. Biol. Technol. Agric.* 4:5. doi: 10.1186/s40538-017-0089-5

Yakhin, O. I., Lubyanov, A. A., Yakhin, I. A., and Brown, P. H. (2016). Biostimulants in plant science: a global perspective. *Front. Plant Sci.* 7:2049. doi: 10.3389/fpls.2016.02049

Zeng, D., Luo, X., and Tu, R. (2012). Application of bioactive coatings based on chitosan for soybean seed protection. *Int. J. Carbohydr. Chem.* 2012, 1–5. doi: 10.1155/2012/104565

Conflict of Interest Statement: The authors declare that the research was conducted in the absence of any commercial or financial relationships that could be construed as a potential conflict of interest.

frontiers in Plant Science

ORIGINAL RESEARCH
published: 20 September 2018
doi: 10.3389/fpls.2018.01342

Use of Biostimulants for Organic Apple Production: Effects on Tree Growth, Yield, and Fruit Quality at Harvest and During Storage

*Sebastian Soppelsa[1], Markus Kelderer[2], Claudio Casera[2], Michele Bassi[2], Peter Robatscher[2] and Carlo Andreotti[1]**

[1] Faculty of Science and Technology, Free University of Bozen-Bolzano, Bolzano, Italy, [2] Laimburg Research Centre, Vadena, Italy

The experiment was conducted during two consecutive seasons (years 2016 and 2017) in an organic apple orchard of the cultivar Jonathan. Several biostimulants were tested (10 in total), including humic acids, macro and micro seaweed extracts, alfalfa protein hydrolysate, amino acids alone or in combination with zinc, B-group vitamins, chitosan and a commercial product containing silicon. Treatments were performed at weekly intervals, starting from the end of May until mid-August. The macroseaweed extract was effective in stimulate tree growth potential in both years, as shown by a significantly larger leaf area (+20% as compared to control) and by an higher chlorophyll content and leaf photosynthetic rate in year 2016. As for the yield performances and apples quality traits at harvest (average fruit weight, soluble solids content, titratable acidity, and flesh firmness), they were generally affected by the different climatic conditions that characterized the two growing seasons (year 2017 being characterized by higher maximal and average temperatures and by limited rainfalls at the beginning of the season). Treatments with macroseaweed extract, B-group vitamins and alfalfa protein hydrolysate were able to significantly improve the intensity and extension of the red coloration of apples at harvest. Correspondingly, the anthocyanin content in the skin of apples treated with the same biostimulants resulted significantly higher than control, highlighting the potential influence of these substances on the synthesis of secondary metabolites in apple. The incidence of physiological disorders was also monitored during apple storage period. Amino acids plus zinc application was effective in reducing (more than 50%) the incidence of the "Jonathan spot," the main post-harvest disorder for this cultivar.

Keywords: *Malus* × *domestica*, seaweed extract, photosynthesis, phenolic compounds, anthocyanins, physiological disorders, organic production

OPEN ACCESS

Edited by:
Youssef Rouphael, Università degli Studi di Napoli Federico II, Italy

Reviewed by:
Riccardo Lo Bianco, Università degli Studi di Palermo, Italy
Sophie Colombie, Institut National de la Recherche Agronomique (INRA), France
Lechaudel Mathieu, Centre de Coopération Internationale en Recherche Agronomique pour le Développement (CIRAD), France

****Correspondence:***
Carlo Andreotti
carlo.andreotti@unibz.it

Specialty section:
This article was submitted to Crop and Product Physiology, a section of the journal Frontiers in Plant Science

Received: *30 May 2018*
Accepted: *24 August 2018*
Published: *20 September 2018*

Citation:
Soppelsa S, Kelderer M, Casera C, Bassi M, Robatscher P and Andreotti C (2018) Use of Biostimulants for Organic Apple Production: Effects on Tree Growth, Yield, and Fruit Quality at Harvest and During Storage. Front. Plant Sci. 9:1342. doi: 10.3389/fpls.2018.01342

INTRODUCTION

Organic farming, including organic apple production, is generally characterized by lower crop yield as compared with conventional production systems mainly because of the limitation imposed on fertilization (no use of chemical fertilizers) and on plant defense (no use of pesticides) (Amarante et al., 2008; de Ponti et al., 2012; Seufert et al., 2012; Orsini et al., 2016). In order to reduce

this gap of productivity, the organic agriculture sector is therefore constantly seeking for new agroecological practices to integrate in the management of the cultivation systems. Biostimulants are considered among the most innovative and promising solutions to improve sustainability and profitability of organic agriculture (Povero et al., 2016). Biostimulants are defined as "*any substance or microorganism applied to plants with the aim to enhance nutrition efficiency, abiotic stress tolerance and/or crop quality traits, regardless of its nutrients content*" (du Jardin, 2015). The main categories of plant biostimulants include natural substances such as humic and fulvic acids, protein hydrolysates, seaweed extracts (Battacharyya et al., 2015; Canellas et al., 2015; Colla et al., 2017), beneficial fungi (e.g., arbuscular mycorrhizal fungi and *Trichoderma* spp.) (Rouphael et al., 2015) and plant growth promoting rhizobacteria (Ruzzi and Aroca, 2015). Other substances (e.g., vitamins, chitosan and other biopolymers, inorganic compounds) can have biostimulant properties, but their classification within the group of biostimulants is still under consideration.

Organic farming can benefit from the use of biostimulants because these substances can enhance plant resilience to the nutrient limitation typical of this production system, therefore reducing the gap between organic and conventional yields (De Pascale et al., 2017). The increase of nutrient uptake and assimilation by biostimulant substances can follow different mechanisms. Biostimulants such as humic substances and protein hydrolysates can enhance nutrient availability by changing the physico-chemical properties of soils (i.e., increasing the cation exchange capacity of sandy soils) and by forming complexes with micronutrients more available to plants (Canellas et al., 2015; Colla et al., 2017). Moreover, the use of biostimulants (e.g., humic acids, protein hydrolysates, and seaweed extracts) can promote root growth and development, allowing a better soil exploration and consequently nutrient uptake (Battacharyya et al., 2015; Hernández-Herrera et al., 2016; Scaglia et al., 2016; Colla et al., 2017). The nutrient assimilation process can be also positively affected by biostimulants as shown by the increased activity of key enzymes (e.g., nitrate reductase) following the application of bioactive substances (protein hydrolysates and seaweed extracts) to roots and leaves (Schiavon et al., 2008; Ertani et al., 2009; Zhang et al., 2010). Despite the large and increasing number of publications dealing with biostimulants (Colla and Rouphael, 2015), scientific-based information on their optimal use, crop specificity, and interaction with the growing conditions is anyway still incomplete. Studies on the effect of biostimulants on the growth and yield potential of plants have been conducted primarily on vegetable crops. Rouphael et al. (2017) and Ertani et al. (2015) on tomato and pepper, respectively, demonstrated how protein hydrolysates were able to increase plant productivity, probably because of a stimulation of the plant primary metabolism trigged by signaling molecules (peptides, oligopeptides, and free amino acids) contained in the hydrolysate. This fostering effect on the primary metabolism is particularly significant when plants are under stress conditions, as demonstrated for tomato and spinach suffering of drought and treated with seaweed extracts (Xu and Leskovar, 2015; Goñi et al., 2018). Similar studies are more complex when conducted on woody plants, also due to the role played by nutrient reserves stored in the permanent woody structure of the tree for its growth metabolism. This could be one of the reasons explaining the current lack of solid evidences connecting the use of biostimulant compounds with the final growth and yield of fruit crops.

Biostimulants have been found active in promoting final crop quality and, more in detail, researches had highlighted the relevance of biostimulant applications for selected functional quality traits. The concentration of secondary metabolites such as phenols, flavonoids, and ascorbic acid were enhanced after the application of protein hydrolysates or seaweed extract in tomato (Rouphael et al., 2017), pepper (Ertani et al., 2015), onion, and potato (Lola-Luz et al., 2014). Giving the high antioxidant activity of phenolic compounds, the nutraceutical value of those vegetables was also improved. The physiological mechanism behind these results is the up-regulation of genes responsible for the secondary metabolism in plants treated with the biostimulants as demonstrated with the microarrays technique on tomato (Ertani et al., 2017). Color is another crop quality trait considered as very relevant because of its tight connection with the consumer-choice behavioral mechanism. In the case of apple fruits, color intensity and extension is largely related to the anthocyanin biosynthesis and accumulation in skin tissue (Liu et al., 2013). In order to promote apple final coloration at harvest, several cultivation techniques have been tested such as defoliation, reflective mulches in the inter-row space prior to harvest, overhead sprinkler irrigation, and fruit bagging (Whale et al., 2008; Blanke and Kunz, 2016). In addition, also the use of synthetic growth regulators (ethephon) to stimulate pigments biosynthesis can be an option, even though not allowed by the organic production system (Ubi, 2004). Selected biostimulants have been found able to improve final coloration of different fruit crops (Roussos et al., 2009; Portu et al., 2015; Blanke and Kunz, 2016) probably thanks to their capacity to modulate the activity of endogenous plant hormones (Wally et al., 2013), leading to an induction of the anthocyanin biosynthetic pathway at fruit skin level.

Biostimulants could also be considered for their implementation in the post-harvest management of fruits. Biostimulants containing mineral nutrients such as zinc and silicon might contribute with calcium to the strengthening of cell wall structure (Ferguson et al., 1999), therefore allowing the preservation of fruit quality attributes for longer period. This is of particular interest for the organic apple production system that is presently lacking of any useful means to manage apple physiological disorders during storage.

Our field experiment was conducted in an organic apple orchard located in the Alto-Adige/South Tyrol Province, northeast of Italy. This area is the major apple-growing district of Italy, accounting for approximately 65% of the total national and 10% of EU production (FAOSTAT, 2017, ISTAT, 2017). Pushed by the concern of the public opinion about the intensive use of pesticides in this area and by the favorable market conditions, the organic apple production sector has gained relevance constantly during the last years. Today around 10% of the apple orchard surface in Alto-Adige/South Tyrol is cultivated according to the organic protocol and about one third of all

organic apples in Europe are harvested in this province (Alto-Adige/South Tyrol Province, 2017). To sustain further the growth and profitability of the organic apple sector, the implementation of new agroecological means, such as the biostimulant products, in the management of the organic horticultural systems is highly requested by the growers. The use of these new tools must follow anyway information deriving from scientifically sound research about their effects on plant physiological and biochemical responses. With this goal, this work aimed to investigate the effects of biostimulant applications on the growth, yield, and fruit quality of organically cultivated apple trees, belonging to the cv. Jonathan. Some of the selected substances were tested for the first time on apple crop and, to the best of our knowledge, this was the first study where the efficacy of several biostimulants was evaluated simultaneously and during two consecutive growing seasons. In addition, their effect was considered also during the storage period of fruits by measuring the incidence of the main post-harvest physiological disorder of "Jonathan" apples.

MATERIALS AND METHODS

Experimental Site and Biostimulant Applications

The experiment was conducted over two growing seasons (years 2016 and 2017) in an experimental apple orchard located at the Laimburg Research Centre, in the municipality of Ora/Auer (46° 22′ North; 11° 17′ East; 237 m a.s.l.) in Alto Adige/South Tyrol, Italy. Meteorological conditions during the growing seasons (from April to August 2016 and 2017) are reported in **Figure 1**. The 8-year-old "Jonathan" apple trees (*Malus*× *domestica* Borkh. "Red Jonathan") were grafted on M.9 rootstock, spaced 1.0 m × 3.0 m (3,333 trees ha^{-1}), and trained to spindle system. The orchard received standard horticultural cares in accordance with the regulation governing organic production.

The experiment set up was organized as a completely randomized block design with four replications per treatment and five trees per replicate. To avoid any contamination between treatments, replicates on the same row were separated by an interval of 10 untreated trees, whereas a buffer row was used to separate plots on adjacent rows. Trees were selected according to their uniformity as for flowering and growth, by estimating the number of flowers per tree and measuring trunk circumference at 20 cm from the ground, respectively. The same set of trees was selected for the experiment in both the considered growing seasons. Biostimulant applications to the tree canopy started 40 days after full bloom (DAFB) at the end of May and were performed at weekly intervals until the end of August (1 week before harvest).

Details on the names, abbreviations, and physico-chemical characteristics of the utilized biostimulants, including the way of application are reported in **Table 1** and **Supplementary Table S1**. All treatments were performed with a total volume of 1,500 L ha^{-1}; each tree was sprayed until the run-off point using a pulled sprayer under favorable weather forecast (no rainfalls expected in the following 24 h).

Vegetative Growth and Leaf Gas Exchanges

Two shoots per plant were selected at a similar canopy height and position and tagged in order to measure the shoot elongation at 2-week interval, from May until growth cessation. Two fully expanded leaves from the tagged shoots were used for the indirect evaluation of the chlorophyll content with a SPAD-502 Chlorophyll Meter (Konica Minolta, Tokyo, Japan) and the measurements conducted at 2-week interval. At mid-July of both years two leaves per plant, chosen at an intermediate position along the same tagged apple shoots, were collected and leaf area was determined with a LI-COR 3000 Leaf Area Meter (LI-COR Inc., Lincoln, NE, United States). In mid-summer (28th, 30th of July and 1st of August of both years), when trees had already received nine applications of all the treatments, net assimilation (A, $\mu mol\ m^{-2}\ s^{-1}$) and transpiration (E, $mmol\ m^{-2}\ s^{-1}$) rates of leaves were evaluated using a portable gas exchange analyzer (LCpro ADC, Hoddesdon Bioscientific, Ltd., United Kingdom). The gas exchange evaluations were conducted at time 0 (T0, immediately before the tenth application of biostimulants), time 1 (T1, 48 h), and time 2 (T2, 96 h) after application. Measurements were performed on a young, fully expanded leaf of five randomly selected shoots per treatment and were taken under saturating light conditions (1,800 μmol photons $m^{-2}\ s^{-1}$), around midday (11:00–13:00 h) using a broad leaf gas chamber with a window size of 6.25 cm^2 and a flow rate of 400 ml min^{-1}.

Yield and Fruit Quality

Apples were harvested on the 1st of September of both years, approximately 140 DAFB, based on the starch-iodine maturity test. Starting from the 10th of August (approximately 20 days prior the expected time of harvest), 10 apples per replicate (40 per treatments) were randomly sampled and evaluated for their starch index (**Supplementary Figure S1**). The harvest was performed when the apples reached uniformly a value of the starch index around 2.5, which is considered the optimal picking time for the cv. Jonathan. Yield per tree was determined by collecting all fruits from three out of the five trees per replicate (those located in the central position of each replicate). Fruits from each tree were placed in tagged boxes in order to keep track of the tree they come from, and then transported in the laboratory where they were automatically sorted with a sorting machine (Aweta, Nootdorp, Netherlands). This device delivers the following parameters: number of fruits and total yield per tree, average fruit weight and red overcolor extension in percentage of apple fruit surface (four classes: <33%, 33–50%, 51–75%, and >75%). The colorimetric coordinates (L*, a*, and b*) were determined with a colorimeter (Minolta, model CR-400, Tokyo, Japan) by measuring 10 fruits randomly selected among those belonging to the same replicate at five different positions around the equatorial side of each fruit. Values are presented as color index [CI = (1000*a)/(L*b)], with higher CI value indicating a more intense red color in the fruit (Tessmer et al., 2016). The total soluble solids (TSS

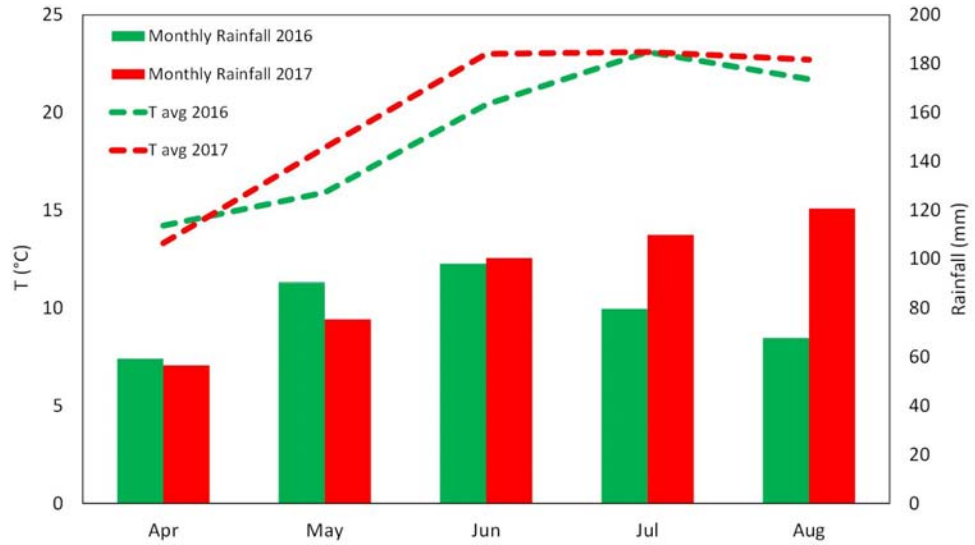


FIGURE 1 | Meteorological conditions during the growing season 2016 and 2017 at the Laimburg Research Centre in Ora (Italy). Average temperature (dotted lines) and cumulated monthly rainfalls (columns).

FIGURE 2 | Symptoms of "Jonathan spot" disorder (**Left**: healthy fruit, **Right**: symptomatic fruit).

as °Brix), titratable acidity (TA as g L^{-1} of malic acid) and flesh firmness (FF as kg cm^{-2}) of 10 fruits per replicate (40 per treatment) were determined at harvest with the automatic measuring device "Pimprenelle" (Satop Giraud Technologie, Cavillon, France).

Physiological Disorder After Storage

The tagged boxes containing around one hundred apples per tree (around 300 per replicate) were kept in cold room (2°C and RH 85–90%) and sampled regularly (every 2 months) to monitor the post-harvest ripening process. Moreover, the incidence of the physiological disorder "Jonathan spot" was evaluated during storage period by counting the number of symptomatic fruits per plant. "Jonathan spot" symptoms are characterized by irregular small black spots on the skin of apples as shown in **Figure 2**. Number of spots on each fruit (severity) can be very variable and, differently from those of the more studied apple bitter pit, the necrosis rarely involve cells of the inner pulp tissue of the apple.

Biochemical Analysis of Apple Fruits

Chemicals

Ethanol (96%) was obtained from J.T. Baker (Center Valley, PA, United States). Acetic acid (96%), potassium chloride, and hydrochloric acid (36%) were from Merck (Kenilworth, NJ, United States) and Fisher Chemical (Thermo Fisher Scientific, Waltham, MA, United States), respectively. Phosphoric acid (≥99%), Folin-Ciocalteu's phenol reagent, sodium carbonate, 2,2′-azino-bis(3-ethylbenzothiazoline-6-sulfonic acid) diammonium salt (ABTS), sodium fluoride, ascorbic acid (99%) and Trolox (6-hydroxy-2,5,7,8-tetramethylchroman-2-carboxylic acid) were purchased from Sigma-Aldrich (St. Louis, MO, United States). Sodium acetate (anhydrous) was purchased from Fisher Chemical (Thermo Fisher Scientific, Waltham, MA, United States). Potassium persulfate ($K_2S_2O_8$) and gallic acid (≥99%) were purchased from Carl Roth (Karlsruhe, Germany). Methanol (HPLC-grade) was purchased from VWR Chemicals (Milan, Italy), and meta-phosphoric acid (≥99%) and monopotassium phosphate (≥ 99%) were from Thermo Fisher Scientific Inc. (Waltham, MA, United States). The ultrapure water was prepared with a Milli-Q-water purification system (EMD Millipore Corporation, Billerica, MA, United States).

Sample Preparation and Extraction Procedure

Six fruits per replicate (24 per treatment) were randomly collected at harvest. Apples were peeled and pulp and skin samples were sampled, immediately frozen in liquid nitrogen and stored at −80°C. Extraction was conducted using 25 mg of lyophilized sample which were homogenized and extracted in 1.8 mL of extraction solution (80% methanol acidified with H_3PO_4, pH 1.0) and in 30 μL of 0.1 M NaF solution for 15 min at 5°C. The extract was then filtered with PTFE filters (0.45 μm, Thermo

TABLE 1 | Biostimulant characterization, properties, and mode of application.

Treatment	Active ingredients	Moisture (%)	Ash (%)	Density (kg dm^{-3})	Organic matter (%)	pH	Electrical conductivity (ds m^{-1})	Total organic carbon (% w w^{-1})	Total organic nitrogen (% w w^{-1})	Free amino acids (% w w^{-1})	Total amino acids (% w w^{-1})	Other characteristics	Concentration
CON	Water												
HAL	Humic acids	–	–	1.1	–	9.2	1.2	7.5	0.1	–	–	–	1.0 kg ha^{-1}
APH	Alfalfa protein hydrolysate	70.0	7.0	1.2	23.0	5.5	1.6	–	–	1.5	5.1	More details in the annex	3.0 kg ha^{-1}
SEA	Macroseaweed extract	84.0	1.5	1.0	14.5	4.5	0.4	3	≤0.1	–	–	From *A. nodosum*	4.0 kg ha^{-1}
SPI	Microalga hydrolysate	–	–	1.2	–	5.5	1.5	16.8	3.9	6.5	–	From *Spirulina* spp. (37% hydrol. degree)	4.0 kg ha^{-1}
MAA	Mix of amino acids	45.0	5.0	1.2	50.0	5.5	0.8	24.5	9	1.5	55.0	More details in the annex	3.0 kg ha^{-1}
PHE	MAA combined with pure phenylalanine	55.0	5.0	1.2	40.0	5.5	–	19.6	7.2	1.8	45.0	Phenylalanine (1%)	3.0 kg ha^{-1}
ZIN	MAA combined with zinc	55.0	7.0	1.2	38.0	5.5	–	19.6	7.2	0.8	44.0	Zn (2%)	3.0 kg ha^{-1}
VIT	B-group vitamins (Sigma-Aldrich, United States)	–	–	–	–	–	–	–	–	–	–	B1-thiamine (33.3%), B2-riboflavin (33.3%), B6-pyridoxine (33.3%)	1.5 kg ha^{-1}
CHI	chitosan – ChitoPlant Solution (Agritalia, Italy)	98.3	0.01	–	1.7	5.2	–	–	–	–	–	–	15 kg ha^{-1}
SIL	Siliforce (ILSA S.p.A., Italy)	–	–	1.2	–	2.0	–	–	–	–	–	Si (8 g kg^{-1}), Zn (1.8%), Mo (0.2%)	300 ml ha^{-1}

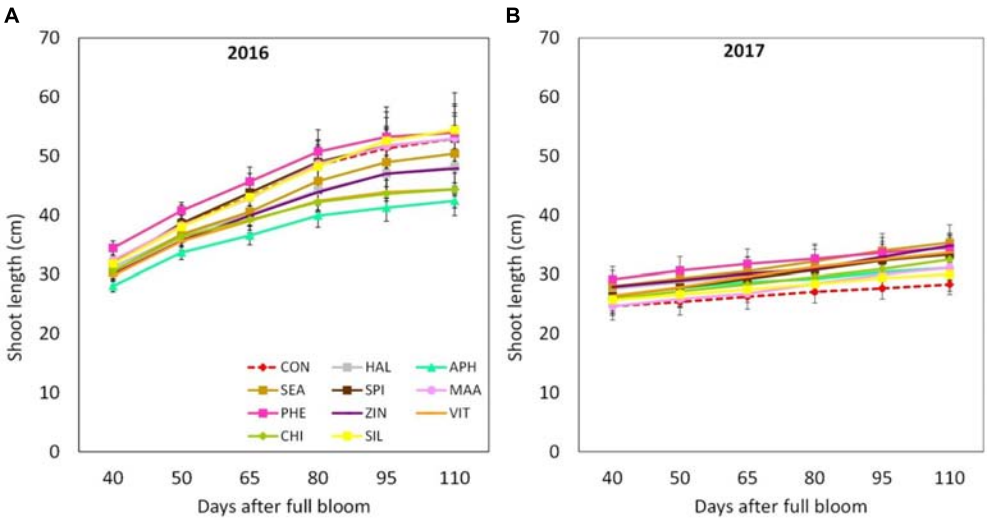


FIGURE 3 | Shoot growth dynamics (from 40 to 110 DAFB) in apple plants treated with different biostimulant products and water (control) for year 2016 **(A)** and 2017 **(B)**. Vertical bars indicate mean ± SE, *n* = 4. Treatments' legend: CON, control; HAL, humic acids; APH, alfalfa protein hydrolysate; SEA, macroseaweed extract; SPI, microalga hydrolysate; MAA, mix of amino acids; PHE, MAA combined with pure phenylalanine; ZIN, MAA combined with zinc; VIT, B-group vitamins; CHI, chitosan; SIL, Siliforce®.

Fisher Scientific) and the filtrate was stored at −80°C for analyses. Extraction procedure for ascorbic acid analysis is described in Section "Ascorbic Acid Quantification."

Determination of Total Phenolic Content (TPC)

Total phenolic content (TPC) in peel and pulp extracts was quantified using the Folin-Ciocalteu assay following the methodology described in Wolfe et al. (2003). Briefly, a 60 μL aliquot of the sample extract was diluted with 250 μL of deionized water. Then, 60 μL Folin-Ciocalteu reagents were added and the mixture was allowed to react for 6 min at 20°C. Afterward, 630 μL of Na_2CO_3 (7.5% w/v) was added and incubated for 90 min at 20°C. The absorbance of samples and standards was read at 740 nm on a spectrophotometer Cary 60 UV-Vis (Agilent Technologies, Palo Alto, CA, United States) and the results were expressed as milligrams of gallic acid equivalents (GAE) per

TABLE 2 | Leaf area and fruit yield parameters at harvest as affected by biostimulants and growth season.

	Leaf area (cm^2)	Yield (kg $tree^{-1}$)	Number fruits $tree^{-1}$ (N.)	Fruit weight (g)	Fruit diameter (mm)
Treatment					
CON	26.30 ± 0.78[1]	14.61 ± 1.62	96.67 ± 13.54	157.45 ± 6.27	72.51 ± 0.87
HAL	25.92 ± 0.79	15.44 ± 0.93	97.50 ± 6.88	161.21 ± 4.00	73.00 ± 0.59
APH	28.80 ± 1.92	13.79 ± 0.78	86.92 ± 4.93	161.35 ± 4.86	73.10 ± 0.71
SEA	32.22 ± 0.97**	12.75 ± 0.82	78.21 ± 5.78	165.20 ± 4.35	73.73 ± 0.64
SPI	30.25 ± 0.99	13.99 ± 1.02	88.67 ± 7.67	160.11 ± 3.80	73.00 ± 0.54
MAA	28.16 ± 0.84	13.50 ± 0.88	90.33 ± 8.25	158.48 ± 6.88	72.81 ± 1.05
PHE	26.91 ± 1.05	15.43 ± 1.33	95.67 ± 9.65	165.53 ± 3.99	73.91 ± 0.60
ZIN	30.24 ± 1.30	13.08 ± 0.79	81.42 ± 5.59	163.79 ± 5.42	73.62 ± 0.80
VIT	29.82 ± 1.35	12.76 ± 0.96	76.33 ± 5.98	169.99 ± 4.05	74.68 ± 0.54
CHI	27.20 ± 0.82	13.79 ± 1.36	88.54 ± 10.30	160.03 ± 4.41	73.01 ± 0.71
SIL	27.83 ± 1.34	15.52 ± 1.11	100.21 ± 10.45	159.05 ± 5.42	72.70 ± 0.81
Significance	**	ns	ns	ns	ns
Year					
2016	27.79 ± 1.34	14.38 ± 0.90	84.64 ± 5.59	171.67 ± 2.44	74.59 ± 0.38
2017	29.24 ± 1.16	13.74 ± 1.24	93.62 ± 10.35	152.36 ± 4.18	71.97 ± 0.68
Significance	*	ns	***	***	*
T × Y	ns	ns	ns	ns	ns

[1]*Mean ± SE. Values followed by asterisk indicate significant differences between a single treatment group and control group, according to Dunnett's test (n = 4). ***P < 0.001; **P < 0.01; *P < 0.05, ns, not significant.*

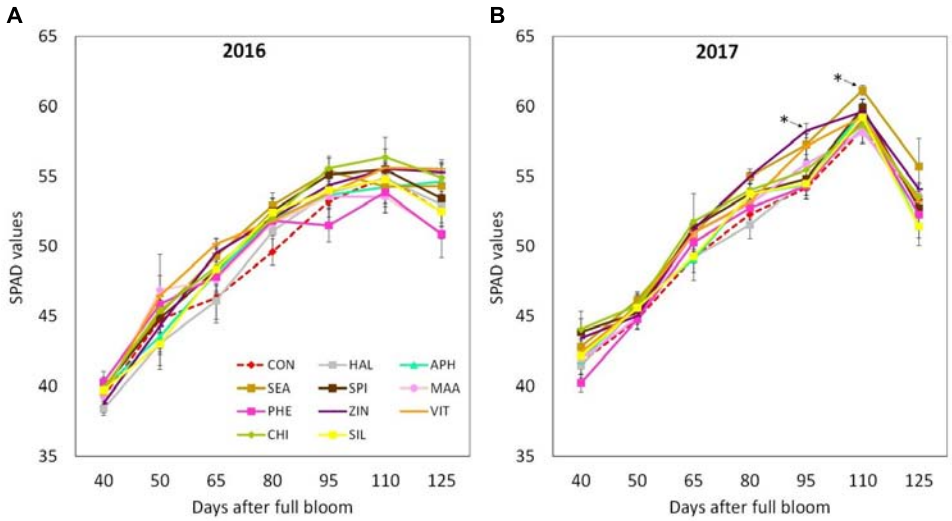


FIGURE 4 | Chlorophyll content (as SPAD values) dynamics (from 40 to 125 DAFB) in apple plants treated with different biostimulant products and water (control) for year 2016 **(A)** and 2017 **(B)**. Vertical bars indicate mean ± SE, $n = 4$. *Indicates significant differences according to Dunnett's test. ***$P < 0.001$; **$P < 0.01$; *$P < 0.05$.

100 grams of dry weight (mg GAE 100 g^{-1} DW) referred to a standard curve of gallic acid (range 5–500 mg L^{-1}, $r^2 = 0.999$).

Determination of Total Anthocyanin Content (TAC)

Total anthocyanin content (TAC) in peel extracts was determined using the spectrophotometric pH differential method as described in Lee et al. (2005). Briefly, two dilutions of the same sample were prepared by adding 200 μL of extract to 800 μL of potassium chloride (0.25 M, pH 1) and to 800 μL of sodium acetate (0.4 M, pH 4.5), respectively. The absorbances were measured at 520 and 700 nm on the Cary 60 UV-Vis spectrophotometer. Total anthocyanins content was calculated using Lambert–Beer law (ε = 26900 L/mol/cm, MW = 449.2 g/mol) as mg cyanidin 3-glucoside equivalents (CGE) per 100 g of dry weight.

Antioxidant Activity (ABTS)

The antioxidant activity was determined using the ABTS assay as described in Re et al. (1999) with some modifications. Briefly, ABTS radical cation ($ABTS^+$) was generated by reacting 7 mM of ABTS with 2.45 mM of potassium persulfate ($K_2S_2O_8$). The mixture was incubated in a darkroom at 4°C for 16 h. $ABTS^+$ solution was diluted with water until the absorbance was 0.700 ± 0.02 at 734 nm. For the assay, 30 μL of sample extract or standard were added and mixed to 1.97 mL of diluted $ABTS^+$ solution. The absorbance was measured at 734 nm on the Cary 60 UV–Vis spectrophotometer after 10 min in dark conditions. A calibration curve was prepared using Trolox standard at different concentrations. The antioxidant activity results were expressed as milligrams Trolox equivalents per 100 grams of dry weight (mg Trolox 100 g^{-1} DW) using an external calibration (Trolox, range 15.6–500 mg L^{-1}, $r^2 = 0.999$).

Ascorbic Acid Quantification

The quantification of ascorbic acid was determined using the methodology described in Bassi et al. (2018). Briefly, an aliquot of ca. 50 mg of freeze-dried apple pulp or peel was extracted using 1 mL of extraction solution [700 μL deionized H_2O containing 8% (v/v) acetic acid and 3% (w/v) metaphosphoric acid added with 300 μL of methanol] (AOAC, 2005), mixed using a Vortex-Genie 2 at 3200 rpm for about 20 s at room temperature and filtered through a 0.20 μm PTFE filter. An HPLC Agilent 1260 Infinity (Santa Clara, CA, United States) with a diode array (1260 DAD VL) detector was used for the analysis of the ascorbic acid. Chromatographic separation was carried out at 25°C using a Kinetex 5 μ C18 100 Å column (150 mm × 4.6 mm, 5 μm particle size; Phenomenex, Torrance, CA, United States) and a pre-column (4.6 mm, Security Card, Phenomenex, Torrance, CA, United States). The detection wavelength was fixed at 260 nm. The mobile phases used were 5 mM KH_2PO_4, pH 4.8 (solvent A) and methanol (solvent B). The analytical gradient was the follow: 0 min, 100% A; 2.5 min, 100% A; 6 min, 80% A; 8 min, 100% A, and 13 min, 100% A. The flow rate was set at 1.0 mL min^{-1}. The temperature of the autosampler was 4°C and injection volume was 5 μL. The Agilent ChemStation™ (ver. C.01.03) (Agilent Technologies, Palo Alto, CA, United States) was used for system control and data processing.

Mineral Elements in Fruit Skin

At harvest of both years, six fruits per replicate (four replicates per treatment) were randomly selected and peeled. Skin samples were immediately frozen in liquid nitrogen and stored at −80°C. Subsequently, samples were lyophilized, ground and homogenized for mineral element content analyses. Nitrogen (N) content was determined by Kjeldahl method and the other macro (P, K, Ca, and Mg) and microelements (S, Fe, Cu, B, Zn, Mn, Na, and Si) were analyzed by using the

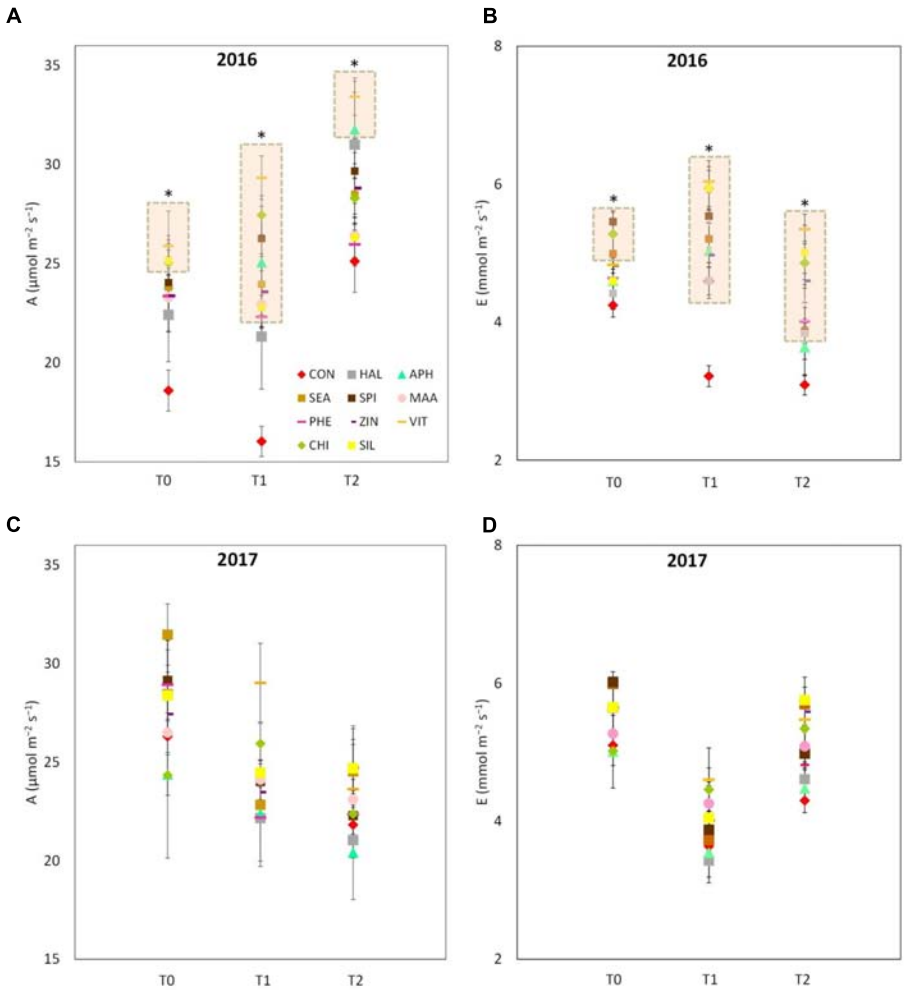


FIGURE 5 | Photosynthetic and transpiration rates measured in 2016 (**A,B**, respectively) and 2017 (**C,D**, respectively) as affected by biostimulant applications (T0: immediately before spray; T1: 48 h after spray; T2: 96 h after spray). Asterisk (*) and the pink rectangle in the background indicate the group of treatments that significantly differed from control according to Dunnett's test ($P < 0.05$).

inductively coupled plasma optical emission spectrometry (ICP-OES).

Statistical Analysis

A two-way analysis of variance (ANOVA) on the complete randomized block design was performed on the data, using the factors "treatment" and "year" as fixed and the factor "block" as random. Mean separation of variables with equal variance was performed by the Dunnett's test, contrasting each biostimulant group mean against the control group mean. This procedure is recommended when working with several experimental treatments (Dunnett, 1985). Data expressed in percentage (classes of red overcolor extension and "Jonathan spot" incidence) were arcsine-transformed prior to the application of the ANOVA. In case of significant interaction between the factor "treatment" and the factor "year," results were presented separately for the 2 years in dedicated figures as vertical grouped bars with standard error per treatment combination. Data gathered from repeated measurements during both seasons (shoot length, SPAD index, leaf gas exchanges, and incidence of post-harvest disorder) were subjected to one-way ANOVA for single year, using the Dunnett's test to compare the means at each data point.

RESULTS

Vegetative Growth

Final shoot growth resulted significantly affected by the factor "year," 2016 being generally characterized by longer shoots (40–50 cm) at the end of the growing season as compared to year 2017 (25–35 cm) (**Figures 3A,B**). Treatments with biostimulants did not induce any significant modification of the growth dynamic in both years (**Figure 3**). Leaf area was generally larger in year 2017 than in 2016 (**Table 2**). Independently from the considered year, SEA applications were found able to increase the average leaf area by around 20%. Chlorophyll content (measured as SPAD

TABLE 3 | Fruit quality traits (TA, titratable acidity; CI, color index; TAC, total anthocyanin content; AA, ascorbic acid in the pulp) as affected by biostimulants and growth season.

	TA (g L^{-1})	CI	TAC (mg CGE 100 g^{-1} DW)	AA (mg 100 g^{-1} DW)
Treatment				
CON	6.16 ± 0.23[1]	12.54 ± 1.51	58.07 ± 8.37	19.56 ± 3.86
HAL	6.86 ± 0.20	13.91 ± 1.95	75.05 ± 12.79	20.49 ± 4.33
APH	6.54 ± 0.29	31.15 ± 2.17***	125.50 ± 12.89***	15.76 ± 2.62
SEA	6.93 ± 0.32	31.44 ± 2.27***	186.11 ± 9.29***	16.25 ± 2.28
SPI	7.30 ± 0.12	16.49 ± 2.32	126.26 ± 12.02***	16.33 ± 2.94
MAA	6.74 ± 0.35	13.61 ± 2.02	74.73 ± 6.90	20.43 ± 3.70
PHE	6.98 ± 0.17	21.35 ± 3.40	106.84 ± 15.59**	19.09 ± 3.25
ZIN	6.81 ± 0.27	20.99 ± 2.37	113.76 ± 19.16**	20.12 ± 4.26
VIT	7.14 ± 0.38	22.67 ± 3.76*	137.92 ± 10.57***	14.07 ± 2.21
CHI	6.18 ± 0.90	21.31 ± 2.21	124.68 ± 14.37***	15.94 ± 2.71
SIL	6.65 ± 0.31	15.84 ± 2.42	78.59 ± 8.57	21.76 ± 4.90
Significance	*ns*	***	***	*ns*
Year				
2016	7.26 ± 0.15	20.38 ± 2.65	126.31 ± 13.33	25.53 ± 2.98
2017	6.24 ± 0.44	19.85 ± 3.73	93.24 ± 18.42	10.80 ± 0.66
Significance	***	*ns*	***	***
T × Y	*ns*	*ns*	*ns*	*ns*

[1]*Mean ± SE. Values followed by asterisk indicate significant differences between a single treatment group and control group, according to Dunnett's test (n = 4). ***P < 0.001; **P < 0.01; *P < 0.05, ns, not significant.*

index) reached slightly higher values in year 2017 than in year 2016 (**Figures 4A,B**). Treatments did not induced any significant modification of the chlorophyll content in year 2016, whereas leaves treated with ZIN and SEA showed higher SPAD values than control in year 2017 at 95 and 110 DAFB, respectively (**Figure 4B**).

Leaf Gas Exchanges

VIT, SIL and CHI showed higher leaf photosynthetic rates as compared with control at T0 of year 2016 (**Figure 5A**). Forty-eight hours after the sprays (T1), leaves from all treatments (with the exception of HAL) presented a higher photosynthetic rate (values ranging from 20 to 27 μmol m^{-2} s^{-1}) as compared to control (15 μmol m^{-2} s^{-1}). Four day after the application of the biostimulants (T2), the differences in the leaf photosynthetic rates among treatments were reduced, even though APH and VIT were still significantly higher than control. Similarly to the photosynthetic rate, leaf transpiration of treated trees resulted significantly higher than control in 2016, especially 48 and 96 h after the sprays (**Figure 5B**). In 2017, photosynthetic rate and transpiration presented values within the same range as in 2016, but no significant differences were detected between control leaves and leaves previously treated with the biostimulants (**Figures 5C,D**).

Yield and Fruit Quality

Fruits per tree were less numerous in 2016 than in 2017, but of significantly higher weight (170 vs. 150 grams per fruit approximately, **Table 2**). Overall, the final yield per tree did not differ between years, the average value being around 14 kg $tree^{-1}$. As for the effect of biostimulants on yield and fruit biometric characteristics (weight and diameter), no statistically significant differences were shown (**Table 2**).

Apples were harvested at the same ripening stage in year 2016 and 2017 as shown by very similar values of the average starch index for both seasons (**Supplementary Figure S1**). As for FF and TA, their average values were found higher in 2016 than in 2017, whereas biostimulant applications were ineffective on these parameters (**Table 3** and **Figure 6A**). TSS at harvest presented values ranging from 12 and 13.5°Brix. Apples from most of the biostimulant-treated trees (HAL, APH, SEA, SPI, MAA, PHE, ZIN, and SIL) were characterized by values of TSS approximately 1–1.5 degree lower than control in year 2016 only (no differences in 2017) (**Figure 6B**). Treatments with selected biostimulants had a visible and significant effect on the final fruit color index, independently from the considered year (**Figure 7**). More in detail, apples previously treated with the SEA, APH, and, to a less extent, by VIT, were characterized by a most intense red over coloration and presented values of the color index that were significantly higher than control (**Table 3**). Red overcolor was significantly more pronounced in 2017 than in 2016 with more than 50% of the apples belonging to the most colored class (>75%) (**Figures 8A,B**). Among the tested biostimulants, SEA was significantly effective in enhancing the percentage of fruits presenting more than 75% overcoloration (+87% and +50% as compared to control in 2016 and 2017, respectively). As for the other treatments, APH and ZIN slightly improved red overcolor of fruits, even though not significantly.

Total phenolic content (TPC) evaluated at skin level was significantly affected by both factors ("treatment" and "year") and by their interaction. The total amount was significantly higher in

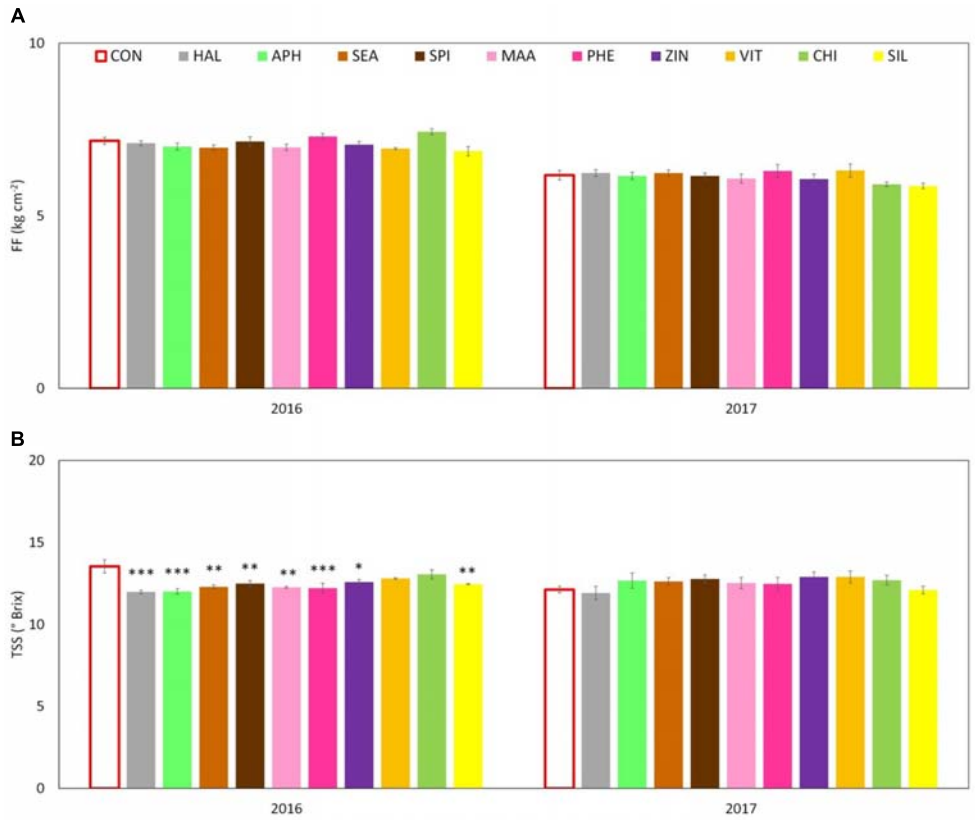


FIGURE 6 | Effect of the biostimulants on flesh firmness – FF **(A)** and total soluble solids – TSS **(B)** compared with control for year 2016 and 2017. Vertical bars indicate mean ± SE, $n = 4$. *Indicates significant differences according to Dunnett's test. ***$P < 0.001$; **$P < 0.01$; *$P < 0.05$.

FIGURE 7 | Apple color at harvest as affected by treatment applications (CON, control; APH, alfalfa protein hydrolysate; SEA, macroseaweed extract; VIT, B-group vitamins; ZIN, zinc plus amino acids).

2017 than in 2016 with average concentrations of around 2,400 and 1,800 mg GAE 100 g^{-1} DW, respectively. Treatments with SEA, SPI, and ZIN significantly enhanced TPC in apple skin at harvest in both years, whereas CHI and VIT were effective in 2016 only (**Figure 9A**). TPC at apple pulp level was also higher in 2017 than in 2016, without any significant effect of the treatments in both years (**Figure 9B**). TAC of apple skin was higher in year 2016 as compared to year 2017 (**Table 3**). TAC was also significantly enhanced by selected biostimulants, independently from the considered year (no significant interaction "T × Y"). More in detail, in apples treated with APH, SEA, SPI, VIT, and CHI the final anthocyanin concentration was more than the double of that of control. The antioxidant potential (ABTS) of apple skin and pulp tissues was linked to the phenolic content of these tissues. Similarly to the TPC parameter, ABTS was significantly higher in 2017 with values that were almost double than those of the year 2016 in both skin and pulp (**Figures 10A,B**). Treatments with SEA and VIT were able to significantly increase ABTS at skin level in both years, whereas other biostimulants (PHE, CHI, SPI, and ZIN) enhanced the antioxidant potential of apple skin in only one of the two considered years (**Figure 10A**). Similarly to the skin tissue, pulp antioxidant potential was significantly affected by the factors "treatment," "year" and their interaction. Pulp antioxidant potential was enhanced by treatments in 2016 only (**Figure 10B**), with CHI and SIL among the most effective biostimulants in promoting this feature. Treatments did not affect the final ascorbic acid concentration in both apple skin and pulp (**Figure 11** and **Table 3** for skin and pulp, respectively). The factor "year" was the only significantly relevant for final ascorbic acid accumulation, year 2016 showing approximately a twofold concentration in both skin and pulp tissues as compared to 2017.

Incidence of Physiological Disorder After Cold Storage

"Jonathan spot" incidence was significantly higher in 2017 apples, when around 20% of the fruits collected from each considered

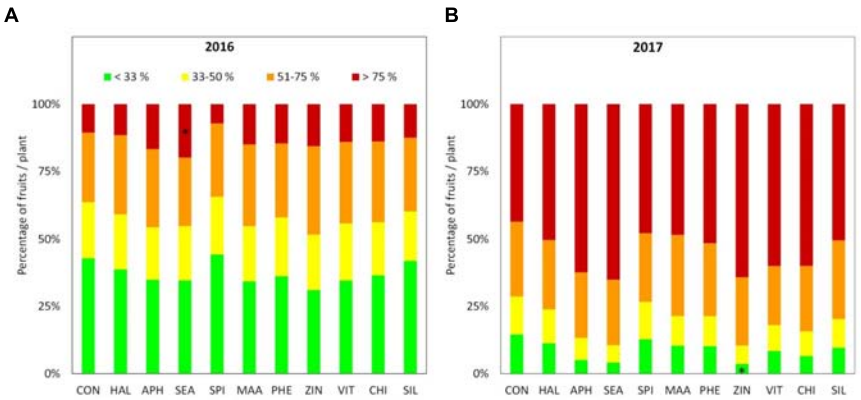


FIGURE 8 | Percentage of fruits according to red overcolor extension classes at harvest 2016 **(A)** and 2017 **(B)**, $n = 4$. *Indicates significant differences according to Dunnett's test. ***$P < 0.001$; **$P < 0.01$; *$P < 0.05$.

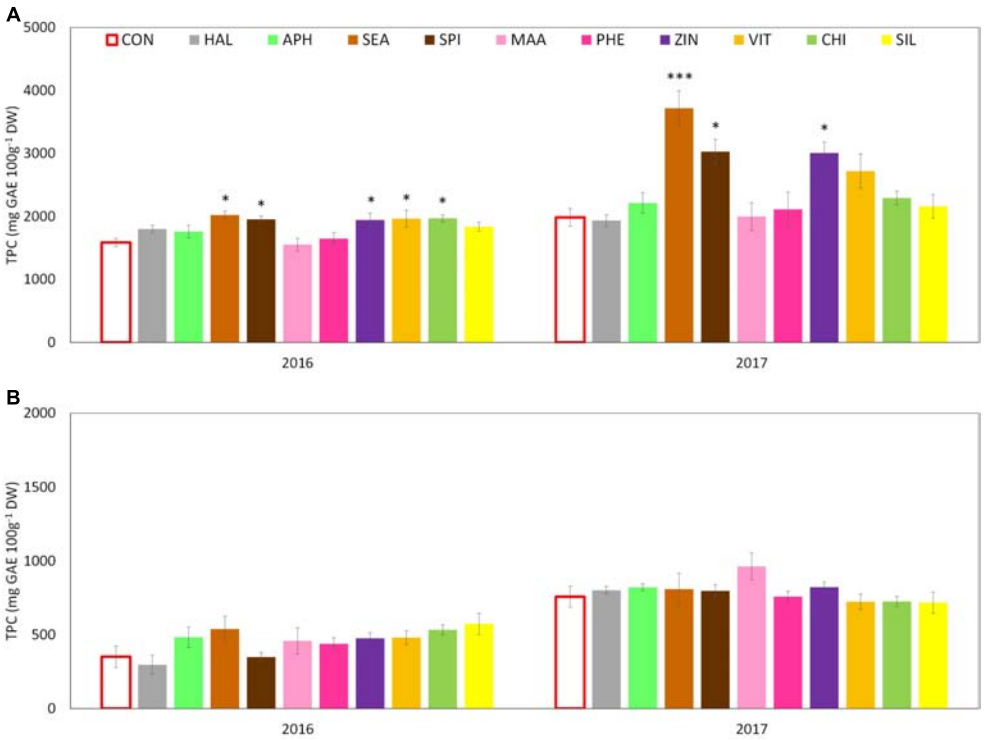


FIGURE 9 | Effect of the biostimulants on total phenolic content (TPC) in apple skin **(A)** and pulp **(B)** compared with control for year 2016 and 2017. Vertical bars indicate mean ± SE, $n = 4$. *Indicates significant differences according to Dunnett's test. ***$P < 0.001$; **$P < 0.01$; *$P < 0.05$.

tree were symptomatic after 4 months (**Figures 12A,B**). In 2016, selected biostimulants (SPI, CHI, SIL, and SEA) were able to significantly reduce the incidence of the "Jonathan spot" disorder during different phases of the post-harvest of apples (+21 and +60 days from harvest). At the end of the storage period (4 months after harvest) apples treated with ZIN were the only ones showing a significantly lower incidence of the disorder, with a reduction of approximately 60% as compare to control (**Figure 12A**). In 2017 biostimulant applications were not as effective as in 2016. ZIN, SIL, and SPI slightly reduced the disorder incidence at the end of the 4 months storage even though differences with control were not statistically significant (**Figure 12B**).

DISCUSSION

The vegetative growth behavior of apple trees was mainly determined by the seasonal climatic conditions. Despite trees were drip irrigated during both seasons, warmer meteorological

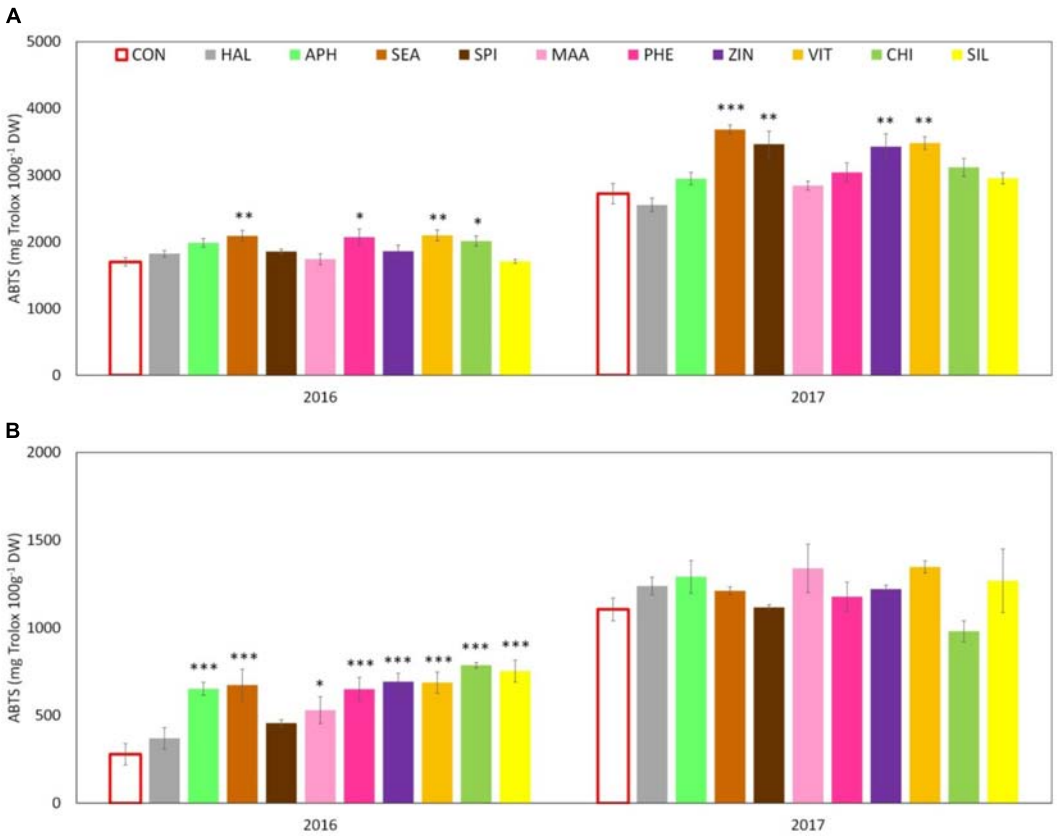


FIGURE 10 | Effect of the biostimulants on antioxidant activity (as ABTS) in apple skin **(A)** and pulp **(B)** compared with control for year 2016 and 2017. Vertical bars indicate mean ± SE, $n = 4$. *Indicates significant differences according to Dunnett's test. ***$P < 0.001$; **$P < 0.01$; *$P < 0.05$.

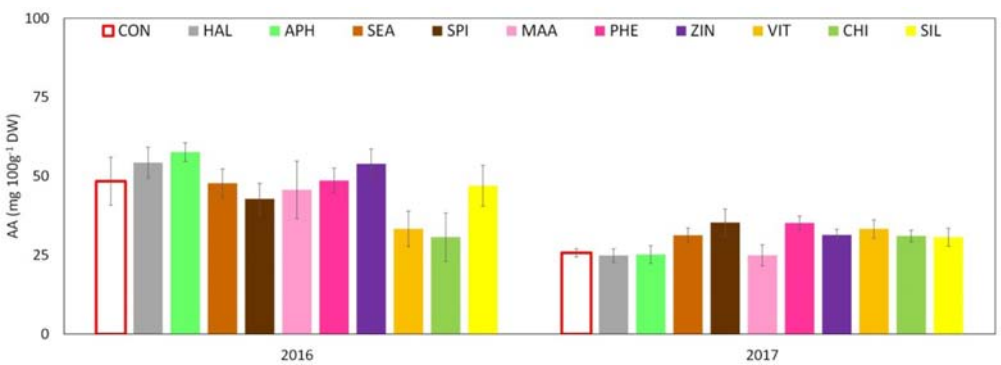


FIGURE 11 | Effect of the biostimulants on ascorbic acid (AA) level in apple skin compared with control for year 2016 and 2017. Vertical bars indicate mean ± SE, $n = 4$. *Indicates significant differences according to Dunnett's test. ***$P < 0.001$; **$P < 0.01$; *$P < 0.05$.

conditions during May–July 2017 (high maximal and average temperatures, limited rainfalls in May, **Figure 1**) might have determined a stressful status for trees, which then reduced shoot growth (**Figure 3**). In such conditions, trees treated with SEA were characterized by similar shoot growth but larger leaf area as compared to untreated trees (**Figure 3** and **Table 2**). Seaweed extracts were found able to promote shoot length in apple cv. Fuji (Spinelli et al., 2009) and to contrast drought stress effects on vegetable crops such as tomato and spinach by enhancing their growth and foliar density (Xu and Leskovar, 2015; Goñi et al., 2018). Another effect of SEA application was the higher concentration of chlorophyll (measured with the SPAD index) shown by treated leaves as compared to the untreated ones in 2017 (**Figure 4B**). This result is in agreement with the outcomes of other experiments conducted with seaweeds on apple (Spinelli et al., 2009) and on other crops such as

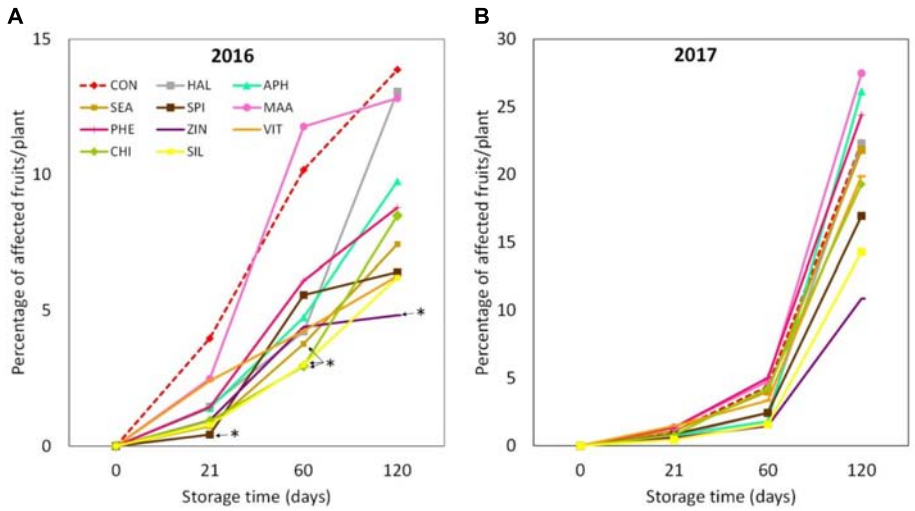


FIGURE 12 | "Jonathan spot" incidence during apple storage in 2016 **(A)** and 2017 **(B)**, $n = 4$. *Indicates significant differences according to Dunnett's test. ***$P < 0.001$; **$P < 0.01$; *$P < 0.05$.

grapevine (Sabir et al., 2014), cabbage (Rengasamy et al., 2016), and tomato (Goñi et al., 2018). This represents therefore a further evidence of a possible role of seaweed extracts in the reduction of chlorophyll degradation and in delaying leaf senescence (Battacharyya et al., 2015). As for the gas exchanges at leaf level, the first measurement was conducted when trees had already received nine biostimulant applications (every week starting from 40 DAFB). This might explain the higher A and E rates shown by the biostimulant-treated leaves before (T0) and after (T1 and T2) the 10th spray in year 2016 (**Figures 5A,B**). Photosynthetic and transpiration rates did not differ between biostimulants and control in 2017 (**Figures 5C,D**). Moreover, despite the higher values of chlorophyll concentration detected in SEA-treated leaves at the end of the 2017 season, their photosynthetic activity was not enhanced on that year. These results are partially different from those reported by Spinelli et al. (2009) on "Fuji" apple trees, where a consistent increase of chlorophyll content and photosynthetic activity was detected after the application of a commercial seaweed extract. Beside the different conditions that could have characterized the plants at the time of the measurements (leaf temperature, leaf water status, stomatal conductance, etc.), also the different methodology used for the analysis of the gas exchanges (punctual measurements at leaf level vs. continuous measurements at whole canopy level) might explain this partial inconsistency. In addition, according to values reported in the literature (Jakopic et al., 2007), the chlorophyll content was above the sufficiency threshold for apple leaves, leaving the photosynthetic rate more dependent from other environmental or endogenous factors. The only biostimulant showing a rather consistent effect on photosynthetic rate was the B-group vitamins, which was able to increase the photosynthetic potential of treated leaves in both years (**Figures 5A,C**). The use of single vitamin B1 (thiamine) was found ineffective on photosynthetic rate of rice leaves (Bahuguna et al., 2012); comparison with the outcome of the present research is anyway difficult giving that a complex mix of vitamins (including B1, B2, and B6) and not the single thiamine was used in our study.

At harvest, no difference was found on tree productivity in both years (**Table 2**). This outcome partially differs from another research performed on apple (Spinelli et al., 2009) where the use of a similar seaweed extract (from *Ascophyllum nodosum*) was found able to induce a higher final yield in a year of low crop load. The protein hydrolysate from alfalfa resulted also ineffective on final yield (**Table 2**). Differently, other studies conducted on vegetable crops (Rouphael et al., 2017 and Polo and Mata, 2018 on tomato; Ertani et al., 2015 on hot pepper) showed an increment of plant productivity, probably as the result of a stimulation mechanism of the plant primary metabolism triggered by signaling molecules (peptides, oligopeptides, and free amino acids) contained in the hydrolysate.

The effect of the biostimulants on primary apple quality traits (FF, TSS, and TA) was limited (**Figure 6** and **Table 3**). Only in 2016, the fruit TSS was found lower in treated apples (**Figure 6B**). According to the available literature, biostimulants can have different effect on final sugar accumulation in fruits. Protein hydrolysate-based substances were found able to enhance final sugar content in hot pepper and tomato (Ertani et al., 2015; Rouphael et al., 2017). Differently, seaweed extracts did not changed or slightly reduced final Brix value in strawberry fruits (Roussos et al., 2009) and grapevine berries (Frioni et al., 2018). In our conditions, the lower TSS could be the result of an internal trade-off at fruit level for carbon skeleton between sugars and secondary metabolites (i.e., phenolic compounds) that might had occurred in year 2016, as also suggested by the works of Lux-Endrich et al. (2000) and Rühmann et al. (2002) on apple.

One of the major effect of treatments application was the significant change in final apple coloration obtained with SEA, APH, and VIT. This outcome was confirmed by the colorimetric coordinates (color index), by the total anthocyanin concentration

measured on fruit samples taken from different replications and by the evaluation of the red overcolor extension performed on all fruits harvested from all the considered trees under evaluation (**Table 3** and **Figures 7**, **8**). These results confirm those described by Malaguti et al. (2002) on apple "Gala" and those by Frioni et al. (2018) on red grapevine cultivars evaluated in different cultivation areas. The boosted final red over color of apples might be ascribed to a modulation of the metabolism of plant endogenous growth regulators (mainly cytochins and abscisic acid) obtained with the application of the biostimulant substances (i.e., *A. nodosum*; Wally et al., 2013), leading to an induction of anthocyanin biosynthesis and accumulation in fruit skin prior to harvest (**Table 3**).

Another relevant effect of the biostimulant applications was the higher concentration of phenolic compounds detected in the skin tissue of apples treated with SEA, SPI, and ZIN in the 2 years (**Figure 9A**). Since no difference was found for the ascorbic acid concentration, this increase in phenolic compounds was likely responsible for the higher ABTS values showed by skin samples of fruits previously treated with the biostimulants mentioned above (**Figure 10A**). Phenolic compounds are biologically active metabolites showing antioxidant potential (Rice-Evans et al., 1997) and therefore highly considered as health-promoting substances in fruits (Slavin and Lloyd, 2012). Similar health-promoting responses were found in onion and potato after the application of *A. nodosum* (Lola-Luz et al., 2014). Protein hydrolysates of different origin (legume and alfalfa) promoted the antioxidant capacity of tomato (Rouphael et al., 2017) and green or red pepper (Ertani et al., 2015) similarly to what was detected for the apple pulp in our study (**Figure 10B**). It has been shown that the protein hydrolysate mode of action involves the up-regulation of a number of genes responsible for the secondary metabolism of plants leading to the synthesis and accumulation of phenolics and terpenes which are responsible for the enhanced antioxidant activity and for the increased tolerance to biotic and abiotic stresses (Ertani et al., 2017). Moreover apple treated with APH were also characterized by a higher total anthocyanin and a lower nitrogen content at skin level (**Table 3** and **Supplementary Table S2**). A negative correlation between the concentration of nitrogen and the concentration of anthocyanin and total flavonoids was often found when measured in apple skin tissue (Awad and de Jager, 2002). Low nutrients concentration at skin level are often positive for the final coloration of fruits, probably as a result of the internal trade-off between the synthesis of secondary substances and the growth primary metabolism as also described by Veberic (2016). Higher phenolic concentration and antioxidant activity were also detected in apple skin after B-group vitamins application (**Figures 9A**, **10A**). Similar results were obtained with vitamin B1 (thiamine) on grapevine. It was found that thiamine was able to elicit different genes belonging to the phenylpropanoid pathway (including phenylalanine ammonia lyase, chalcone synthase, and stilbene synthase) leading to a higher accumulation of secondary metabolites and antioxidant activity with positive effect on tolerance to downy mildew in grapevine (Boubakri et al., 2013). Zinc applications also induced phenolic compounds accumulation and antioxidant potential in apple (**Figures 9A**, **10A,B**). This result partially conflicts with those of Aglar et al. (2016) which described a negative effect of the application of zinc on both phenols and antioxidant potential in apple. Studies on the role of zinc on the apple phenolic metabolism are currently not available in the literature. An interpretation of this conflicting result could consider the different product formulation used in the present study (a mix of zinc and amino acids), whereas it was zinc sulfate alone in the case of Aglar et al. (2016). Moreover, differences in the cultivars studied and in the experimental conditions could also have played a role on the outcomes of the experiments. Anyhow, the role of genotype in plant response to zinc treatments seems to be of relevance, considering that the phenolic accumulation after zinc sulfate application was found enhanced in grapevine (Song et al., 2015) and in aromatic herbs extracts such as dill and anise, but reduced in other *species* such as fennel (Majdoub et al., 2017).

Nutrient concentration at the skin tissue level has often been linked to the incidence of physiological disorders in apple (Amarante et al., 2013; Baugher et al., 2017). In our experiment, the application of zinc (in combination with a mix of amino acids) was found effective in lowering the incidence of the physiological disorder of apple during storage (**Figure 12**). This effect was consistent in both years, even though incidence percentage was significantly higher in 2017 probably because of the higher rainfalls that characterized the month of July and August of that year. Deficit in nutrient concentration in fruit skin (calcium in particular) as well as adverse meteorological conditions are commonly associated with post-harvest disorders (Ferguson et al., 1999). The role of other mineral nutrients (such as zinc and silicon) for fruit storability is anyway still unclear. Pais and Petho (1970) found that foliar applications of calcium plus zinc retarded the development of post-harvest disorder in apple cv. Jonathan. Zinc applications at 10-day intervals from 8 weeks after full bloom to harvest were found to reduce loss of mechanical properties of apples after storage (Johnson and Dover, 2002). The authors explained this result as a possible indirect effect of zinc applications on the enhanced calcium concentration of fruits, which likely had a positive consequence on apple storability. In our study, apples treated with zinc in the form of zinc + amino acids were found significantly richer in zinc and showed a tendency to higher calcium concentration (**Supplementary Tables S2**, **S3**). Both these elements may have contributed to strengthen the middle lamella and primary cell wall structure (Henriques et al., 2012; Guerriero et al., 2016), therefore contrasting the development of the post-harvest disorder "Jonathan spot" during storage period.

CONCLUSION

This study is the first comprehensive investigation of the use of different classes of biostimulants on organic apple production over a period of two consecutive years. The results of the study indicate that products based on alfalfa protein hydrolysate, seaweed extracts and B-group vitamins improved final red coloration of apple "Jonathan" in both years, therefore enhancing their market potential. Moreover, the same biostimulants had a positive effect on fruit functional traits as shown by the

higher phenolic compounds concentration, total anthocyanin and antioxidant potential of treated apples. Taken together, these findings suggest a role for selected biostimulants in promoting the secondary metabolism of treated plants, leading to an improvement of fruit quality, appearance, and nutritional value. The research has also shown that biostimulants containing zinc are effective in reducing the incidence of physiological disorders in cold stored apples, strengthening the idea of a positive role of this element on the structure and resistance of cell walls and membranes at fruit level. Giving the current lack of effective means for the post-harvest management of organic fruits, this finding might have significant implication on the practices presently used for organic apple conservation and commercialization. As for the fine tuning of the use of biostimulants under orchard conditions, further research needs to be done to deepen our understanding on the way of application of the available products (number of applications, period, and concentrations) for the different apple cultivars on the market.

AUTHOR CONTRIBUTIONS

SS contributed in the set-up of the experimental protocol, performed the agronomic study, processed the fruit samples, analyzed the data, and participated to the writing of the paper. MK contributed to the set-up of the experiment and to the interpretation of the results. CC contributed to the agronomic implementation of the study. MB contributed to the analysis of phenols, anthocyanins, antioxidant potential, and ascorbic acid. PR contributed to the set-up of the analytical work related to the quality indexes of the fruits. CA coordinated the research, worked on the statistical analysis, and contributed to the writing of several sections of the manuscript.

FUNDING

The research was co-founded by the Free University of Bozen-Bolzano (Project BIO_TOOL TN 1B07), Laimburg Research Centre, and ILSA S.p.A. Laimburg Research Centre was funded by the Autonomous Province of Bolzano, financial support for the "Technology Park" (Decision no. 1472, 07.10.2013) is gratefully acknowledged. This work was also supported by the Open Access Publishing Fund provided by the Free University of Bozen-Bolzano. SS was funded by a Ph.D. grant from the Free University of Bozen-Bolzano.

ACKNOWLEDGMENTS

The authors would like to thank Dr. Aldo Matteazzi for technical assistance.

SUPPLEMENTARY MATERIAL

The Supplementary Material for this article can be found online at: https://www.frontiersin.org/articles/10.3389/fpls.2018.01342/full#supplementary-material

REFERENCES

Aglar, E., Yildiz, K., Ozkan, Y., Ozturk, B., and Erdem, H. (2016). The effects of aminoethoxyvinylglycine and foliar zinc treatments on pre-harvest drops and fruit quality attributes of jersey mac apples. *Sci. Hortic.* 213, 173–178. doi: 10.1016/j.scienta.2016.10.026

Alto-Adige/South Tyrol Province (2017). *WWW Document*. Available at: http://www.provincia.bz.it/agricoltura-foreste/agricoltura/relazione-agraria-forestale.asp [accessed August 03.2018].

Amarante, C. V. T., do Steffens, C. A., Mafra, ÁL., and Albuquerque, J. A. (2008). Yield and fruit quality of apple from conventional and organic production systems. *Pesqui. Agropecuária Bras* 43, 333–340. doi: 10.1590/S0100-204X2008000300007

Amarante, C. V. T., Silveira, J. P. G., Steffens, C. A., Paes, F. N., and Argenta, L. C. (2013). Tissue sampling method and mineral attributes to predict bitter pit occurrence in apple fruit: a multivariate approach. *Acta Hortic.* 1012, 1133–1139. doi: 10.17660/ActaHortic.2013.1012.153

AOAC (2005). "Vitamin C in Juices and Vitamin Preparations," in *Proceedings of the 18th AOAC Official Methods of Analysis* (Gaithersburg, MD: Association of Official Analytical Chemists).

Awad, M. A., and de Jager, A. (2002). Relationships between fruit nutrients and concentrations of flavonoids and chlorogenic acid in 'Elstar' apple skin. *Sci. Hortic.* 92, 265–276. doi: 10.1016/S0304-4238(01)00290-4

Bahuguna, R. N., Joshi, R., Shukla, A., Pandey, M., and Kumar, J. (2012). Thiamine primed defense provides reliable alternative to systemic fungicide carbendazim against sheath blight disease in rice (*Oryza sativa* L.). *Plant Physiol. Biochem.* 57, 159–167. doi: 10.1016/j.plaphy.2012.05.003

Bassi, M., Lubes, G., Bianchi, F., Agnolet, S., Ciesa, F., Brunner, K., et al. (2018). Ascorbic acid content in apple pulp, peel, and monovarietal cloudy juices of 64 different cultivars. *Int. J. Food Prop.* 20, S2626–S2634. doi: 10.1080/10942912.2017.1381705

Battacharyya, D., Babgohari, M. Z., Rathor, P., and Prithiviraj, B. (2015). Seaweed extracts as biostimulants in horticulture. *Sci. Hortic.* 196, 39–48. doi: 10.1016/j.scienta.2015.09.012

Baugher, T. A., Marini, R., Schupp, J. R., and Watkins, C. B. (2017). Prediction of bitter pit in 'Honeycrisp'apples and best management implications. *HortScience* 52, 1368–1374. doi: 10.21273/HORTSCI12266-17

Blanke, M. M., and Kunz, A. (2016). Alternatives to phosphonates for fruit colouration. *Sci. Hortic.* 198, 434–437. doi: 10.1016/j.scienta.2015.12.019

Boubakri, H., Poutaraud, A., Wahab, M. A., Clayeux, C., Baltenweck-Guyot, R., Steyer, D., et al. (2013). Thiamine modulates metabolism of the phenylpropanoid pathway leading to enhanced resistance to *Plasmopara viticola* in grapevine. *BMC Plant Biol.* 13:31. doi: 10.1186/1471-2229-13-31

Canellas, L. P., Olivares, F. L., Aguiar, N. O., Jones, D. L., Nebbioso, A., Mazzei, P., et al. (2015). Humic and fulvic acids as biostimulants in horticulture. *Sci. Hortic.* 196, 15–27. doi: 10.1016/j.scienta.2015.09.013

Colla, G., Hoagland, L., Ruzzi, M., Cardarelli, M., Bonini, P., Canaguier, R., et al. (2017). Biostimulant action of protein hydrolysates: unraveling their effects on plant physiology and microbiome. *Front. Plant Sci.* 8:2202. doi: 10.3389/fpls.2017.02202

Colla, G., and Rouphael, Y. (2015). Biostimulants in horticulture. *Sci. Hortic.* 196, 1–2. doi: 10.1016/j.scienta.2015.10.044

De Pascale, S., Rouphael, Y., and Colla, G. (2017). Plant biostimulants: innovative tool for enhancing plant nutrition in organic farming. *Eur. J. Hortic. Sci.* 82, 277–285. doi: 10.17660/eJHS.2017/82.6.2

de Ponti, T., Rijk, B., and van Ittersum, M. K. (2012). The crop yield gap between organic and conventional agriculture. *Agric. Syst.* 108, 1–9. doi: 10.1016/j.agsy.2011.12.004

du Jardin, P. (2015). Plant biostimulants: definition, concept, main categories and regulation. *Sci. Hortic.* 196, 3–14. doi: 10.1016/j.scienta.2015.09.021

Dunnett, C. W. (1985). "Multiple Comparisons between several treatments and a specified treatment," in *Linear Statistical Inference*, eds T. Caliński and W. Klonecki (New York, NY: Springer), 39–47.

Ertani, A., Cavani, L., Pizzeghello, D., Brandellero, E., Altissimo, A., Ciavatta, C., et al. (2009). Biostimulant activity of two protein hydrolyzates in the growth and nitrogen metabolism of maize seedlings. *J. Plant Nutr. Soil Sci.* 172, 237–244. doi: 10.1002/jpln.200800174

Ertani, A., Sambo, P., Nicoletto, C., Santagata, S., Schiavon, M., and Nardi, S. (2015). The use of organic biostimulants in hot pepper plants to help low input sustainable agriculture. *Chem. Biol. Technol. Agric.* 2:11. doi: 10.1186/s40538-015-0039-z

Ertani, A., Schiavon, M., and Nardi, S. (2017). Transcriptome-wide identification of differentially expressed genes in Solanum lycopersicon L. *in response to an alfalfa-protein hydrolysate using microarrays*. *Front. Plant Sci.* 8:1159. doi: 10.3389/fpls.2017.01159

FAOSTAT. (2017). *WWW Document*. Available at: http://www.fao.org/faostat/en/#data/QC [accessed August 03 2018].

Ferguson, I., Volz, R., and Woolf, A. (1999). Preharvest factors affecting physiological disorders of fruit. *Postharvest Biol. Technol.* 15, 255–262. doi: 10.1016/S0925-5214(98)00089-1

Frioni, T., Sabbatini, P., Tombesi, S., Norrie, J., Poni, S., Gatti, M., et al. (2018). Effects of a biostimulant derived from the brown seaweed *Ascophyllum nodosum* on ripening dynamics and fruit quality of grapevines. *Sci. Hortic.* 232, 97–106. doi: 10.1016/j.scienta.2017.12.054

Goñi, O., Quille, P., and O'Connell, S. (2018). *Ascophyllum nodosum* extract biostimulants and their role in enhancing tolerance to drought stress in tomato plants. *Plant Physiol. Biochem.* 126, 63–73. doi: 10.1016/j.plaphy.2018.02.024

Guerriero, G., Hausman, J.-F., and Legay, S. (2016). Silicon and the plant extracellular matrix. *Front. Plant Sci* 7:463. doi: 10.3389/fpls.2016.00463

Henriques, A. R., Chalfun-Junior, A., and Aarts, M. (2012). Strategies to increase zinc deficiency tolerance and homeostasis in plants. *Braz. J. Plant Physiol.* 24, 3–8. doi: 10.3389/fpls.2013.00144

Hernández-Herrera, R. M., Santacruz-Ruvalcaba, F., Zañudo-Hernández, J., and Hernández-Carmona, G. (2016). Activity of seaweed extracts and polysaccharide-enriched extracts from *Ulva lactuca* and *Padina gymnospora* as growth promoters of tomato and mung bean plants. *J. Appl. Phycol.* 28, 2549–2560. doi: 10.1007/s10811-015-0781-4

ISTAT. (2017). *WWW Document*. Available at: https://www.istat.it (accessed August 03.2018)

Jakopic, J., Veberic, R., Zupancic, K., and Stampar, F. (2007). Influence of nitrogen on the contents of carbohydrates and organic acids in apples (*Malus domestica* Borkh.) cv. 'Golden Delicious'. *Eur. J. Hortic. Sci.* 72:66.

Johnson, D. S., and Dover, C. J. (2002). The effect of calcium and zinc sprays on the texture of "Cox's Orange Pippin" apples in controlled atmosphere storage. *Acta Horticulturae* 594, 427–433. doi: 10.17660/ActaHortic.2002.594.55

Lee, J., Durst, R. W., and Wrolstad, R. E. (2005). Determination of total monomeric anthocyanin pigment content of fruit juices, beverages, natural colorants, and wines by the pH differential method: collaborative study. *J. AOAC Int.* 88, 1269–1278.

Liu, Y., Che, F., Wang, L., Meng, R., Zhang, X., and Zhao, Z. (2013). Fruit coloration and anthocyanin biosynthesis after bag removal in non-red and red apples (*Malus* x *domestica* Borkh.). *Molecules* 18, 1549–1563. doi: 10.3390/molecules18021549

Lola-Luz, T., Hennequart, F., and Gaffney, M. (2014). Effect on health promoting phytochemicals following seaweed application, in potato and onion crops grown under a low input agricultural system. *Sci. Hortic.* 170, 224–227. doi: 10.1016/j.scienta.2014.03.022

Lux-Endrich, A., Treutter, D., and Feucht, W. (2000). Influence of nutrients and carbohydrate supply on the phenol composition of apple shoot cultures. *Plant Cell Tissue Organ Cult.* 60, 15–21. doi: 10.1023/A:1006406527242

Majdoub, N., el-Guendouz, S., Rezgui, M., Carlier, J., Costa, C., Kaab, L. B. B., et al. (2017). Growth, photosynthetic pigments, phenolic content and biological activities of *Foeniculum vulgare* Mill., *Anethum graveolens* L. and *Pimpinella anisum* L. (Apiaceae) in response to zinc. *Ind. Crops Prod* 109, 627–636. doi: 10.1016/j.indcrop.2017.09.012

Malaguti, D., Rombolà, A. D., Gerin, M., Simoni, G., Tagliavini, M., and Marangoni, B. (2002). Effect of seaweed extracts-based leaf sprays on the mineral status, yield and fruit quality of apple. *Acta Horticulturae* 594, 357–359. doi: 10.17660/ActaHortic.2002.594.44

Orsini, F., Maggio, A., Rouphael, Y., and De Pascale, S. (2016). "Physiological quality" of organically grown vegetables. *Sci. Hortic.* 208, 131–139. doi: 10.1016/j.scienta.2016.01.033

Pais, L., and Petho, F. (1970). Investigations on causes of Jonathan spot. *I. Szolo Gyumolcstermesztes* 6, 31–42.

Polo, J., and Mata, P. (2018). Evaluation of a biostimulant (pepton) based in enzymatic hydrolyzed animal protein in comparison to seaweed extracts on root development, vegetative growth, flowering, and yield of gold cherry tomatoes grown under low stress ambient field conditions. *Front. Plant Sci* 8:2261 doi: 10.3389/fpls.2017.02261

Portu, J., López-Alfaro, I., Gómez-Alonso, S., López, R., and Garde-Cerdán, T. (2015). Changes on grape phenolic composition induced by grapevine foliar applications of phenylalanine and urea. *Food Chem.* 180, 171–180. doi: 10.1016/j.foodchem.2015.02.042

Povero, G., Mejia, J. F., Di Tommaso, D., Piaggesi, A., and Warrior, P. (2016). A systematic approach to discover and characterize natural plant biostimulants. *Front. Plant Sci* 7:435. doi: 10.3389/fpls.2016.00435

Re, R., Pellegrini, N., Proteggente, A., Pannala, A., Yang, M., and Rice-Evans, C. (1999). Antioxidant activity applying an improved ABTS radical cation decolorization assay. *Free Radic. Biol. Med.* 26, 1231–1237. doi: 10.1016/S0891-5849(98)00315-3

Rengasamy, K. R. R., Kulkarni, M. G., Papenfus, H. B., and Van Staden, J. (2016). Quantification of plant growth biostimulants, phloroglucinol and eckol, in four commercial seaweed liquid fertilizers and some by-products. *Algal Res.* 20, 57–60. doi: 10.1016/j.algal.2016.09.017

Rice-Evans, C., Miller, N., and Paganga, G. (1997). Antioxidant properties of phenolic compounds. *Trends Plant Sci.* 2, 152–159. doi: 10.1016/S1360-1385(97)01018-2

Rouphael, Y., Colla, G., Giordano, M., El-Nakhel, C., Kyriacou, M. C., and De Pascale, S. (2017). Foliar applications of a legume-derived protein hydrolysate elicit dose-dependent increases of growth, leaf mineral composition, yield and fruit quality in two greenhouse tomato cultivars. *Sci. Hortic.* 226, 353–360. doi: 10.1016/j.scienta.2017.09.007

Rouphael, Y., Franken, P., Schneider, C., Schwarz, D., Giovannetti, M., Agnolucci, M., et al. (2015). Arbuscular mycorrhizal fungi act as biostimulants in horticultural crops. *Sci. Hortic.* 196, 91–108. doi: 10.1016/j.scienta.2015.09.002

Roussos, P. A., Denaxa, N.-K., and Damvakaris, T. (2009). Strawberry fruit quality attributes after application of plant growth stimulating compounds. *Sci. Hortic.* 119, 138–146. doi: 10.1016/j.scienta.2008.07.021

Rühmann, S., Leser, C., Bannert, M., and Treutter, D. (2002). Relationship between growth, secondary metabolism, and resistance of apple. *Plant Biol.* 4, 137–143. doi: 10.1007/s00203-016-1207-7

Ruzzi, M., and Aroca, R. (2015). Plant growth-promoting rhizobacteria act as biostimulants in horticulture. *Sci. Hortic.* 196, 124–134. doi: 10.1016/j.scienta.2015.08.042

Sabir, A., Yazar, K., Sabir, F., Kara, Z., Yazici, M. A., and Goksu, N. (2014). Vine growth, yield, berry quality attributes and leaf nutrient content of grapevines as influenced by seaweed extract (*Ascophyllum nodosum*) and nanosize fertilizer pulverizations. *Sci. Hortic.* 175, 1–8. doi: 10.1016/j.scienta.2014.05.021

Scaglia, B., Nunes, R. R., Rezende, M. O. O., Tambone, F., and Adani, F. (2016). Investigating organic molecules responsible of auxin-like activity of humic acid fraction extracted from vermicompost. *Sci. Total Environ.* 562, 289–295. doi: 10.1016/j.scitotenv.2016.03.212

Schiavon, M., Ertani, A., and Nardi, S. (2008). Effects of an alfalfa protein hydrolysate on the gene expression and activity of enzymes of the tricarboxylic acid (TCA) cycle and nitrogen metabolism in Zea mays L. *J. Agric. Food Chem.* 56, 11800–11808. doi: 10.1021/jf802362g

Seufert, V., Ramankutty, N., and Foley, J. A. (2012). Comparing the yields of organic and conventional agriculture. *Nature* 485, 229–232. doi: 10.1038/nature11069

Slavin, J. L., and Lloyd, B. (2012). Health benefits of fruits and vegetables. *Adv. Nutr.* 3, 506–516. doi: 10.3945/an.112.002154

Song, J., Du, L., Li, L., Kalt, W., Palmer, L. C., Fillmore, S., et al. (2015). Quantitative changes in proteins responsible for flavonoid and anthocyanin biosynthesis in strawberry fruit at different ripening stages: a targeted quantitative proteomic investigation employing multiple reaction monitoring. *J. Proteomics* 122, 1–10. doi: 10.1016/j.jprot.2015.03.017

Spinelli, F., Fiori, G., Noferini, M., Sprocatti, M., and Costa, G. (2009). Perspectives on the use of a seaweed extract to moderate the negative effects of alternate bearing in apple trees. *J. Hortic. Sci. Biotechnol.* 84, 131–137. doi: 10.1080/14620316.2009.11512610

Tessmer, M. A., Appezzato-da-Glória, B., and Antoniolli, L. R. (2016). Influence of growing sites and physicochemical features on the incidence of lenticel breakdown in 'Gala' and 'Galaxy' apples. *Sci. Hortic.* 205, 119–126. doi: 10.1016/j.scienta.2016.04.027

Ubi, B. E. (2004). External stimulation of anthocyanin biosynthesis in apple fruit. *J. Food Agric. Environ.* 2, 65–70.

Veberic, R. (2016). The impact of production technology on plant phenolics. *Horticulturae* 2:8. doi: 10.3390/horticulturae2030008

Wally, O. S. D., Critchley, A. T., Hiltz, D., Craigie, J. S., Han, X., Zaharia, L. I., et al. (2013). Erratum to: regulation of phytohormone biosynthesis and accumulation in arabidopsis following treatment with commercial extract from the marine macroalga *Ascophyllum nodosum*. *J. Plant Growth Regul.* 32, 340–341. doi: 10.1007/s00344-012-9311-7

Whale, S. K., Singh, Z., Behboudian, M. H., Janes, J., and Dhaliwal, S. S. (2008). Fruit quality in 'Cripp's Pink' apple, especially colour, as affected by preharvest sprays of aminoethoxyvinylglycine and ethephon. *Sci. Hortic.* 115, 342–351. doi: 10.1016/j.scienta.2007.10.015

Wolfe, K., Wu, X., and Liu, R. H. (2003). Antioxidant activity of apple peels. *J. Agric. Food Chem.* 51, 609–614. doi: 10.1021/jf020782a

Xu, C., and Leskovar, D. I. (2015). Effects of A. nodosum seaweed extracts on spinach growth, physiology and nutrition value under drought stress. *Sci. Hortic.* 183, 39–47. doi: 10.1016/j.scienta.2014.12.004

Zhang, X., Wang, K., and Ervin, E. H. (2010). Optimizing dosages of seaweed extract-based cytokinins and zeatin riboside for improving creeping bentgrass heat tolerance. *Crop Sci.* 50:316. doi: 10.2135/cropsci2009.02.0090

Conflict of Interest Statement: ILSA S.p.A. provided part of the biostimulants used for the experiment. Collection of data, analysis and interpretation, as well as article writing, was carried out by the authors independently.

The authors declare that the research was conducted in the absence of any commercial or financial relationships that could be construed as a potential conflict of interest.

frontiers in Plant Science

OPINION
published: 24 September 2018
doi: 10.3389/fpls.2018.01407

Functional Complementarity of Arbuscular Mycorrhizal Fungi and Associated Microbiota: The Challenge of Translational Research

*Alessandra Turrini, Luciano Avio, Manuela Giovannetti and Monica Agnolucci**

Department of Agriculture, Food and Environment, University of Pisa, Pisa, Italy

Keywords: arbuscular mycorrhizal fungi, mycorrhizosphere, beneficial soil bacteria, plant growth promoting bacteria, mycorrhiza helper bacteria, P solubilization

OPEN ACCESS

Edited by:
Youssef Rouphael,
Università degli Studi di Napoli Federico II, Italy

Reviewed by:
Giulia Conversa,
University of Foggia, Italy
Stefano Mocali,
Consiglio per la Ricerca in Agricoltura e l'Analisi dell'Economia Agraria (CREA), Italy

****Correspondence:***
Monica Agnolucci
monica.agnolucci@unipi.it

Specialty section:
This article was submitted to Crop and Product Physiology, a section of the journal Frontiers in Plant Science

Received: *12 July 2018*
Accepted: *05 September 2018*
Published: *24 September 2018*

Citation:
Turrini A, Avio L, Giovannetti M and Agnolucci M (2018) Functional Complementarity of Arbuscular Mycorrhizal Fungi and Associated Microbiota: The Challenge of Translational Research. Front. Plant Sci. 9:1407. doi: 10.3389/fpls.2018.01407

ARBUSCULAR MYCORRHIZAL FUNGI AND THE ASSOCIATED MICROBIOTA

One of the main challenge for humanity in the years to come is represented by the production of enough food for a growing global population, while reducing the use of pesticides and chemical fertilizers and maintaining environmental quality and natural resources for future generations. The new paradigm in agriculture—sustainable intensification—may be implemented by the efficient use of practices enhancing the activity of beneficial soil microorganisms, essential elements of soil nutrient flows, at the basis of long-term soil productivity and health. There is a growing awareness of the essential roles played by soil microorganisms in human nutrition and welfare and of the economic importance of ecosystem services they provide in agriculture, forestry, and society (Philippot et al., 2013; Avio et al., 2018).

Among beneficial microorganisms, arbuscular mycorrhizal (AM) fungi (AMF) have been long known for their positive impact on plant growth and health. AMF are obligate biotrophs, establishing mutualistic symbioses with the roots of the majority of land plants, including the most important food crops, such as cereals, pulses, potatoes, fruit trees, vegetables, and medicinal plants. They obtain carbon from the host plants, and, in exchange, facilitate the uptake and transfer of mineral nutrients—phosphorus (P), nitrogen (N), sulfur (S), potassium (K), calcium (Ca), copper (Cu), and zinc (Zn)—from the soil, by means of an extensive network of hyphae spreading from colonized roots into the surrounding environment. Beyond improving mineral nutrition, AMF increase plant tolerance to biotic and abiotic stresses, and provide multiple ecosystem services in natural and agricultural environments, from the completion of biogeochemical cycles to the maintenance of biological soil fertility (Smith and Read, 2008). In addition to such multifunctional roles, AMF induce changes in plant secondary metabolism, stimulating the biosynthesis of phytochemical compounds, such as polyphenols and carotenoids, thus leading to the production of safe and high-quality foods, able to promote human health (Sbrana et al., 2014).

A number of multimodal investigations showed the occurrence of diverse assemblages of bacterial communities living strictly associated with AMF spores, extraradical mycelium and mycorrhizal roots, in the mycorrhizosphere. The use of transmission electron microscope allowed the detection of unculturable endobacteria inside the spores of some AMF species (Mosse, 1970; Bianciotto et al., 1996) and of free living bacteria embedded in the spore wall layers or in the microniches formed by the peridial hyphae interwoven around the spores in the sporocarps (Ames et al., 1989; Filippi et al., 1998). Molecular studies confirmed the occurrence of either Mollicutes-related and unculturable endosymbionts (Desirò et al., 2014) or bacteria strictly associated with the spores of different AMF taxa, affiliated with *Cellvibrio*, *Chondromyces*, *Flexibacter*, *Lysobacter*,

and *Pseudomonas* (Roesti et al., 2005), Proteobacteria and Actinobacteria (Long et al., 2008), and Actinomycetales, Bacillales, Rhizobiales, Pseudomonadales, Burkholderiales, including *Arthrobacter*, *Streptomyces*, *Bacillus*, *Paenibacillus*, *Pseudomonas*, *Herbaspirillum*, *Massilia*, *Rhizobium,* and *Sinorhizobium* (Agnolucci et al., 2015).

Overall, the ultrastructural and molecular studies revealed the complexity and diversity of bacterial communities living associated with AMF, suggesting putative important functional roles as plant growth promoting (PGP) bacteria and mycorrhiza helper bacteria (MHB), able to promote AMF activity and development. However, in order to verify such hypothesis and to unravel the physiological interactions between AMF and associated bacteria possibly leading to positive synergistic effects on plant nutrition and health, it is crucial to isolate the bacteria in pure culture. Using culture-dependent approaches many authors obtained a number of bacterial strains from the sporosphere of *Glomus versiforme* and *Glomus clarum* NT4 (Mayo et al., 1986; Xavier and Germida, 2003), *Gigaspora margarita* (Cruz et al., 2008), *Glomus mosseae,* and *Glomus intraradices* (syn. *Rhizophagus intraradices*) (Bharadwaj et al., 2008b; Battini et al., 2016b), most of which were further studied for PGP activities, with the aim of their possible utilization, either as individual strains or consortia, as biocontrol agents, biofertilisers, and bioenhancers (Rouphael et al., 2015).

A NETWORK OF FUNCTIONAL INTERACTIONS AMONG AMF AND ASSOCIATED MICROBIOTA

As early as 1959 soil bacteria were studied for their ability to affect AMF spore germination and hyphal growth (Mosse, 1959). Subsequent works confirmed their functional role in the promotion of mycorrhizal activity (Azcón, 1989), and proposed the term "mycorrhiza helpers" for such bacteria (Frey-Klett et al., 2007). Many actinobacteria isolated from the soil, in particular species belonging to the genera *Streptomyces* and *Corynebacterium*, as well as species of *Pseudomonas* increased the germination of *G. versiforme, G. mosseae*, and *G. margarita* spores (Mayo et al., 1986; Tylka et al., 1991; Carpenter-Boggs et al., 1995), while *Klebsiella pneumoniae* and *Trichoderma* sp. enhanced germlings growth in *Glomus deserticola* and *G. mosseae* (Will and Sylvia, 1990; Calvet et al., 1992). Bacteria isolated from the mycorrhizosphere, either from mycorrhizal roots or from AMF spores and hyphae, were able to enhance spore germination, germling growth, and AMF root colonization (Mayo et al., 1986; Xavier and Germida, 2003; Giovannetti et al., 2010). One of the possible mechanistic explanations of the phenomenon is based on the ability of several bacteria to decompose insoluble biopolymers like chitin and chitosan, the two main constituents of AMF spore walls, thus boosting germination. Such hypothesis is supported by the frequent isolation of chitinolytic bacteria from spores of *Glomus macrocarpum*, *G. mosseae,* and *R. intraradices* (Ames et al., 1989; Filippi et al., 1998; Battini et al., 2016b). Mycorrhizosphere and sporosphere bacteria may act as "mycorrhiza helper" also by improving the growth of extraradical mycelium (ERM), the fine absorbing network of hyphae extending around the roots. *In vitro* studies showed that ERM length of *G. intraradices* and *Rhizophagus irregularis* was increased by *Paenibacillus rhizosphaerae*, *Rhizobium etli,* and several strains of *Azospirillum* and *Pseudomonas* (Bidondo et al., 2011; Ordoñez et al., 2016). Accordingly, *in vivo* investigations confirmed large ERM growth, boosted by *Pseudomonas fluorescens*, *Burkholderia cepacia*, *Sinorhizobium meliloti,* and *Streptomyces* spp. in *Glomus caledonium, G. intraradices,* and *R. irregularis* (Ravnskov and Jakobsen, 1999; Battini et al., 2017). Moreover, a number of indole acetic acid-producing bacteria were isolated from *G. mosseae* and *R. irregularis* spores, including several actinobacteria species, *Paenibacillus favisporus, S. meliloti*, and *Fictibacillus barbaricus* (Bidondo et al., 2011; Battini et al., 2017). However, the mechanistic explanation of ERM development promotion remains to be investigated.

Beyond "mycorrhizal helper" activity, AMF associated microbiota is fundamental for the maintenance of plant health, as it can protect plants from soilborne diseases and abiotic stresses. Actually, some strains were able to inhibit the growth of fungal pathogens by either producing antibiotics (Li et al., 2007; Bharadwaj et al., 2008a) or competing for iron nutrition by secreting siderophores, high-affinity iron-chelating compounds able to bind soluble Fe^{3+} (Whipps, 2001; Battini et al., 2016b). Interestingly, the AMF associated bacterial strains *Massilia* sp. RK4 and *Pseudomonas koreensis* S2CB35 were reported to improve maize tolerance to salinity (Krishnamoorthy et al., 2016; Selvakumar et al., 2018).

Another essential role played by AMF associated microbiota is represented by the solubilization of P, a key mineral nutrient which is poorly available to plants in most agricultural soils, as the result of its immobilization and precipitation with other soil minerals—iron and aluminum in acid and calcium in alkaline soils. P-mobilizing bacteria were isolated from AMF spores of *G. mosseae* and *R. intraradices*, and were ascribed to *Streptomyces* and *Leifsonia* species (Mohandas et al., 2013) and *Streptomyces* spp., *Bacillus pumilus*, *Lisinobacillus fusiformis,* and *S. meliloti* (Battini et al., 2016b). Such bacteria represent a sustainable strategy for the mobilization of the soil P pool and the facilitation of P uptake by mycorrhizal plants (Battini et al., 2017).

Also the acquisition of N, a major plant nutrient, may be mediated by AMF associated bacteria, as different species of the N-fixers *Rhizobium* and *Sinorhizobium*, isolated from AMF spores (Bharadwaj et al., 2008a; Agnolucci et al., 2015; Battini et al., 2016b), promoted mycorrhizal functioning, and plant mineral nutrition (Battini et al., 2017). Moreover, in recent years evidence suggested that bacteria isolated from AMF spores may improve the concentration of the health-promoting compound rosmarinic acid in basil plants, by modulating the expression of genes involved into its biosynthesis (Battini et al., 2016a).

The multifunctional activities of AMF associated microbiota described so far clearly show the complex and previously unimagined network of interactions involving AMF and their associated microbiota, that may encompass not univocal outcomes, depending on the identity of the taxa active in the mycorrhizosphere. In order to exploit the

functional complementarity of the diverse AMF-bacteria combinations, extensive studies should be carried out to answer basic questions concerning not only the isolation and identification of bacterial strains involved in specific functional activities, but also the spatio-temporal and environmental conditions affecting their behavior and its mechanistic explanation.

THE CHALLENGE OF TRANSLATIONAL RESEARCH

One of the main problems to be tackled when trying to translate the findings of fundamental research and laboratory studies into new tools and meaningful innovations in agricultural practices is represented by the absence of a systematic collection of data enabling scientists to gather information on the source of AMF associated beneficial bacteria, i.e., the different AMF genera, species and strains and where they were isolated from. Indeed, most data were obtained using AMF whose taxonomy was repeatedly and widely changed, emended and subverted in recent years (Oehl et al., 2011; Redecker et al., 2013; Sieverding et al., 2014). As an example, the ubiquitous species *F. mosseae* was named *Endogone mosseae* in 1968 and *G. mosseae* in 1974, while *Racocetra coralloidea* was known as *Gigaspora coralloidea* since 1974 and as *Scutellospora coralloidea* since 1986. As long known AMF species and genera were renamed, it is very difficult to link the taxon currently under investigation with its very properties, including the diverse spore-associated bacterial communities, whose composition differs in the diverse AMF isolates (Agnolucci et al., 2015).

In addition, very rarely scientists named the strains used in their experiments and deposited them in a germplasm bank, thus hindering the possibility of retrieving both AMF and the relevant associated microbiota for further investigations on their functional properties. On the other hand, such strains were not elite strains, i.e., the result of a fine tuned selection aimed at detecting the best performing ones, but just those able to produce, in pot-cultures, large quantities of mycelium, and spores from which bacteria could be easily isolated. Thus, the great majority of AMF are still to be studied as the home and source of beneficial bacteria. Such flaws are currently mirrored by the low number of AMF genotypes utilized as inoculants available on the market globally, and by the lack of innovative products or new formulations, developed from the isolation of AMF associated bacterial strains, selected for their specific activities to be applied as single strains or combined consortia. Moreover, the efficacy of the available AMF and associated bacteria should be deeply studied after inoculation in the field.

Another major problem that hindered the understanding of the diverse properties of mycorrhizospheric microbiota and its functional complementarity with AMF, leading to the promotion of mycorrhizal activity and host plant performance, is represented by the lack of studies on gene expression changes regulated by the presence of AMF associated bacterial isolates. Actually, while transcriptome studies, recently carried out by RNAseq technology, obtained the expression profiles of genes specifically regulated in mycorrhizal model and crop plants (Handa et al., 2015; Vangelisti et al., 2018), only few data are available on the expression levels of transcripts modulated by AMF associated beneficial bacteria (Battini et al., 2016a). Functional genomics studies are needed in order to advance our understanding of the multifunctionality and complementarity of AMF associated microbiota, and to boost translational research on the selection of the best performing strains and consortia, showing the most desirable functional activities relevant for plant growth, nutrition and health. Bridging the gap between basic science and the formulation of innovative products for the sustainable intensification of food production systems, represents the real next challenge in the years to come.

AUTHOR CONTRIBUTIONS

All authors listed have made a substantial, direct and intellectual contribution to the work, and approved it for publication.

FUNDING

The authors gratefully acknowledge the financial support by the University of Pisa (Fondi di Ateneo).

REFERENCES

Agnolucci, M., Battini, F., Cristani, C., and Giovannetti, M. (2015). Diverse bacterial communities are recruited on spores of different arbuscular mycorrhizal fungal isolates. *Biol. Fertil. Soils* 51, 379–389. doi: 10.1007/s00374-014-0989-5

Ames, R. N., Mihara, K. L., and Bayne, H. G. (1989). Chitin-decomposing actynomycetes associated with a vesicular–arbuscular mycorrhizal fungus from a calcareous soil. *New Phytol.* 111, 67–71. doi: 10.1111/j.1469-8137.1989.tb04219.x

Avio, L., Turrini, A., Giovannetti, M., and Sbrana, C. (2018). Designing the ideotype mycorrhizal symbionts for the production of healthy food. *Front. Plant Sci.* 9:1089. doi: 10.3389/fpls.2018.01089

Azcón, R. (1989). Selective interaction between free-living rhizosphere bacteria and vesicular arbuscular mycorrhizal fungi. *Soil Biol. Biochem.* 21, 639–644. doi: 10.1016/0038-0717(89)90057-6

Battini, F., Bernardi, R., Turrini, A., Agnolucci, M., and Giovannetti, M. (2016a). *Rhizophagus intraradices* or its associated bacteria affect gene expression of key enzymes involved in the rosmarinic acid biosynthetic pathway of basil. *Mycorrhiza* 26, 699–707. doi: 10.1007/s00572-016-0707-2

Battini, F., Cristani, C., Giovannetti, M., and Agnolucci, M. (2016b). Multifunctionality and diversity of culturable bacterial communities strictly associated with spores of the plant beneficial symbiont *Rhizophagus intraradices*. *Microbiol. Res.* 183, 68–79. doi: 10.1016/j.micres.2015.11.012

Battini, F., Grønlund, M., Agnolucci, M., Giovannetti, M., and Jakobsen, I. (2017). Facilitation of phosphorus uptake in maize plants by mycorrhizosphere bacteria. *Sci. Rep.* 7:4686. doi: 10.1038/s41598-017-04959-0

Bharadwaj, D. P., Lundquist, P. O., and Alström, S. (2008a). Arbuscular mycorrhizal fungal spore-associated bacteria affect mycorrhizal colonization, plant growth and potato pathogens. *Soil Biol. Biochem.* 40, 2494–2501. doi: 10.1016/j.soilbio.2008.06.012

Bharadwaj, D. P., Lundquist, P. O., Persson, P., and Alstro¨m, S. (2008b). Evidence for specificity of cultivable bacteria associated with arbuscular mycorrhizal fungal spores. *FEMS Microbiol. Ecol.* 65, 310–322. doi: 10.1111/j.1574-6941.2008.00515.x

Bianciotto, V., Bandi, C. D., Minerdi, M., Sironi, H., Tichy, V., and Bonfante, P. (1996). An obligately endosymbiotic mycorrhizal fungus itself harbors obligately intracellular bacteria. *Appl. Environ. Microbiol.* 62, 3005–3010.

Bidondo, L. F., Silvani, V., Colombo, R., Peérgola, M., Bompadre, J., and Godeas, A. (2011). Pre-symbiotic and symbiotic interactions between *Glomus intraradices* and two *Paenibacillus* species isolated from AM propagules. *In vitro* and *in vivo* assays with soybean (AG043RG) as plant host. *Soil Biol. Biochem.* 43, 1866–1872. doi: 10.1016/j.soilbio.2011.05.004

Calvet, C., Barea, J. M., and Pera, J. (1992). *In vitro* interactions between the vesicular-arbuscular mycorrhizal fungus *Glomus mosseae* and some saprophytic fungi isolated from organic substrates. *Soil Biol. Biochem.* 24, 775–780. doi: 10.1016/0038-0717(92)90252-S

Carpenter-Boggs, L., Loynachan, T. E., and Stahl, P. D. (1995). Spore germination of *Gigaspora margarita* stimulated by volatiles of soil-isolated actinomycetes. *Soil Boil. Biochem.* 27, 1445–1451. doi: 10.1016/0038-0717(95)00075-P

Cruz, A. F., Horii, S., Ochiai, S., Yasuda, A., and Ishii, T. (2008). Isolation and analysis of bacteria associated with spores of *Gigaspora margarita*. *J. Appl. Microbiol.* 104, 1711–1717. doi: 10.1111/j.1365-2672.2007.03695.x

Desirò, A., Salvioli, A., Ngonkeu, E. L., Mondo, S. J., Epis, S., Faccio, A., et al. (2014). Detection of a novel intracellular microbiome hosted in arbuscular mycorrhizal fungi. *ISME J.* 8, 257–270. doi: 10.1038/ismej.2013.151

Filippi, C., Bagnoli, G., Citernesi, A. S., and Giovannetti, M. (1998). Ultrastructural spatial distribution of bacteria associated with sporocarps of *Glomus mosseae*. *Symbiosis* 24, 1–12.

Frey-Klett, P., Garbaye, J. A., and Tarkka, M. (2007). The mycorrhiza helper bacteria revisited. *New Phytol.* 176, 22–36. doi: 10.1111/j.1469-8137.2007.02191.x

Giovannetti, M., Avio, L., and Sbrana, C. (2010). "Fungal spore germination and pre-symbiotic mycelial growth–physiological and genetic aspects," in *Arbuscular Mycorrhizas: Physiology and Function*, eds H. Koltai and Y. Kapulnik (Dordrecht: Springer), 3–32.

Handa, Y., Nishide, H., Takeda, N., Suzuki,Y., Kawaguchi, M., and Saito, K. (2015). RNA-seq transcriptional profiling of an arbuscular mycorrhiza provides insights into regulated and coordinated gene expression in *Lotus japonicus* and *Rhizophagus irregularis*. *Plant Cell Physiol.* 8, 1490–1511. doi: 10.1093/pcp/pcv071

Krishnamoorthy, R., Kim, K., Subramanian, P., Senthilkumar, M., Anandham, R., and Sa, T. (2016). Arbuscular mycorrhizal fungi and associated bacteria isolated from salt-affected soil enhances the tolerance of maize to salinity in coastal reclamation soil. *Agric. Ecosyst. Environ.* 231, 233–239. doi: 10.1016/j.agee.2016.05.037

Li, B., Ravnskov, S., Xie, G., and Larsen, J. (2007). Biocontrol of *Pythium* damping-off in cucumber by arbuscular mycorrhiza-associated bacteria from the genus *Paenibacillus*. *Biocontrol* 52, 863–875. doi: 10.1007/s10526-007-9076-2

Long, L., Zhu, H., Yao, Q., and Ai, Y. (2008). Analysis of bacterial communities associated with spores of *Gigaspora margarita* and *Gigaspora rosea*. *Plant Soil* 310, 1–9. doi: 10.1007/s11104-008-9611-7

Mayo, K., Davis, R. E., and Motta, J. (1986). Stimulation of germination of spores of *Glomus versiforme* by spore-associated bacteria. *Mycologia* 78, 426–431. doi: 10.2307/3793046

Mohandas, S., Poovarasan, S., Panneerselvam, P., Saritha, B., Upreti, K. K., Kamal, R., et al. (2013). Guava (*Psidium guajava* L.) rhizosphere *Glomus mosseae* spores harbor actinomycetes with growth promoting and antifungal attributes. *Sci. Hortic.* 150, 371–376. doi: 10.1016/j.scienta.2012.11.019

Mosse, B. (1959). The regular germination of resting spores and some observations on the growth requirements of an *Endogone* sp. causing vesicular-arbuscular mycorrhiza. *Tr. Br. Myc. Soc.* 42, 273–286. doi: 10.1016/S0007-1536(56)80033-8

Mosse, B. (1970). Honey-coloured sessile Endogone spores. II. Changes in fine structure during spore development. *Arch. Mikrobiol.* 74, 146–159. doi: 10.1007/BF00446901

Oehl, F., Sieverding, E., Palenzuela, J., Ineichen, K., and Alves da Silva, G. (2011). Advances in glomeromycota taxonomy and classification. *IMA Fungus* 2, 191–199. doi: 10.5598/imafungus.2011.02.02.10

Ordoñez, Y. M., Fernandez, B. R., Lara, L. S., Rodriguez, A., Uribe-Vélez, D., and Sanders, I. R. (2016). Bacteria with phosphate solubilizing capacity alter mycorrhizal fungal growth both inside and outside the root and in the presence of native microbial communities. *PLoS ONE* 11:e0154438. doi: 10.1371/journal.pone.0154438

Philippot, L., Raaijmakers, J. M., Lemanceau, P., and Van Der Putten, W. H. (2013). Going back to the roots: the microbial ecology of the rhizosphere. *Nat. Rev. Microbiol.* 11, 789–799. doi: 10.1038/nrmicro3109

Ravnskov, S., and Jakobsen, I. (1999). Effects of *Pseudomonas fluorescens* DF57 on growth and P uptake of two arbuscular mycorrhizal fungi in symbiosis with cucumber. *Mycorrhiza* 8, 329–334. doi: 10.1007/s005720050254

Redecker, D., Schüßler, A., Stockinger, H., Stürmer, S. R., Morton, J. B., and Walker, C. (2013). An evidence-based consensus for the classification of arbuscular mycorrhizal fungi (Glomeromycota). *Mycorrhiza* 23, 515–531. doi: 10.1007/s00572-013-0486-y

Roesti, D., Ineichen, K., Braissant, O., Redecker, D., Wiemken, A., and Aragno, M. (2005). Bacteria associated with spores of the arbuscular mycorrhizal fungi *Glomus geosporum* and *Glomus constrictum*. *Appl. Environ. Microbiol.* 71, 6673–6679. doi: 10.1128/AEM.71.11.6673-6679.2005

Rouphael, Y., Franken, P., Schneider, C., Schwarz, D., Giovannetti, M., Agnolucci, M., et al. (2015). Arbuscular mycorrhizal fungi act as biostimulants in horticultural crops. *Sci. Hortic.* 196, 91–108. doi: 10.1016/j.scienta.2015.09.002

Sbrana, C., Avio, L., and Giovannetti, M. (2014). Beneficial mycorrhizal symbionts affecting the production of health-promoting phytochemicals. *Electrophoresis* 35, 1535–1546. doi: 10.1002/elps.201300568

Selvakumar, G., Shagol, C. C., Kim, K., Han, S., and Sa, T. (2018). Spore associated bacteria regulates maize root K^+/Na^+ ion homeostasis to promote salinity tolerance during arbuscular mycorrhizal symbiosis. *BMC Plant Biol.* 18:109. doi: 10.1186/s12870-018-1317-2

Sieverding, E., Silva, G. A., Berndt, R., and Oehl, F. (2014). *Rhizoglomus*, a new genus in the Glomeraceae. *Mycotaxon* 129, 373–386. doi: 10.5248/129.373

Smith, S. E., and Read, D. J. (2008). *Mycorrhizal Symbiosis*. London: Academic Press.

Tylka, G. L., Hussey, R. S., and Roncadori, R. W. (1991). Axenic germination of vesicular–arbuscular mycorrhizal fungi: effects of selected *Streptomyces* species. *Phytopathology* 81, 754–759. doi: 10.1094/Phyto-81-754

Vangelisti, A., Natali, L., Bernardi, R., Sbrana, C., Turrini, A., Hassani-Pak, K., et al. (2018). Transcriptome changes induced by arbuscular mycorrhizal fungi in sunflower (*Helianthus annuus* L.) roots. *Sci. Rep.* 8:4. doi: 10.1038/s41598-017-18445-0

Whipps, J. M. (2001). Microbial interactions and biocontrol in the rhizosphere. *J. Exp. Bot.* 52, 487–511. doi: 10.1093/jexbot/52.suppl_1.487

Will, M. E., and Sylvia, D. M. (1990). Interaction of rhizosphere bacteria, fertilizer, and vesicular-arbuscular mycorrhizal fungi with sea oats. *Appl. Environ. Microbiol.* 56, 2073–2079.

Xavier, L. J. C., and Germida, J. J. (2003). Bacteria associated with *Glomus clarum* spores influence mycorrhizal activity. *Soil Biol. Biochem.* 35, 471–478. doi: 10.1016/S0038-0717(03)00003-8

Conflict of Interest Statement: The authors declare that the research was conducted in the absence of any commercial or financial relationships that could be construed as a potential conflict of interest.

frontiers in Plant Science

ORIGINAL RESEARCH
published: 09 October 2018
doi: 10.3389/fpls.2018.01480

The Association With Two Different Arbuscular Mycorrhizal Fungi Differently Affects Water Stress Tolerance in Tomato

*Veronica Volpe[1†], Walter Chitarra[2†], Pasquale Cascone[3], Maria Grazia Volpe[4], Paola Bartolini[3], Gloriano Moneti[5], Giuseppe Pieraccini[5], Claudia Di Serio[6], Biancaelena Maserti[3], Emilio Guerrieri[3] and Raffaella Balestrini[3]**

[1] Department of Life Sciences and Systems Biology, University of Turin, Turin, Italy, [2] Council for Agricultural Research and Economics, Centre of Viticulture and Enology Research, Conegliano, Italy, [3] National Research Council, Institute for Sustainable Plant Protection, Turin-Florence-Portici (NA) Units, Portici, Italy, [4] CNR, Institute of Food Sciences, Avellino, Italy, [5] Department of Health Sciences, University of Florence, Florence, Italy, [6] Geriatric Intensive Care Unit, Experimental and Clinical Medicine Department, University of Florence, AOU Careggi, Florence, Italy

OPEN ACCESS

Edited by:
Youssef Rouphael,
Università degli Studi di Napoli Federico II, Italy

Reviewed by:
Graziella Berta,
Università degli Studi del Piemonte Orientale, Italy
Rosa Hermosa,
Universidad de Salamanca, Spain

***Correspondence:**
Raffaella Balestrini
raffaella.balestrini@ipsp.cnr.it

[†] These authors have contributed equally to this work

Specialty section:
This article was submitted to Plant Microbe Interactions, a section of the journal Frontiers in Plant Science

Received: 05 July 2018
Accepted: 21 September 2018
Published: 09 October 2018

Citation:
Volpe V, Chitarra W, Cascone P, Volpe MG, Bartolini P, Moneti G, Pieraccini G, Di Serio C, Maserti B, Guerrieri E and Balestrini R (2018) The Association With Two Different Arbuscular Mycorrhizal Fungi Differently Affects Water Stress Tolerance in Tomato. Front. Plant Sci. 9:1480. doi: 10.3389/fpls.2018.01480

Arbuscular mycorrhizal (AM) fungi are very widespread, forming symbiotic associations with ∼80% of land plant species, including almost all crop plants. These fungi are considered of great interest for their use as biofertilizer in low-input and organic agriculture. In addition to an improvement in plant nutrition, AM fungi have been reported to enhance plant tolerance to important abiotic and biotic environmental conditions, especially to a reduced availability of resources. These features, to be exploited and applied in the field, require a thorough identification of mechanisms involved in nutrient transfer, metabolic pathways induced by single and multiple stresses, physiological and eco-physiological mechanisms resulting in improved tolerance. However, cooperation between host plants and AM fungi is often related to the specificity of symbiotic partners, the environmental conditions and the availability of resources. In this study, the impact of two AM fungal species (*Funneliformis mosseae* and *Rhizophagus intraradices*) on the water stress tolerance of a commercial tomato cultivar (San Marzano nano) has been evaluated in pots. Biometric and eco-physiological parameters have been recorded and gene expression analyses in tomato roots have been focused on plant and fungal genes involved in inorganic phosphate (Pi) uptake and transport. *R. intraradices*, which resulted to be more efficient than *F. mosseae* to improve physiological performances, was selected to assess the role of AM symbiosis on tomato plants subjected to combined stresses (moderate water stress and aphid infestation) in controlled conditions. A positive effect on the tomato indirect defense toward aphids in terms of enhanced attraction of their natural enemies was observed, in agreement with the characterization of volatile organic compound (VOC) released. In conclusion, our results offer new insights for understanding the molecular and physiological mechanisms involved in the tolerance toward water deficit as mediated by a specific AM fungus. Moreover, they open new perspectives for the exploitation of AM symbiosis to enhance crop tolerance to abiotic and biotic stresses in a scenario of global change.

Keywords: aphid, arbuscular mycorrhizal symbiosis, phosphate transporter, plant tolerance, *Solanum lycopersicum*, volatile organic compound, water deficit

INTRODUCTION

In the last decade, climatic change heavily influenced environmental conditions and a negative impact on plant development and productivity has been recorded. Plant responses to the environmental stresses include morphological, physiological, cellular and molecular changes (reviewed in Gray and Brady, 2016). One of the major abiotic threats on agriculture productivity is the progressive diffusion of water deficit in different areas of world (Dai, 2011), which induces a reduction in plant growth and development. Water deficit causes stomatal closure with a consequent decrease of CO_2 adsorption followed by a reduction in photosynthetic activity and carbon partitioning (Osakabe et al., 2014; Chitarra et al., 2016). Additionally, drought has a negative impact on nutrient supply, leading to a decrease in phosphate availability (Sardans and Peñuelas, 2004). Root growth and development are important traits for plant survival in water stressed soil. A deep and extensive root system enables plants to access moisture in water-absorbed zones of soils, and is thus considered as a vital strategy for drought adaptation (Zou et al., 2017). In many crops (e.g., rice, wheat, and maize) an alteration of root system architecture has been observed under water deficit conditions, with a consistent increase in the elongation of primary roots, in the total root biomass and in the production of lateral roots, along with a change in the root growth angle and a reduction of root diameter (Comas et al., 2013). Root responses to drought stress depend on the induction of abscisic acid (ABA) pathway. This hormone is involved in different root cellular responses such as the activation of enzymes involved in cell wall remodeling (Wu et al., 1994), the regulation of cell type-specific development (Enstone et al., 2002) and the increase of lateral roots (De Smet et al., 2003). However, in agro-ecosystems, in addition to water limitation, crops have often to face the concurrent presence of other abiotic and biotic stresses, e.g., insect pests and pathogens (Bai et al., 2018). Hence, plants have to modulate a multifaceted defense by activating a number of molecular, biochemical and morphological changes, which can differ from the responses to a single stress factor (Atkinson and Urwin, 2012). Different plant defense responses lead to the release of volatile organic compounds (VOC) blends having different composition in term of presence or quantity of single compounds, depending on the stress features (Holopainen and Gershenzon, 2010). VOC released in response to pest attack can have direct and indirect defensive effect on insect performance. For example, methyl salicylate and the *cis*-3-hexen-1-ol, released by tomato plants in response to aphid attack (Sasso et al., 2007), have negative effects on subsequent aphid fixation (Digilio et al., 2012). These same compounds are powerful attractant of aphid natural enemies (Sasso et al., 2007, 2009). Moreover, the response of different genotypes, i.e., commercial genotypes or traditional varieties/landraces adapted to local environmental conditions, to stresses may be different. In this scenario, there is a consolidated evidence of the positive role played by "biostimulants," e.g., organic (humic acids and seaweed extracts) and inorganic (silicium) substances, and/or microorganisms, which can be applied to improve plant nutrition/growth and to protect against stresses (du Jardin, 2015). Among them, great interest has been developed in the last years to beneficial soil microorganisms, including arbuscular mycorrhizal (AM) fungi, with a role to improve plant nutrition and tolerance to several environmental stresses. AM symbiosis is one of the most ancient interactions between the roots of more than 80% of terrestrial plants, including several crops (Balestrini and Lumini, 2018). During this interaction, in addition to the improvement of plant nutritional status, AM fungi can promote plant performance and protection from various stresses, including water deficit (Lenoir et al., 2016; Balestrini et al., 2018). In addition to an improved root system capacity to absorb nutrients and water due to the presence of external fungal hyphae, it has been also demonstrated that AM colonization influences the architecture of the host root system, leading to a better adaptation of morphology in response to water stress (Gutjahr et al., 2009; Fusconi, 2014; Zou et al., 2017). Recently, Chitarra et al. (2016) showed that the AM symbiosis positively affects the tomato tolerance to severe water deficit and how the adaptive plant response is dependent on the AM fungi species involved, underlying the importance to identify the optimal genotype/microorganism(s) combination to maximize plant resilience. Considering the role of isoprenoids in resistance to stress and plant defense, Asensio et al. (2012) tested the interaction between tomato plants and a mixed AM fungal inoculum under a drought stress conditions or jasmonic acid (JA) application, demonstrating an impact of the AM colonization in the pathways involved in isoprenoid production mainly under the considered treatments (drought and JA).

However, although the effects of AM symbiosis on the interactions with aphids have been already reported (Babikova et al., 2013, 2014), scarce information is available on these interactions under an abiotic stress condition such as water stress.

In this work, a multidisciplinary approach, involving eco-physiological, morphometric, biochemical and molecular analyses, and targeted metabolomics, has been used. Particularly, the mechanisms and the species-specificity of AM symbiosis in plant tolerance to moderate (MS) and severe (SS) water stress have been evaluated in comparison with unstressed plants. For this purpose, we tested two AM fungi, *Funneliformis mosseae* and *Rhizophagus intraradices,* in their association with a cultivated tomato cultivar (i.e., San Marzano nano). In particular, root gene expression analyses were focused on plant and fungal genes involved in phosphate (Pi) uptake and transport because of the importance of phosphorous use efficiency (PUE) in plants grown under abiotic stress (reviewed in Wang et al., 2017). It has been recently reported that, Pi transporter genes are regulated by a drought stress, both in poplar (Zhang et al., 2016) and apple (Sun et al., 2017). Here, we aimed to assess whether this applies to tomato and mainly how different AM fungi alter the expression of these genes under moderate (MS) and severe (SS) water stress conditions. In addition, considering that in the field plants are usually subjected to multiple stresses, the AM fungus (*R. intraradices*), resulting more efficient in increasing water stress tolerance, was selected to assess its role on tomato resistance to a combination of abiotic (water deficit) and biotic (aphid attack) stresses.

MATERIALS AND METHODS

Plant, Fungal Material, and Experimental Design

Solanum lycopersicum cv. "San Marzano nano" (i.e., dwarf) seeds were surface-sterilized in sodium hypochlorite 5% (NaOCl) for 20 min, washed five times in sterile water, and germinated on wet paper. Seedlings were then moved to pots (10 × 10 × 12) containing a mixture of quartz sand (50%), sterile pumice (20%), and an inoculum (30%) of either *Funneliformis mosseae* (formerly *Glomus mosseae*; BEG 12) or *Rhizophagus intraradices* (FR 121), both purchased from MycAgro (Bretenière, France[1]). Propagules of AM fungi (i.e., a mix of spores, mycelium and mycorrhizal root pieces) are minimum equal to 10 propagules/g. For non-colonized plants (Ctrl), sterile inoculum carrier only (i.e., mix of inert mineral predominantly made with zeolite) was used instead of the specific inoculum (30%). For each pot, a quantity of about 650 g of sand plus inoculum/carrier has been used. Plants were grown in controlled conditions, at 23/21 ± 1°C (day/night), 16/8 h light/dark photoperiod, and 65 ± 10% relative humidity. From transplanting to the beginning of the experiment (after about 6/7 weeks), all the plants were watered twice a week with tap water and, once a week, with a modified Long-Ashton nutrient solution (Hewitt, 1966) containing 300 μM [Pi]. Two independent experiments (Experiment 1 and 2) were performed to determine the role of AM symbiosis under water deficit.

Experiment 1

This experiment was devoted to verify the impact of the two different AM fungi on the regulation of Pi transporter genes in roots and on the production of specific metabolites in leaves. Biometric and eco-physiological parameters have been also registered to verify the plant stressed status. Considered treatments were: (i) AM fungal colonization [non-colonized (Ctrl), *F. mosseae*-colonized (Fmos) and *R. intraradices*-colonized (Rin)] and (ii) water stress [none (NS), moderate (MS), and severe (SS)]. For each condition, 12 plants were maintained in a well-watered state (at container capacity, irrigated or unstressed, NS). The remaining 24 plants were subjected to two water stress levels. Irrigation was withheld about 6/7 weeks after fungal inoculation: moderate stress (MS) was achieved in about 2 weeks (leaf water potential of about −0.9 MPa), whilst 3 weeks were necessary to produce a severe water stress (SS) status (petiole water potential lower than −1.0 MPa). From the beginning of the water deficit, plants were moved (at least one time a week) inside the climatic chamber to avoid positional differences. Root colonization has been assessed at the beginning and at the end of the water stress imposition (not shown).

Biometric and Physiological Measurements

At the end of the water stress experiment (about 10 weeks after the beginning of the experiment), plants were harvested and plant height and shoot diameter were recorded. Parameters were taken as reported by Chitarra et al. (2016). Briefly, leaf water potential (Ψ_{leaf}) was measured on one transpiring leaf per plant, using a Scholander-type pressure chamber (Soil Moisture Equipment Corp., Santa Barbara, CA, United States). Measurements of transpiration rate (E), stomatal conductance (g_s), and net photosynthetic rate (A_N) were performed on adult, non-senescing, leaves at the same physiological age (in the middle part of the plant, considering the third-fourth leaf form the shoot apex). Intrinsic water use efficiency (iWUE) was calculated as the ratio between A_N and g_s. Measurements were taken with an infrared gas analyzer ADC-LCPro+ system (The Analytical Development Company Ltd., Hoddesdon, United Kingdom). During measurement, light intensity in the leaf chamber was set at 1200 μmol photons m^{-2} s^{-1}, temperature was 25°C, and concentration of CO_2 was maintained at 450–470 ppm. Measurements were taken between 10:00 and 13:00 h. The chlorophyll content index (CCI) was determined at the end of the experiment (16 or 20 days after treatment, DAT) using the portable chlorophyll meter SPAD 502 (CCM-200, Opti-Sciences, Inc., Hudson, NH, United States). Readings were collected from the second or third fully developed leaves from the top on five randomly selected tomato plants for each experimental condition.

Phosphorus Determination

Analytical grade reagents and Phosphorus (P) standard (1000 mg/L) were purchased from Sigma-Aldrich chemical. The digestion of leaf samples was performed according to Olowu et al. (2015) with some modifications. 0.10 g of each sample was weighed and 3 mL of a mixture of nitric acid: perchloric (1: 1; v: v) was added to each. The mixture was placed in a digester at 220°C for about 60 min. After 45 min, 500 μL of H_2O_2 (30%) were added in order to facilitate the oxidation reaction. After cooling the whole was transferred into a 10 mL volumetric flask and brought to volume with H_2O. An Inductively Coupled Argon Plasma Optical Emission Spectrometers (ICP-OES iCAP 7000 Series Thermo Scientific), equipped with ASX-520 Autosampler (CETAC^{TM}, Thermo Scientific) was used. To prepare the calibration curve with the Phosphorus concentrations equal to 0.5; 1; 2; 4 and 5 mg/L, the Multi-Element Test Solution ICAP 6000 with P concentration of 10 mg/L in 1% v/v HNO_3 was used. The element content was calculated by using standard curves and the final concentrations of samples were expressed as g/kg dry weight of P. The emission line for the analysis by ICP OES was chosen according to previous interference studies. The line that exhibited low interference and high analytical signal and background ratios was selected. The emission line that was employed was 185.942 nm. The ICP-OES measurements were performed in triplicate, with R^2 = 0.9994 and BEC (Background Equivalent Concentration) = 0.002 ppm.

RNA Extraction and RT-qPCR

Expression changes of target transcripts were quantified on root samples (three independent biological replicates) by quantitative real-time PCR (RT-qPCR). Roots from two plants from each treatment were pooled to form a biological replicate, immediately frozen in liquid nitrogen and stored at −80°C. Total RNA was isolated from each biological replicate and cDNA synthesis was

[1]http://www.mycagrolab.com/

performed as described in Chitarra et al. (2016). Genomic DNA contamination was checked before proceeding with the cDNA synthesis by PCR reactions using *LeEF* specific primers of tomato (**Supplementary Table S1**). RT-qPCR experiments were carried out in a final volume of 15 μl containing 7.5 μl of Rotor-Gene™ SYBR® Green Master Mix (Qiagen), 1 μl of 3 μM specific primers and about 10 ng of cDNA. Samples were run in the Rotor Gene apparatus (Qiagen) using the following program: 10 min pre-incubation at 95°C, followed by 40 cycles of 15 s at 95°C, and 30 s at 60°C. Each amplification was followed by melting curve analysis (60–94°C) with a heating rate of 0.5°C every 15 s. All reactions were performed with three technical replicates and only Ct values with a standard deviation that did not exceed 0.3 were considered. The comparative threshold cycle method (Rasmussen, 2001) was used to calculate relative expression levels using plant *LeEF* and *LeUBI* reference genes. Oligonucleotide sequences are listed in **Supplementary Table S1**. In detail, genes encoding for plant (*LePT1*, *LePT2*, *LePT3*, *LePT4*, and *LePT5*) and fungal (*RiPT* and *FmPT*) phosphate transporters have been considered.

Targeted Analysis of Leaf Volatiles

Leaf VOC analyses (isolation, identification and quantification) were done by headspace solid-phase microextraction-gas chromatography-mass spectrometry (HS-SPME-GC-MS). For sample preparation, 0.1 g of tomato leaf stored in a 2 ml screw cap headspace glass vial at −80°C were transferred at room temperature for 5 min and 0.5 ml of 30% NaCl solution containing the internal standard (2-hexanone at 1 ng/μl) was added to the vial. After mixing, samples were pre-incubated for 30 min at room temperature. A 65 μm PDMS/DVB SPME fiber (Supelco, Sigma Aldrich, Milan, Italy) was used for extraction procedure: the fiber was introduced into the vial headspace for 5 min.

A Hewlett Packard GC-MS system composed by a HP 5890 Series II Gas Chromatograph coupled to a HP 5971A Mass Selective Detector single quadrupole mass spectrometer was used. The GC-MS was equipped with a ZB-5MS column with column guard (Phenomenex) (30 m × 0.25 μm i.d.; film thickness 0.50 μm). Oven temperature was set up with following parameters: initial temperature 45°C maintained for 3 min, then to 220°C at 12°C/min, held isothermally at 220°C for 2.5 min. The volatiles trapped on the fiber were desorbed for 2 min at 250°C in the injection port of the GC; the injection was performed in splitless mode for 1 min. To prevent cross-contamination between successive samples, the fiber was cleaned by exposure in a different GC injection port for 5 min at 250°C before a new sampling process. Ultra high purity helium was used as the carrier gas at a constant head pressure of 12 psi (corresponding to 1 ml/min at 45°C). Transfer line temperature was 280°C.

Data were recorded in full scan mode from 40 to 550 m/z, using an electron ionization (EI, 70 eV) source, with 1.53 scan/s. Data acquisition and processing were performed using HP ChemStation software (version D.02.00).

Quantitative analysis by GC-MS was performed recording the signals in selected ion monitoring (SIM) mode; the areas of the quantifier ion peak of each analyte were measured and compared to that of the internal standard 2-hexanone. In **Supplementary Table S2** the retention time and principal ion of each compound are reported.

Calibration curves constructed with pure standards – trans-2-hexenal, methyl salicylate, eugenol, α–phellandrene, β–phellandrene (Sigma Aldrich) – in the ranges of the interest concentrations allowed the calculation of the compound concentrations in the samples.

Experiment 2

Experiment 2 was devoted to evaluate the impact of AM symbiosis on a combined abiotic (moderate water stress) and biotic (aphid infestation) stress condition. To this aim, aphid survival (a measure of direct plant defense), parasitoid attractiveness (a measure of indirect defense) and VOC emissions were considered in unstressed and water stressed conditions. Considered treatments were: (i) AM fungus [non-colonized (Ctrl) and *R. intraradices*-colonized (Rin)], (ii) water stress [none (NS) and moderate (WS)], and (iii) Aphid infestation (Aph) and their combinations. In detail, for aphid survival, 20 plants non-colonized (Ctrl) and 20 colonized with *R. intraradices* (Rin) were prepared for the aphid survival experiment: 10 plants for each condition were maintained under a well water conditions (CtrlNS and RinNS) and 10 were subjected to a moderate water stress (WS and WS + Rin). By contrast, for parasitoid attractiveness and VOC collection, 6 plants for the following 8 conditions were considered: (NS, Aph, Rin, WS, Rin + Aph, WS + Rin, WS + Aph, and WS + Rin + Aph), totalling 48 plants.

Insect Rearings

Both the herbivore and its parasitoid were reared at CNR-Institute for Sustainable Plant Protection (Portici, NA, Italy) as follows:

- the potato aphid *Macrosiphum euphorbiae* Thomas (Hemiptera: Aphididae) was reared on tomato plants *cv*. San Marzano nano (i.e., dwarf) from material collected in the field (Scafati, SA, Italy) on the same plant cultivar in 2001 and periodically refreshed by adding field collected aphids. Rearing conditions were: 20 ± 1°C, 18 h light/6 h dark photoperiod, 65 ± 5% relative humidity.
- the aphid parasitoid *Aphidius ervi* Haliday (Hymenoptera: Braconidae) was permanently reared on the pea aphid *Acyrthosiphon pisum* reproduced on broad bean plants (*cv*. Aquadulce) from material collected in the field (Battipaglia, SA, Italy) in 2001 on alfalfa and periodically refreshed by adding field collected aphids. Rearing conditions were: 20 ± 1°C, 18/6 h light/dark photoperiod, 65 ± 5% relative humidity (Guerrieri et al., 2002).

Aphid Survival Assays

For each experimental condition 10 plants were infested with one newly born nymph of *M. euphorbiae*, transferred with a soft brush. All plants were checked daily to assess presence of aphids, presence of exuviae (evidence of molting), presence and number of newly laid nymphs and dead aphids. When reproduction

started, the offspring were counted and removed daily. The mortality of the aphids was recorded daily until the death of the last individual. The parameters of the bioassay were set as indicated above.

Wind-Tunnel Bioassay

Plants were tested in a wind-tunnel bioassay for their attractiveness toward the aphid parasitoid wasp *A. ervi*. For the aphid infestation fifty mixed-aged individuals of *M. euphorbiae* (mimicking a natural population) were gently transferred by paintbrush on tomato leaves allowing them to feed for 1 week. For the double stress thesis, the aphids were added at the onset of water deficit imposition. These treatments were performed using both inoculated with *R. intraradices* and non-inoculated tomato plants. For each experimental condition, a total of 6 plants was used and offered individually every day for 6 consecutive days in a random order to reduce any bias related to the time of the experiments. One hundred parasitoid females were tested singly for each target in no-choice experiments, and observed for a maximum of 5 min. The percentage of response (oriented flights, landings on the target) to each target plant was calculated. The parameters of the bioassay were set as follows: 20 ± 1°C; 65 ± 5% relative humidity; 25 ± 5 cm s −1 wind speed; 50 cm distance between releasing vial and plant target.

VOC Collection and Analysis

Immediately after the bioassay in the wind tunnel, each plant was used for the collection of head-space volatiles by an airtight entrainment system. The system consisted of a bell jar containing a single potted plant and the jar was connected to a circulation pump forcing the air in an adsorbent trap made of Tenax TA, 60–80 mesh (Sigma-Aldrich). Each collection lasted 3 h under controlled conditions (24 ± 2°C, 18/6 h light/dark photoperiod, 70 ± 10% relative humidity, 700 μmol m 2 s 1 PPFD). Collected volatiles were analyzed by an integrated system including thermal desorber (Tekmar TD-800) mounted on a gas chromatograph (column: RTX-200, 60 m, 0.25 mm ID, 0.25 μm, carrier gas: He) coupled to a mass spectrometer detector. The resulting peaks were compared with a compound database library (National Institute of Standards and Technology) and the following available authentic standards: anisole-p-allyl, camphor, 3-carene, (E)-β-caryophyllene, chlorobenzene, α-copaene, α-cubebene, p-cymene, decane, p-dichlorobenzene, dodecene, eucalyptol, eugenol, (E)-β-farnesene, α-gurjunene, hexanal, (Z)-3-hexen-1-ol, humulene (=α-caryophyllene), (R)-(+)-limonene, (S)-(-)-limonene, linalool, (+)-longifolene, menthol, 6-methyl-5-hepten-2-one, methyl salicylate, b-myrcene, (Z)-nerolidol,(E)-β-ocimene, (R)-(-)-α-phellandrene, α-pinene, skatol, α-terpinene, γ-terpinene, α-terpineol, and terpinolene.

Statistical Analysis

Analysis of variance (ANOVA) of the experimental data was performed using the SPSS software. When ANOVA indicated that either stress (stress: NS, MS, and SS) or mycorrhizal colonization (myco: Ctrl, Fmos, and Rin) or their interaction was significant, mean separation was performed using the Tukey HSD test, adopting a probability level of $P < 0.05$. The volatile emission patterns, measured as peak areas divided by fresh plant weight, were analyzed by Detrended Correspondence Analysis (DCA). Data were square root transformed to meet normal distribution. Analysis was conducted using the R statistics programming environment 3.3.3 (R Development Core Team, 2012) and the 'vegan' package ver. 2.5-2. Multivariate analysis of variance by permutation (PMANOVA) was used to test for significant differences between group centroids using vegan's adonis functions (Oksanen et al., 2013). The number of parasitoids responding to each target was compared by a G-test for independence with William's correction. The resulting values of G were compared with the critical values of χ2 (Sokal and Rohlf, 1995). To analyze the effect of each experimental condition on aphid survival, we used function survfit and Kaplan–Meier plot to visualize the survival curves, contained in the R package 'survival' version 2.41-3.

RESULTS

Biometric Parameters and Ecophysiology

In our experiments, biometric parameters including plant height, the internodes/height ratio and shoot diameter have been affected by both water treatments and AM colonization, while no significant differences were registered for the chlorophyll content index (CCI; **Supplementary Figures S1A–D**). Plant height and the internodes/height ratio were influenced by stress (S) and myco (M) treatments with no significant S × M interaction, although Fmos-inoculated plants under MS and SS conditions showed higher height when compared with RinMS/SS and CtrlSS plants (**Supplementary Figure S1A** and **Table S3**). Considering the internodes/height ratio, highest values were recorded for RinMS and SS plants with respect to other treatments (**Supplementary Figure S1B** and **Table S3**). Conversely, shoot diameter did not show a different trend among the M variables, while a significant impact of the S variables and S × M interaction was observed (**Supplementary Figure S1C**).

The imposed water stress conditions differently affected leaf water potential (Ψ_{leaf}) levels independently from controls and the AM inoculum used. At the end of the experiment, significant differences were observed. NS plants showed in fact values lower than/equal to −0.5 MPa, while Ψ_{leaf} values of about −0.9 MPa and −1.4 MPa were observed in MS and SS plants, respectively (**Figure 1A**).

Considering A_N rates, a decreasing trend was observed from NS to MS and SS conditions. Significant higher A_N rates were recorded in NS condition, particularly in RinNS plants. Conversely, significant lower values were measured under SS condition with the exception of RinSS plants that were statistically similar to FmosMS ones (**Figure 1B**). A similar trend was also evident for g_s, although a significant influence was observed only for S and M variables (**Figure 1C** and **Supplementary Table S3**). In addition, iWUE index, irrespectively of treatment or water status, showed similar values,

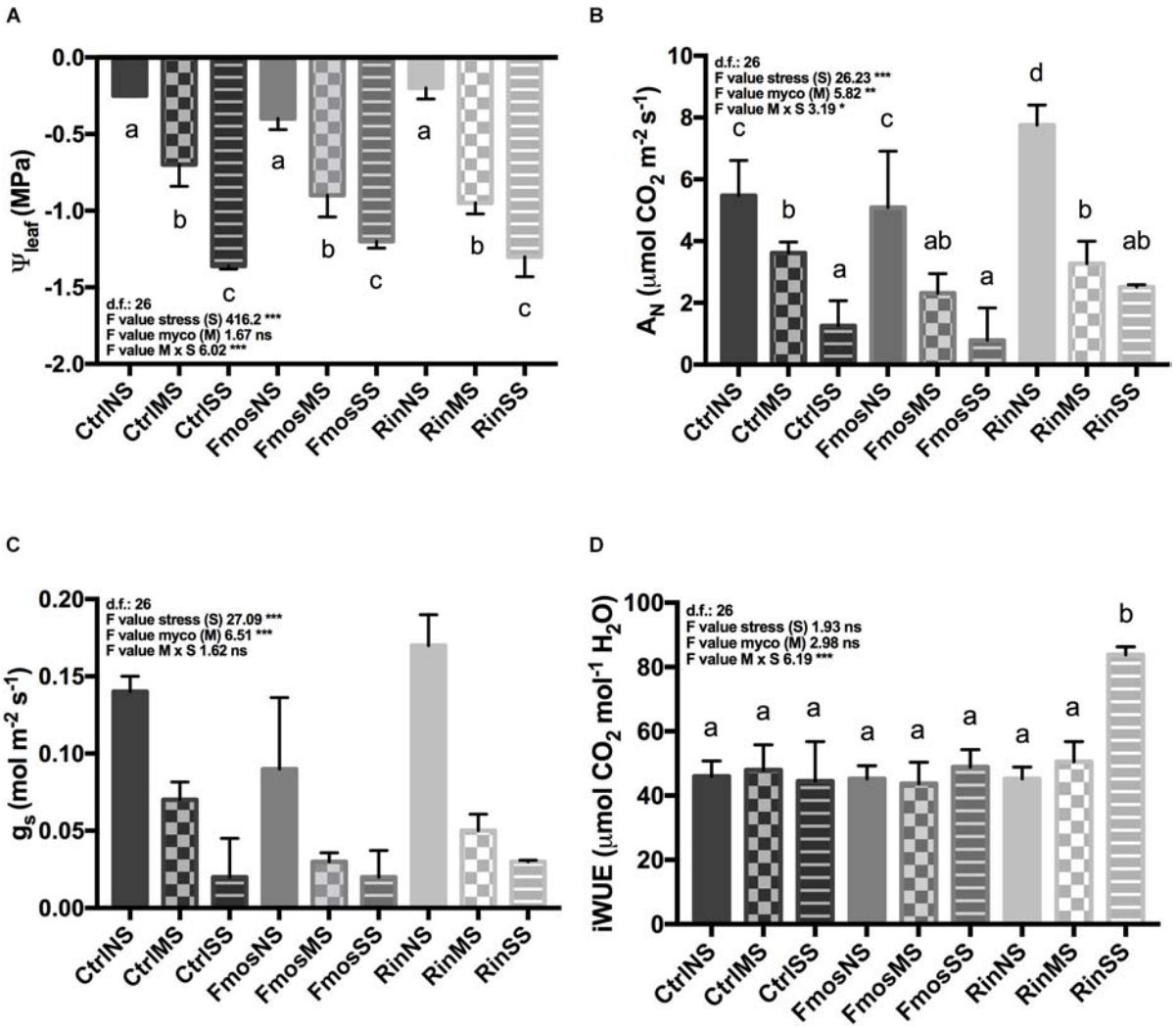


FIGURE 1 | Leaf water potential and gas exchanges in AM– and AM+ plants unstressed (NS) and subjected to two different water deficit levels (moderate stress, MS; severe stress, SS), measured at the end of the experiment. **(A)** Leaf water potential (Ψ_{leaf}); **(B)** net photosynthetic rate (A_N); **(C)** stomatal conductance (g_s); and **(D)** iWUE levels in AM– (Ctrl) and AM+ (Rin and Fmos) plants. All data are expressed as mean ± SE (n = 5). ns, *, **, and ***: non-significant or significant at $P \leq 0.05$, $P \leq 0.01$, and $P \leq 0.001$, respectively. Different letters above the bars indicate significant differences according to Tukey HSD test ($P \leq 0.05$), considering S × M interaction. Analysis of variance on the single variables is reported in **Supplementary Table S3**.

with the exception of RinSS plants where significant higher iWUE was observed (**Figure 1D**).

Phosphorus Determination and Expression of Phosphate Transporter Genes

In no stress condition, no significant differences were detected among the three treatments in phosphorous (P) determination in leaves, i.e., CtrlNS, RinNS, and FmosNS plants, although higher values have been measured in AM-colonized plants in respect to non-colonized ones, with the highest P content (g/kg) recorded in *F. mosseae*-colonized plants. While no strong differences have been registered under a moderate stress condition (MS), under a severe water stress condition (SS) a higher leaf P content was detected in *F. mosseae*-colonized plants mainly respect to Ctrl plants (data not shown).

Additionally, the expression of plant and fungal Pi transporter genes was evaluated in roots from plants at different growth conditions (no stress and water deficit). Expression of PT genes was not influenced by M x S interaction. In detail, although statistical analysis showed an effect of the S variable only for *LePT2,* both *LePT1* and *LePT2* changed their expression under water deficit, but with an opposite trend (**Figures 2A,B** and **Supplementary Table S3**). *LePT1* expression in fact increased under water deficit with the highest value in RinSS, while *LePT2* transcripts progressively decreased with the water deficit. Among the mycorrhizal-inducible PT genes (*LePT3*, *LePT4*, and *LePT5*), *LePT3* resulted to be overexpressed in RinNS roots, while decreased under water stress mainly in AM-colonized

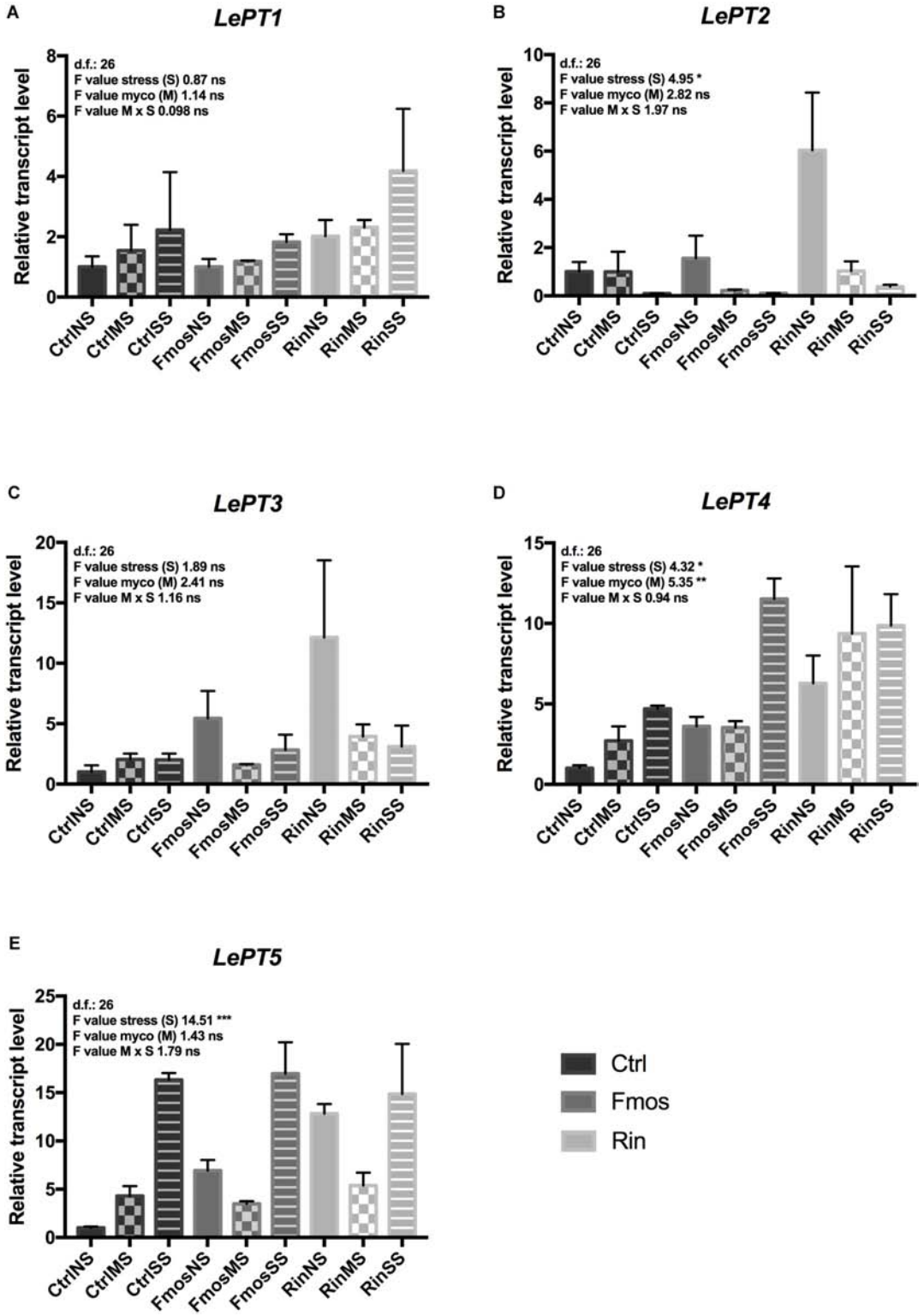


FIGURE 2 | Expression changes of tomato phosphate transporter (PT) genes in roots of AM– and AM+ plants upon irrigation (NS), moderate water stress (MS), and severe water stress (SS) conditions. RT-qPCR analysis of **(A)** *LePT1*, **(B)** *LePT2*, **(C)** *LePT3*, **(D)** *LePT4*, and **(E)** *LePT5*. Lower letters denotes significant differences attested by Tukey HSD test ($P < 0.05$). All data are expressed as mean ± SE ($n = 3$). ns, *, **, ***: non-significant or significant at $P \leq 0.05$, $P \leq 0.01$, and $P \leq 0.001$, respectively. Different letters above the bars indicate significant differences according to Tukey HSD test ($P \leq 0.05$), considering S × M interaction. Analysis of variance on the single variables is reported in **Supplementary Table S3**.

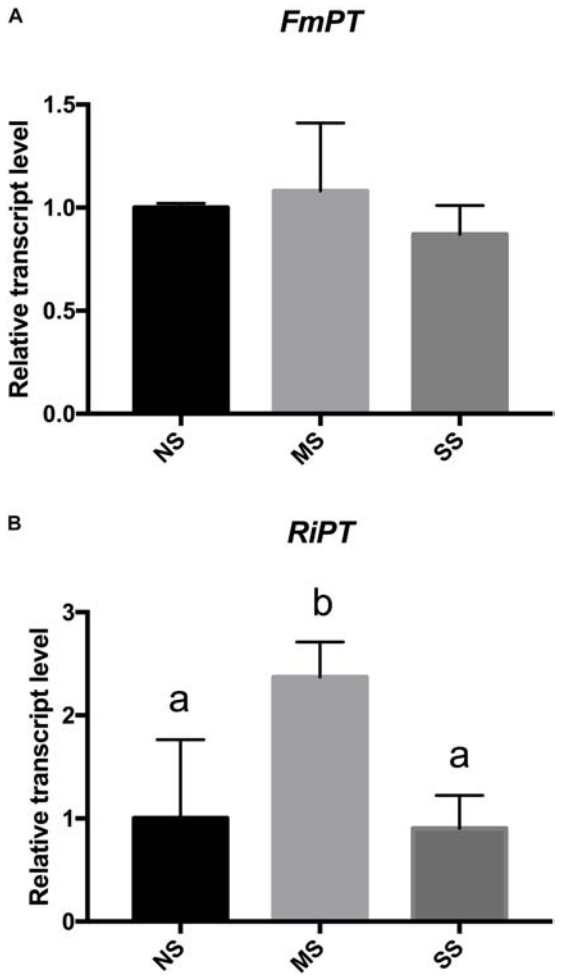


FIGURE 3 | Expression changes of fungal phosphate transporter (PT) genes in AM+ roots upon irrigation (NS), moderate water stress (MS), and severe water stress (SS) conditions. RT-qPCR analysis of **(A)** *F. mosseae* PT (*FmPT*) and **(B)** *R. intraradices* PT (*RiPT*) genes, respectively. Lower letters denotes significant differences attested by Tukey HSD test ($P < 0.05$). Data are expressed as mean ± SE ($n = 3$).

plants (**Figure 2C**). *LePT4* resulted to be influenced by S and M, while *LePT5* only by S (**Figures 2D,E** and **Supplementary Table S3**). In detail, these two last transporters showed a similar expression trend, with increased levels in SS plants (**Figures 2D,E**) with respect to the other treatments (NS and MS). However, *LePT4* resulted overexpressed in RinSS plants, while *LePT5* was in FmosSS ones, suggesting a species-specificity effect. From the fungal side, *F. mosseae* PT gene showed lower expression levels than *R. intraradices* PT in all the considered conditions (**Figures 3A,B**). Under water deficit, the expression of these fungal genes was not significantly affected, except for *RiPT* that is significantly up-regulated in MS (**Figure 3B**).

Target Metabolomics

The target leaf VOCs present in NS and SS plants in the presence or in the absence of either *F. mosseae* or *R. intraradices* have been determined and quantified. The main VOCs, *trans*-2-hexenale, methyl salicylate, eugenol, α-phellandrene and β-phellandrene increased in SS, mainly in the presence of the AM fungus *F. mosseae*, in agreement with statistical analysis that showed a highly significant influence of both S and M variables for all the considered VOCs (**Table 1**). Differently, the S × M interaction resulted to be significant for all the metabolites with the exception of *trans*-2-hexenal (**Table 1**).

Insect Performance and VOC Emission

Root colonization by *R. intraradices* significantly improved the development of *Macrosiphum euphorbiae* on tomato in both conditions tested (NS and WS). When aphid survival curves were calculated, survival probability proved to be higher on colonized plants (**Figure 4**, black lines, log-rank: colonized vs. non-colonized plants, $P = 0.015$). Conversely, water stress (dashed lines) did not affect the development of the aphids (**Figure 4**, log-rank: WS vs. NS, $P = 0.87$).

Root colonization by *R. intraradices* affected the foraging behavior of *A. ervi* in almost all conditions tested (**Figure 5**). Oriented flights significantly increased by root colonization in control, aphid infested and double stressed colonized plants. The same pattern was recorded for the landings on the source with the exception of control plants where root colonization did not affect parasitoid response. The presence of *R. intraradices* did not enhance the foraging behavior of *A. ervi* in condition of water stress.

Several of the collected volatiles were significantly affected by the presence of *R. intraradices* mainly in stressed plants (**Table 2**, **Figure 6**, and **Supplementary Table S4**). The DCA analysis separate plots on the basis of different treatments (**Figure 6** and **Supplementary Table S4**). Distance between centroids (**Supplementary Table S4** and **Figure 6**) indicated that volatile blends differ significantly in all treatments expect for (WS vs. Aph) and in almost all their combinations (double and triple, **Supplementary Table S4**). Whilst in non-stressed plants an increase was recorded in colonized plants in respect to non-colonized ones, in double stressed plants the AM fungus affected the VOCs emission with a significant decrease of most of volatiles, with the exception of methyl salicylate emission that increased under double stress conditions with respect to all other treatments (**Table 2**). Conversely, three terpenes (α-terpinene, α-phellandrene and β-phellandrene) were released at about half rate by *R. intraradices* colonized plants under double stress in respect to non-colonized ones.

DISCUSSION

Although several papers were dedicated to describe the effect of AM symbiosis on tomato response to water deficit (Dell'Amico et al., 2002; Subramanian et al., 2006; Aroca et al., 2008; Wang et al., 2014; Chitarra et al., 2016; Ruiz-Lozano et al., 2016; Rivero et al., 2018), here we have focused on some specific aspects less considered so far. In this study, we have examined the impact of AM symbiosis on the tomato response to water deficit considering some belowground traits

TABLE 1 | Quantification of VOC products in tomato leaves.

Source of variance	Trans-2-hexenal	Methyl salicylate	Eugenol	α-Phellandrene	β-Phellandrene
Stress	***	***	***	***	***
Myco	***	***	***	***	***
Stress * myco	ns	*	*	***	***
Stress					
NS	131.97 ± 47.41a	0.89 ± 0.55a	0.41 ± 0.06a	0.06 ± 0.10a	0.92 ± 0.76a
SS	160.81 ± 47.06b	1.41 ± 0.45b	0.52 ± 0.08b	0.16 ± 0.17b	2.00 ± 1.67b
Myco					
CTRL	127.61 ± 23.70a	1.09 ± 0.47a	0.44 ± 0.09a	0.05 ± 0.04a	0.80 ± 0.30a
Fmos	183.19 ± 64.00b	1.48 ± 0.74b	0.51 ± 0.11b	0.25 ± 0.17b	2.72 ± 1.77b
Rin	126.29 ± 20.32a	0.89 ± 0.17a	0.45 ± 0.06a	0.02 ± 0.02a	0.79 ± 0.22a
Stress * myco					
CTRLNS	117.17 ± 16.08	0.69 ± 0.08a	0.37 ± 0.03a	0.04 ± 0.01a	0.58 ± 0.17a
CTRLSS	138.05 ± 26.38	1.499 ± 0.31bc	0.50 ± 0.08c	0.08 ± 0.05ab	1.01 ± 0.25ab
FmosNS	157.54 ± 76.79	1.19 ± 0.96ab	0.45 ± 0.06ab	0.23 ± 0.13b	1.42 ± 1.25ab
FmosSS	210.75 ± 21.96	1.82 ± 0.20c	0.60 ± 0.07d	0.38 ± 0.09c	4.15 ± 0.96ab
RinNS	125.54 ± 20.98	0.87 ± 0.19a	0.42 ± 0.06ab	0.07 ± 0.003a	0.75 ± 0.13b
RinSS	120.87 ± 16.46	0.94 ± 0.19a	0.47 ± 0.06bc	0.04 ± 0.004a	0.77 ± 0.24c

*All data are expressed as mean ± SD. Ns, *, **, ***: non-significant or significant at $P \leq 0.05$, $P \leq 0.01$, and $P \leq 0.001$, respectively. Different letters within each column indicate significant differences according to Tukey HSD test ($P \leq 0.05$).*

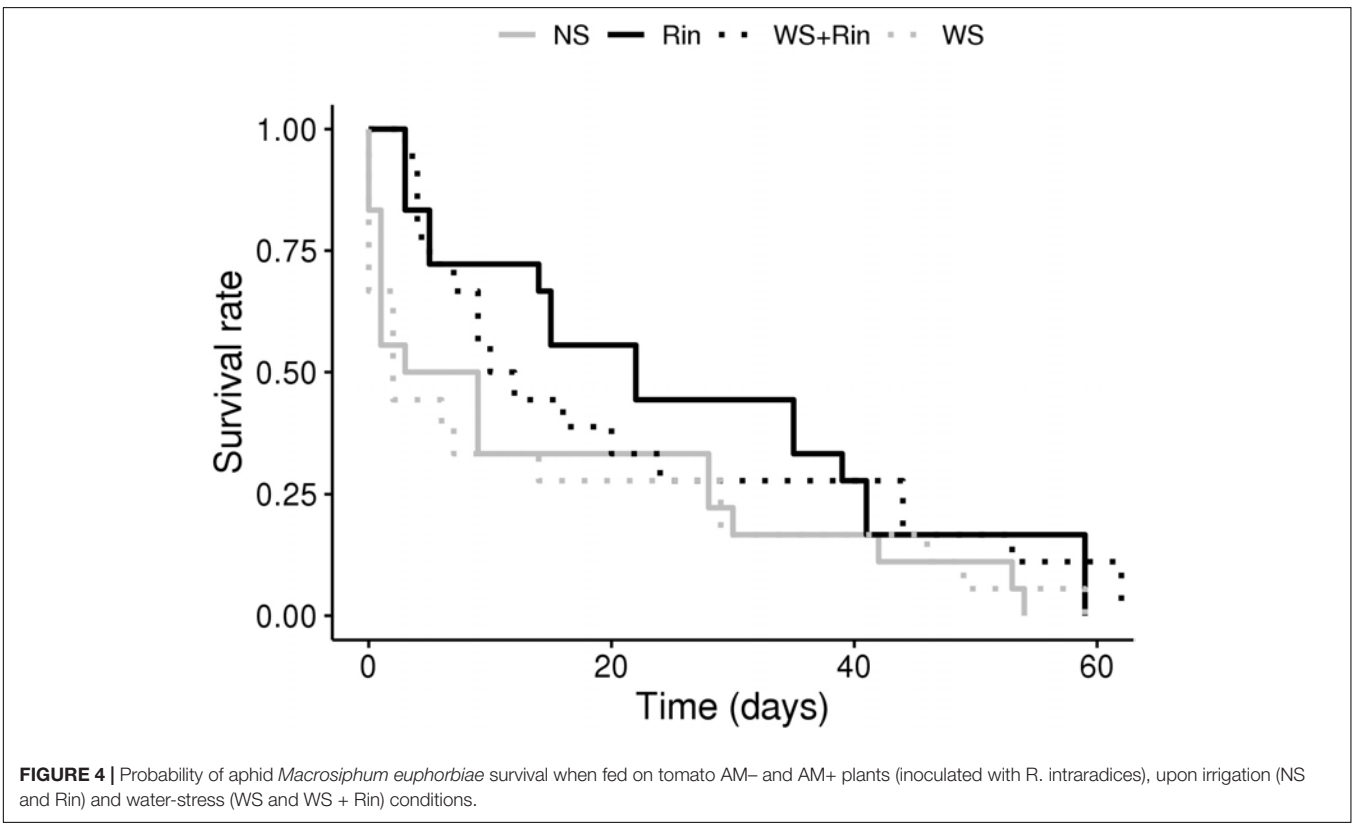


FIGURE 4 | Probability of aphid *Macrosiphum euphorbiae* survival when fed on tomato AM– and AM+ plants (inoculated with R. intraradices), upon irrigation (NS and Rin) and water-stress (WS and WS + Rin) conditions.

related to symbiosis, i.e., the expression of Pi transporter genes, and some aboveground responses, in terms of plant performance traits and changes in metabolites having a role in tolerance/defense. Two different AM fungal species have been used, and results here presented confirmed a species-specific impact on belowground/aboveground interactions in tomato. Previous works already showed the species-specificity of the interactions between plants and AM fungi under stressed conditions (Chitarra et al., 2016; Quiroga et al., 2017; Pollastri et al., 2018). More recently, Rivero et al. (2018) reported an untargeted metabolomic analysis in tomato roots colonized by three AM fungi of different genera, to verify their impact on

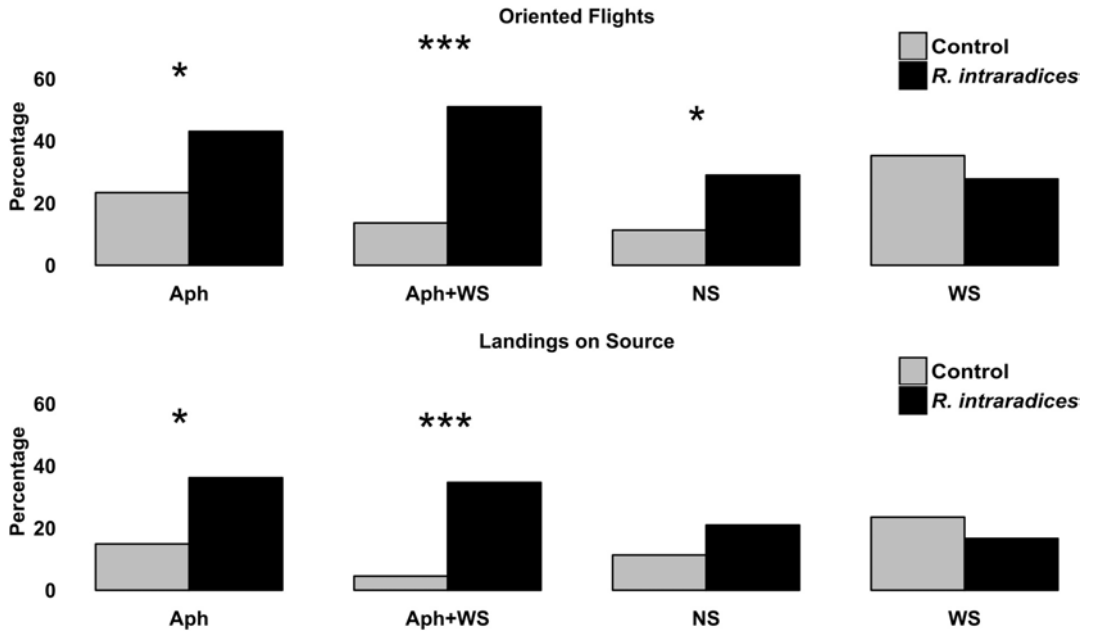


FIGURE 5 | Flight behavior (oriented flights and landing on source) of *Aphidius ervi* females (%) toward tomato plants inoculated with *R. intraradices*, infested by aphids, their combination and relative control. Asterisks assigned by *G*-test for independence (*$P < 0.05$ and ***$P < 0.001$).

tomato tolerance to drought or salt stress. Plant growth responses were also considered and, overall, results showed that whilst some responses were common to all AM fungi tested, others were specifically related to single isolates. The common effect of all AM fungal species tested was a higher biomass (both above- and belowground) in respect to non-colonized plants, correlated with the degree of the stress. Here, tomato plants inoculated with *R. intraradices* showed higher internodes/height ratios, calculated as a measure of plant growth, under water deficit with respect to the other conditions. This result suggests a positive influence of this isolates in regimes of water deficit, probably due to a more compact plant architecture, and thus less subject to water dispersion. As confirmed by statistical analysis (**Supplementary Table S3**), no significant impact on the considered growth parameters has been recorded in tomato plants colonized by *F. mosseae*. The discrepancy with the paper by Rivero et al. (2018) could be due to the different growth conditions and most probably to a different tomato cultivar. A different impact of the two fungi on plant performance under water deficit can be also highlighted, with *R. intraradices* that leads to higher iWUE, as already reported in Chitarra et al. (2016). Overall, these results confirmed the importance to produce *ad hoc* formulates of AM fungi in relation to a specific plant species/cultivar and environmental condition, for a better exploitation/protection in the field (Berruti et al., 2013, 2015).

Two AM Fungal Species Differently Affect Pi Uptake

Limited water resources and increasing soil salinity affect crop performance and productivity worldwide. These abiotic factors can be also accompanied by the low availability of many mineral nutrients including phosphorous (P) (Grattan and Grieve, 1999) and by the inhibition of mineral nutrient uptake and translocation (Wang et al., 2017). P fertilization can increase stress tolerance and productivity in several plant species and it appears mandatory to understand the mechanism/s of Pi acquisition and utilization under salinity and drought, to improve PUE and consequently crop productivity under abiotic stresses (Sawers et al., 2017; Wang et al., 2017). AM symbiosis promotes an improved nutritional status of the host plant (Smith and Smith, 2012), particularly under low nutrient availability. The success in nature of this symbiosis, known for more than 80% terrestrial plants, stays on the bidirectional nutritional exchanges, where the fungus supplies the plants with mineral nutrients and, in turn, receives carbon compounds (Balestrini and Lumini, 2018). Pi uptake, transfer and delivery have been largely investigated in AM roots, resulting in the characterization of a symbiotic Pi uptake pathway (Bucher, 2007; Javot et al., 2007; Smith and Smith, 2011). Particularly, PHT1 transporters, which are mainly involved in Pi uptake from soil and translocation, have been reported to be involved in the acquisition of Pi from AM (Glassop et al., 2005; Javot et al., 2007; Tamura et al., 2012; Yang et al., 2012; Duan et al., 2015; Liu et al., 2016; Tian et al., 2017; Liu et al., 2018). In tomato, eight PHT1 genes are expressed in the roots (Chen et al., 2014) and mycorrhizal-induced PT genes have been identified (Nagy et al., 2005, 2009). Different PT genes can be differentially regulated by drought. For example, several genes encoding Pi transporters from poplar and apple were found to be regulated by drought stress and Pi levels. These genes, especially those up-regulated by drought stress at low Pi level, might contribute to drought tolerance of crops in Pi-limited soils (Zhang et al., 2016).

TABLE 2 | VOCs released by tomato plants that were not stressed (NS), aphid-attacked (Aph), water-stressed (WS), and exposed to both water stress and aphid infestation (WS + Aph).

	Mean value of VOC (ng g^{-1} fr wt) ± SE								Three way ANOVA effects						
	NS	Rin	Aph	Rin + Aph	WS	WS + Rin	WS + Aph	WS + Rin + Aph	Rin (1)	Aph (2)	WS (3)	1 × 2	1 × 3	2 × 3	1 × 2 × 3
2,4-Dimethyl-1-heptene	242.2 ± 26.2	353.3 ± 29.8	285.7 ± 30.6	392.7 ± 18.9	258.2 ± 41.7	276.7 ± 69.5	414.8 ± 35	236.2 ± 44.8					**		
Ethylbenzene	57.2 ± 3.8	74 ± 7.8	72.5 ± 3.4	73.2 ± 4.4	64.2 ± 8.4	75.3 ± 9.5	77.8 ± 7.0	53.3 ± 9.9				*			
p-Xylene	242.2 ± 26.2	353.3 ± 29.8	285.7 ± 30.6	392.7 ± 18.9	258.2 ± 41.7	276.7 ± 69.5	414.8 ± 35	236.2 ± 44.8				*			
o-Xylene	99.7 ± 9.2	144.8 ± 15.6	152.3 ± 10.6	124.8 ± 10.7	114.8 ± 26.6	120.3 ± 24.0	95.8 ± 26.5	100.7 ± 23.3							
α-pinene	134.8 ± 77.3	186 ± 106	236.3 ± 143.7	88.2 ± 7.7	158.5 ± 36	148.3 ± 36.9	153.2 ± 29.8	70.7 ± 18.2							
2-Ethylhexanal	24.2 ± 4.2	34.5 ± 3.2	19.5 ± 2.5	37.7 ± 3.5	17.7 ± 3.5	18 ± 3.8	36.8 ± 6.5	28 ± 9.1					*		
4-Methylnonane	15.8 ± 2.3	39.8 ± 8.5	23.3 ± 3.6	28.7 ± 5.9	14.2 ± 3.1	15.2 ± 8.1	33.5 ± 4.9	12 ± 4.8			*		**		
Benzene, 1-ethyl-3-methyl-	24.2 ± 4.0	25.7 ± 9.2	11.8 ± 5.6	8.3 ± 5.3	2.7 ± 2.7	4.2 ± 4.2	17.5 ± 8.2	22.7 ± 7.6						**	
Benzaldehyde	72.8 ± 4.1	98.5 ± 6.4	104 ± 7.4	96.5 ± 22.2	104.7 ± 18.2	111 ± 19.8	135 ± 12.3	80.3 ± 17.8				*			
1-Decene	151 ± 7.3	200.8 ± 7.8	203.8 ± 15.6	238.8 ± 27.3	183.2 ± 16.4	222.2 ± 40.5	254.8 ± 27.5	174.5 ± 43.6							
Benzene, 1,3,5-trimethyl-	89.3 ± 7.3	113.7 ± 8.6	93.3 ± 3.9	157.2 ± 50.8	18.5 ± 11.9	55.8 ± 29.7	94.7 ± 20.5	86.8 ± 20.1		**	***			*	
α-Terpinene	185.7 ± 15.5	452.5 ± 36.3	874.8 ± 133.3	603.2 ± 196.1	1387.8 ± 288.5	1583.7 ± 442.4	1155.3 ± 248.4	490.3 ± 179.9			***	**		***	
α-phellandrene	84.3 ± 3.5	128.8 ± 13	149.7 ± 33.7	154.5 ± 15.6	50 ± 26.9	295.7 ± 156.7	241.8 ± 61.2	101 ± 24.7				*			
1,4-dichlorobenzene	24.7 ± 1.3	33.7 ± 7.9	53.3 ± 6.2	92.7 ± 30.9	79.7 ± 25.0	108.3 ± 48.1	100.2 ± 26.3	36 ± 8.4						*	
Limonene	384.7 ± 50.9	880 ± 67.6	1435.8 ± 198.4	1232 ± 406.7	2646 ± 612.7	2704.5 ± 555.7	2167.3 ± 269.2	1381 ± 357.2			***	*		**	
β-phellandrene	233.3 ± 49.2	511 ± 46.3	1012.7 ± 97	800 ± 197.3	1407.7 ± 332.6	1409.7 ± 361.8	1424 ± 233.5	579.2 ± 162.8			***	**		***	
Acetophenone	27.7 ± 1.9	39.3 ± 3.0	38.5 ± 1.4	47.7 ± 5.7	40.8 ± 7.8	48.2 ± 12.4	56.8 ± 4.2	30.8 ± 5.2					*		
p-Tolualdehyde	151.7 ± 17.1	218.5 ± 26.2	131.7 ± 23.5	236 ± 32.8	178 ± 28.2	204.7 ± 47.3	334.3 ± 139.7	84.2 ± 22.6					**		*
Methyl benzoate	21.5 ± 1.9	35.3 ± 6.7	25.5 ± 3.0	30 ± 4.7	24 ± 5.8	37.3 ± 6.8	41.8 ± 3.8	27.7 ± 4.0				**			
Nonanal	60 ± 6.0	98.7 ± 9.1	92.5 ± 8.8	102.7 ± 17.4	89 ± 13.7	131.3 ± 25.2	164.2 ± 12.4	101.2 ± 19.7			*	**			
Camphor	19.7 ± 2.0	21.3 ± 4.7	23.7 ± 1.2	21.7 ± 4.8	18.3 ± 3.3	32.3 ± 5.3	46.8 ± 10.4	64 ± 40.9							
(Z)-3-Nonen-1-ol	2.2 ± 2.2	18.5 ± 0.4	15.3 ± 4.9	15.2 ± 4.8	9.8 ± 4.4	20.5 ± 7.2	25.3 ± 1.5	9.5 ± 6.0				**	*		
Naphthalene	410.3 ± 21.9	490.2 ± 21.1	473.2 ± 50.5	579 ± 52.3	350 ± 81.6	549.2 ± 63.9	712.3 ± 37.6	456.3 ± 100.3		*		*			**
Methyl salicylate	15.2 ± 2.8	30.2 ± 1.5	105 ± 2.9	138.3 ± 7.6	41 ± 3.2	65.5 ± 8.3	188.7 ± 5.4	236.2 ± 14.5	***	***	***			*	
1-dodecene	1028.5 ± 49.1	1184.7 ± 48.3	1192.8 ± 117.8	1337.8 ± 189.3	1072.8 ± 71.6	1288.2 ± 174.3	1496.2 ± 100.5	885.5 ± 281.8							
Decanal	151 ± 7.3	200.8 ± 7.8	203.8 ± 15.6	238.8 ± 27.3	183.2 ± 16.4	222.2 ± 40.5	254.8 ± 27.5	174.5 ± 43.6							
2,5-Dimethylbenzaldehyde	13 ± 2.8	26 ± 2.1	21.5 ± 2.7	23 ± 3.5	21.3 ± 4.5	31.8 ± 5.0	41.5 ± 2.2	21.3 ± 7.7				**			
Benzothiazole	35.2 ± 8.8	64.7 ± 6.5	51 ± 11.9	76.2 ± 9.7	50.2 ± 10.1	51 ± 13.3	85.0 ± 7.1	50.2 ± 15.0					**		
Cumin aldehyde	3.7 ± 3.7	26.5 ± 8.0	8.5 ± 5.7	13.3 ± 9.2	36.3 ± 12.0	56.7 ± 12.0	40.5 ± 11.9	13.5 ± 6.4			***	*			
β-caryophyllene	93.3 ± 15.5	157 ± 33.8	196 ± 31.3	167.8 ± 45.3	156.2 ± 41.4	160.2 ± 15.1	190.8 ± 45.5	119 ± 41.6				*			
O-cymene	0 ± 0	13.3 ± 8.5	0 ± 0	0 ± 0	0 ± 0	0 ± 0	0 ± 0	0 ± 0							

*Each experiment was carried out using uninoculated (NS, Aph, WS, and WS + Aph) and inoculated with R. intraradices (Rin, Rin + Aph, WS + Rin, and WS + Rin + Aph) plants. Asterisks indicate statistically significant effects tested by ANOVA (*P < 0.05, **P < 0.01, ***P < 0.001).*

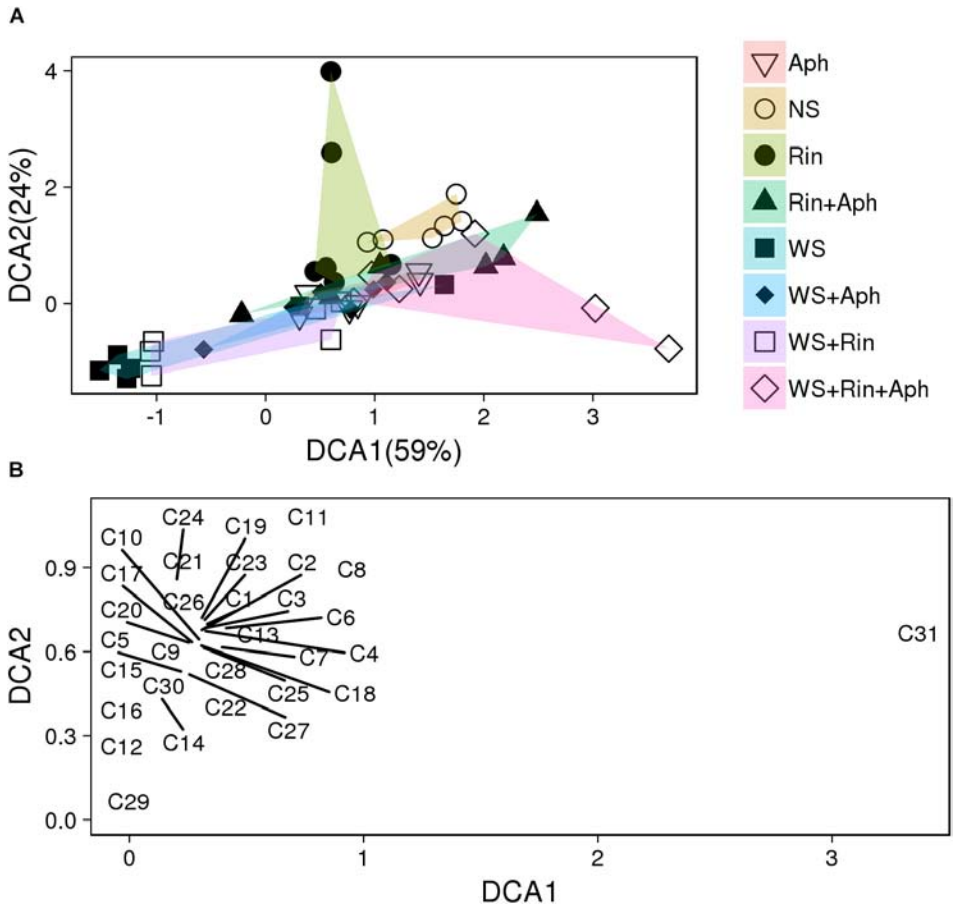


FIGURE 6 | Detrended Correspondence Analysis (DCA) score **(A)** and loading **(B)** plots of the first two components based on volatiles emitted tomato plants that were not stressed (NS), aphid-attacked (Aph), water-stressed (WS), and exposed to both water stress and aphid infestation (WS + Aph). **(A)** Each experiment was carried out using uninoculated (NS, Aph, WS, and WS + Aph) and inoculated with *R. intraradices* (Rin, Rin + Aph, WS + Rin, and WS + Rin + Aph) plants. In brackets the percentage of variation explained is indicated. Multivariate analysis of variance by permutation was used to test for significant differences between group centroids (see **Supplementary Table S4**). **(B)** Loading plot of the DCA first two components, showing the contribution of each VOC (C1–C31, listed in **Table 2**) to the model.

Here, new information on the expression of five tomato PT genes in presence/absence of the AM fungus and in response to two different water deficit conditions (moderate and severe) is reported. As reported in Chitarra et al. (2016), root colonization seemed not to be strongly affected by a severe water stress in this tomato cultivar, independently of the AM fungal species considered. It is worth noting that the proportion of AM root colonization was greater for *R. intraradices*, while the percentage of arbuscules in the colonized portion was significantly greater for *F. mosseae*, both in NS and SS conditions, suggesting a different colonization strategy adopted by the two fungi. Looking at the regulation of PT genes, a significant up-regulation for the AM-inducible *LePT3*, *LePT4,* and *LePT5* has been observed in well-watered conditions, in agreement with previous data (Nagy et al., 2005, 2009; Balestrini et al., 2007). Under water stress, our results showed a different regulation of the considered PT genes. Particularly, it is worth noting the opposite trend for the two genes involved in the direct Pi uptake from soil (*LePT1* and *LePT2*), independently from the presence of the AM fungus. The poplar PHT1;2 has been already reported as induced by drought stress independently from Pi level (Zhang et al., 2016). Among the AM-inducible PT genes, *LePT3* seems not involved in response to water deficit at least in non-colonized plants as indicated by the lowest transcript level recorded in NS, whilst in colonized plants its expression fell independently by the stress level. *LePT4* and *LePT5* transcript levels both increased under water deficit with highest values in FmosSS, in agreement with the higher P content found in the leaves of these plants in respect to control ones. Overall results suggest a major role played by *LePT2* and *LePT4* in promoting tolerance to water deficit, particularly under a severe condition, although this is dependent from the fungal species. Since PT gene induction is also related to Pi-starvation, it is important to note that, in our experimental system, MS plants still received some nutrients during watering, while the nutritional status of SS plants did not received any external inputs even if the P content in leaves does not show a starvation level. We can then speculate that the transcript level for *LePT5* is more affected by a lower Pi content in soil than to water deficit. In

fact, it has been already reported that the expression of some PT genes under drought can be Pi-dependent (Zhang et al., 2016). However, we cannot exclude a relation between our results on PT gene expression and the root architecture modification, which might happen under the different treatments. For example, it has been reported that in legumes phosphate starvation induced a higher number of lateral roots and *PT4* genes, homologs of *LePT4*, can play a role in root tips, creating a link among Pi-perception, root branching, and Pi-signaling mechanisms (Volpe et al., 2013, 2016).

The different behavior of the two AM fungal species tested further highlights the species-specificity of these complex interactions, regulating their final outcome, depending on both fungal and plant species considered (Sawers et al., 2017).

AM Symbiosis Impacts Aphid Survivorship and VOCs Under Water Deficit and Tomato Response to Multiple Stresses

Root-associated microorganisms can alter the development of phytophagous insects in different ways. They can promote defensive responses that hamper the subsequent development of the invasive insect (Guerrieri et al., 2004) or, conversely, by improving plant quality that can enhance plant infestation (Battaglia et al., 2013). In our experiment, *R. intraradices* promoted the survival of *M. euphorbiae* in both control and water stressed plant. The null impact of water stress on aphid survival in *R. intraradices* colonized plants indirectly demonstrates the role of this AM species on plant tolerance to water deficit. Indeed, while water deficit should have had a deep impact on the survival of the sap feeding insects, by reducing the feeding time on sieve elements (Nachappa et al., 2016), this was not recorded for the aphid *M. euphorbiae* in our tests.

Plants synthetize and emit a large variety of VOC that function as important protective and signaling molecules (Guerrieri, 2016). Both biotic and abiotic stresses can induce plant volatile emission (Loreto and Schnitzler, 2010; Dong et al., 2016; Catola et al., 2018), including emissions of lipoxygenase (LOX) pathway products (various C6 aldehydes such *trans*-2-hexenale), shikimate pathway products (e.g., methyl salicylate), specific mono- and sesquiterpenes (such as β-phellandrene) and methanol (Niinemets, 2010; Dong et al., 2016). Additionally, the rate of emissions induced by a specific stress depends on genotype stress tolerance, timing, duration and severity of the stress (Niinemets, 2010; Copolovici et al., 2011; Niederbacher et al., 2015). Our target metabolomics analysis was focused on the impact of AM symbiosis on plant tolerance to a severe water stress, to evaluate an extreme condition that has been still less considered. The data underlined that the SS has an impact on the VOC production, demonstrating that plants, both non-colonized and *F. mosseae*-colonized, showed the highest concentration of the main VOC products with respect to the NS plants. Furthermore, a different effect of the AM fungal species was again evident, with *R. intraradices*-inoculated plants that showed similar level of all targeted metabolites in comparison with NS plants, suggesting an enhanced tolerance to this type of stress promoted by this AM fungal species. In tomato, a vast array of VOCs was found to be emitted under biotic (Digilio et al., 2010) and abiotic stresses, including water deficit (Catola et al., 2018), flooding (Copolovici and Niinemets, 2010), cold and heat (Copolovici et al., 2012; Kask et al., 2016). In our experimental study, where necessarily a MS has been used to permit the combined test with aphids, the emission of a typical tomato VOC bouquet under NS and water deficit conditions was determined. A change in VOC emission was particularly observed between non-colonized and *R. intraradices*-colonized plants both in well-watered condition and under a double stress (water deficit plus the aphid attack). However, the trend emission was opposite between NS and double stress plants. In NS plants colonized by *R. intraradices*, an increase in the release of VOC has been recorded particularly for the methyl salicylate. Significant changes in VOC emission has been registered for the single stress conditions in AM-colonized plants, while the presence of the AM fungus in double stressed plants lead to a decreasing trend in the emission of several compounds with one exception: methyl salicylate. This result strongly supported the behavioral observations given that methyl salicylate plays a major role in the attraction of *A. ervi* (Sasso et al., 2007), being detected by this parasitoid at antennal level at a concentration as low as 0.01 gr/ml (Sasso et al., 2009). On the other hand, the strong reduction in the release of the terpenes in double stressed plants colonized by *R. intraradices* in respect to non-colonized ones could be due to a shift toward the production of non-volatile compounds (essential isoprenoids such as carotenoids) in respect to VOC biosynthesis (not essential isoprenoids). This has been already reported by Asensio et al. (2012) that found that water stressed colonized plants plus exogenous jasmonic acid (JA) treatment (mimicking a wound) have a higher level of carotenoids, which have an important role in photosynthesis, with respect to non-colonized plants. The reduction of monoterpenes recorded in our experiment is also in line with the data of these same authors that explained such a reduction with the high demand of carbon by the AM fungus, particularly under combined stress (drought + JA treatment) in respect to unstressed and non-colonized plant (Asensio et al., 2012). However, a correlation between the VOC emission in colonized plants under double stress and a possible better P availability in the same plants, cannot be excluded, considering that nutritional status might have an impact on plant isoprenoid production (Asensio et al., 2012).

The effect of *R. intraradices* on plant resistance toward aphids resulted bifaceted. AM-colonized plants represented a better substrate for the survival aphid *M. euphorbiae* in respect to non-colonized ones thus reducing plant direct resistance toward insect pests. This negative effect on plant fitness was largely compensated by the significant enhancement of indirect defense as represented by the significant higher attraction of the parasitoid *A. ervi* recorded for colonized plants in respect to non-colonized ones. The increased level of indirect defense persisted in *R. intraradices* colonized plants also in conditions of double stress and was supported by the release of key attractive compounds (methyl salicylate).

CONCLUSION

Our results suggest that the two AM fungi can trigger different adaptation strategies against environmental stresses, with *F. mosseae* that seems more effective on VOC production and *R. intraradices* on the considered plant performance traits, e.g., leading to a significant higher water use efficiency under a severe water stress. Additionally, *R. intraradices* was demonstrated to be effective on response to combined abiotic and biotic stress, the latter in terms of attractiveness toward the natural enemies of aphids. However, although AM fungi enhance crop tolerance to environmental stresses, the exploitation and the practical application in the field of these symbionts require a thorough identification of the mechanisms involved in nutrient transfer, the metabolic pathways induced by single and multiple stresses and the physiological mechanisms leading to improved tolerance. Our results well fit with this aim, adding new tiles to the pieces of information already present about the mechanisms involved in the tolerance to water and biotic stresses in tomato during AM symbiosis, and in nutrition aspects such as Pi uptake. Overall, our results confirmed the importance to develop mixed AM-based products, depending on the environmental conditions to be faced, choosing the right symbiotic partner/s (i.e., plant cultivar, AM fungus) that lead to different outcomes in terms of plant tolerance to stress. This is particularly urgent in a scenario of climate change as characterized by a progressive lack of water that combines, in the field, with other abiotic (e.g., nutritional availability and salinity) and biotic (e.g., insects and pathogens) stresses. However, considering the species-specificity in affecting tolerance/resistance traits, it is relevant to test individually an AM species/isolate before to produce a mixed inoculum, to avoid a null effect on plant response.

AUTHOR CONTRIBUTIONS

RB, EG, and BM conceived and designed the experiment. VV and WC performed the molecular analyses and the eco-physiological measurements. PC performed the multiple stress experiment and VOC collection. MV performed the phosphate determination. WC and PC performed the statistical analysis for all the data. PB participated to the metabolite extraction. GM, GP, CDS, and BM performed the target metabolomics experiment. EG participated to the multiple stress experiment and analyzed the VOC data. RB prepared the plants and performed the molecular analyses with VV. RB and VV wrote the manuscript with the contribution of EG and WC. All authors read and approved the manuscript.

FUNDING

This research was funded by the AQUA project (Progetto Premiale CNR).

SUPPLEMENTARY MATERIAL

The Supplementary Material for this article can be found online at: https://www.frontiersin.org/articles/10.3389/fpls.2018.01480/full#supplementary-material

FIGURE S1 | (A–C) Biometric parameters and **(D)** chlorophyll content index (CCI) and of AM− and AM+ tomato plants upon unstressed and water stressed (MS and SS) conditions. All data are expressed as mean ± SD. ns, *, **, ***: non-significant or significant at $P \leq 0.05$, $P \leq 0.01$, and $P \leq 0.001$, respectively. Different letters above the bars indicate significant differences according to Tukey HSD test ($P \leq 0.05$), considering S × M interaction. Analysis of variance on the single variables is reported in **Supplementary Table S3**.

TABLE S1 | List of the oligonucleotides used in this study.

TABLE S2 | Retention time and principal ion of each compound.

TABLE S3 | Analysis of variance on single variables referred to **Figures 1**, **2** and **Supplementary Figure S1**.

TABLE S4 | Results of pairwise multivariate analysis of variance by permutation among VOCs released by tomato plants (see **Table 2** for treatments). Significant values are in bold.

REFERENCES

Aroca, R., Del Mar Alguacil, M., Vernieri, P., and Ruiz-Lozano, J. M. (2008). Plant responses to drought stress and exogenous ABA application are modulated differently by mycorrhization in tomato and an ABA-deficient mutant (sitiens). *Microb. Ecol.* 56, 704–719. doi: 10.1007/s00248-008-9390-y

Asensio, D., Rapparini, F., and Penuelas, J. (2012). AM fungi root colonization increases the production of essential isoprenoids vs. nonessential isoprenoids especially under drought stress conditions or after jasmonic acid application. *Phytochemistry* 77, 149–161. doi: 10.1016/j.phytochem.2011.12.012

Atkinson, N. J., and Urwin, P. E. (2012). The interaction of plant biotic and abiotic stresses: from genes to the field. *J. Exp. Bot.* 63, 3523–3543. doi: 10.1093/jxb/ers100

Babikova, Z., Gilbert, L., Bruce, T., Dewhirst, S. Y., Pickett, J. A., and Johnson, D. (2014). Arbuscular mycorrhizal fungi and aphids interact by changing host plant quality and volatile emission. *Funct. Ecol.* 28, 375–385. doi: 10.1111/1365-2435.12181

Babikova, Z., Gilbert, L., Bruce, T. J. A., Birkett, M., Caulfield, J. C., Woodcock, C., et al. (2013). Underground signals carried through common mycelial networks warn neighbouring plants of aphid attack. *Ecol. Lett.* 16, 835–843. doi: 10.1111/ele.12115

Bai, Y., Kissoudis, C., Yan, Z., Visser, R. G. F., and van der Linden, G. (2018). Plant behaviour under combined stress: tomato responses to combined salinity and pathogen stress. *Plant J.* 93, 781–793. doi: 10.1111/tpj.13800

Balestrini, R., Chitarra, W., Antoniou, C., Ruocco, M., and Fotopoulos, V. (2018). Improvement of plant performance under water deficit with the employment of biological and chemical priming agents. *J. Agric. Sci.* doi: 10.1017/S002185961800012

Balestrini, R., Gómez-Ariza, J., Lanfranco, L., and Bonfante, P. (2007). Laser microdissection reveals that transcripts for five plant and one fungal phosphate transporter genes are contemporaneously present in arbusculated cells. *Mol. Plant Microbe Interact.* 20, 1055–1062. doi: 10.1094/MPMI-20-9-1055

Balestrini, R., and Lumini, E. (2018). Focus on mycorrhizal symbioses. *Appl. Soil Ecol.* 123, 299–304. doi: 10.1016/j.apsoil.2017.09.001

Battaglia, D., Bossi, S., Cascone, P., Digilio, M. C., Prieto, J. D., Fanti, P., et al. (2013). Tomato below ground–above ground interactions: trichoderma longibrachiatum affects the performance of *Macrosiphum euphorbiae* and its natural antagonists. *Mol. Plant Microbe Interact.* 26, 1249–1256. doi: 10.1094/MPMI-02-13-0059-R

Berruti, A., Borriello, R., Della Beffa, M. T., Scariot, V., and Bianciotto, V. (2013). Application of nonspecific commercial AMF inocula results in poor

mycorrhization in *Camellia japonica* L. *Symbiosis* 61, 63–76. doi: 10.1007/s13199-013-0258-7

Berruti, A., Lumini, E., Balestrini, R., and Bianciotto, V. (2015). Arbuscular mycorrhizal fungi as natural biofertilizers: let's benefit from past successes. *Front. Microbol.* 6:1559. doi: 10.3389/fmicb.2015.01559

Bucher, M. (2007). Functional biology of plant phosphate uptake at root and mycorrhiza interfaces. Tansley review. *New Phytol.* 173, 11–26. doi: 10.1111/j.1469-8137.2006.01935.x

Catola, S., Centritto, M., Cascone, P., Ranieri, A., Loreto, F., Calamai, L., et al. (2018). Effects of single or combined water deficit and aphid attack on tomato volatile organic compound (VOC) emission and plant-plant communication. *Environ. Exp. Bot.* 153, 54–62. doi: 10.1016/j.envexpbot.2018.05.001

Chen, A., Chen, X., Wang, H., Liao, D., Gu, M., Qu, H., et al. (2014). Genome-wide investigation and expression analysis suggest diverse roles and genetic redundancy of Pht1 family genes in response to Pi deficiency in tomato. *BMC Plant Biol.* 14:61. doi: 10.1186/1471-2229-14-6

Chitarra, W., Pagliarani, C., Maserti, B., Lumini, E., Siciliano, I., Cascone, P., et al. (2016). Insights on the impact of arbuscular mycorrhizal symbiosis on tomato tolerance to water stress. *Plant Physiol.* 171, 1009–1023. doi: 10.1104/pp.16.00307

Comas, L. H., Becker, S. R., Cruz, V. M., Byrne, P. F., and Dierig, D. A. (2013). Root traits contributing to plant productivity under drought. *Front. Plant Sci.* 4:442. doi: 10.3389/fpls.2013.00442

Copolovici, L., Kännaste, A., Pazouki, L., and Niinemets, Ü. (2012). Emissions of green leaf volatiles and terpenoids from *Solanum lycopersicum* are quantitatively related to the severity of cold and heat shock treatments. *J. Plant Physiol.* 169, 664–672. doi: 10.1016/j.jplph.2011.12.019

Copolovici, L., Kännaste, A., Remmel, T., Vislap, V., and Niinemets, Ü. (2011). Volatile emissions from *Alnus glutionosa* induced by herbivory are quantitatively related to the extent of damage. *J. Chem. Ecol.* 37, 18–28. doi: 10.1007/s10886-010-9897-9

Copolovici, L., and Niinemets, Ü. (2010). Flooding induced emissions of volatile signalling compounds in three tree species with differing waterlogging tolerance. *Plant Cell Environ.* 33, 1582–1594. doi: 10.1111/j.1365-3040.2010.02166.x

Dai, A. (2011). Drought under global warming: a review. *Wires Clim. Chang.* 2, 45–65. doi: 10.1002/wcc.81

De Smet, I., Signora, L., Beeckman, T., Inzé, D., Foyer, C. H., and Zhang, H. (2003). An abscisic acid-sensitive checkpoint in lateral root development of *Arabidopsis*. *Plant J.* 33, 543–555. doi: 10.1046/j.1365-313X.2003.01652.x

Dell'Amico, J., Torrecillas, A., Rodríguez, P., Morte, A., and Sánchez-Blanco, M. J. (2002). Responses of tomato plants associated with the arbuscular mycorrhizal fungus *Glomus clarum* during drought and recovery. *J. Agric. Sci.* 138, 387–393. doi: 10.1017/S0021859602002101

Digilio, M. C., Cascone, P., Iodice, L., and Guerrieri, E. (2012). Interactions between tomato volatile organic compounds and aphid behaviour. *J. Plant Interact.* 7, 322–325. doi: 10.1080/17429145.2012.727104

Digilio, M. C., Corrado, G., Sasso, R., Coppola, V., Iodice, L., Pasquariello, M., et al. (2010). Molecular and chemical mechanisms involved in aphid resistance in cultivated tomato. *New Phytol.* 187, 1089–1101. doi: 10.1111/j.1469-8137.2010.03314.x

Dong, F., Fu, X., Watanabe, N., Su, X., and Yang, Z. (2016). Recent advances in the emission and functions of plant vegetative volatiles. *Molecules* 21:124. doi: 10.3390/molecules21020124

du Jardin, P. (2015). Plant biostimulants: definition, concept, main categories and regulation. *Sci. Hortic.* 196, 3–14. doi: 10.1016/j.scienta.2015.09.021

Duan, J., Tian, H., Drijber, R. A., and Gao, Y. (2015). Systemic and local regulation of phosphate and nitrogen transporter genes by arbuscular mycorrhizal fungi in roots of winter wheat (*Triticum aestivum* L.). *Plant Physiol. Biochem.* 96, 199–208. doi: 10.1016/j.plaphy.2015.08.006

Enstone, D. E., Peterson, C. A., and Ma, F. (2002). Root endodermis and exodermis: structure, function, and responses to the environment. *J. Plant Growth Regul.* 21, 335–351. doi: 10.1007/s00344-003-0002-2

Fusconi, A. (2014). Regulation of root morphogenesis in arbuscular mycorrhizae: what role do fungal exudates, phosphate, sugars and hormones play in lateral root formation? *Ann. Bot.* 113, 19–33. doi: 10.1093/aob/mct258

Glassop, D., Smith, S. E., and Smith, F. W. (2005). Cereal phosphate transporters associated with the mycorrhizal pathway of phosphate uptake into roots. *Planta* 222, 688–698. doi: 10.1007/s00425-005-0015-0

Grattan, S. R., and Grieve, C. M. (1999). Salinity-mineral nutrient relations in horticultural crops. *Sci. Hortic.* 78, 127–157. doi: 10.1016/S0304-4238(98)00192-7

Gray, S. B., and Brady, S. M. (2016). Plant developmental responses to climate change. *Dev. Biol.* 419, 64–77. doi: 10.1016/j.ydbio.2016.07.023

Guerrieri, E. (2016) "Who's listening to talking plants?" in *Deciphering Chemical Language of Plant Communication. Signaling and Communication in Plants,* eds J. Blande and R. Glinwood (New York, NY: Springer Publishing Company).

Guerrieri, E., Lingua, G., Digilio, M. C., Massa, N., and Berta, G. (2004). Do interactions between plant roots and the rhizosphere affect parasitoid behaviour? *Ecol. Entomol.* 29, 753–756. doi: 10.1111/j.0307-6946.2004.00644.x

Guerrieri, E., Poppy, G. M., Powell, W., Rao, R., and Pennacchio, F. (2002). Plant-to-plant communication mediating in flight orientation of *Aphidius ervi*. *J. Chem. Ecol.* 9, 1703–1715. doi: 10.1023/A:1020553531658

Gutjahr, C., Casieri, L., and Paszkowski, U. (2009). *Glomus intraradices* induces changes in root system architecture of rice independently of common symbiosis signaling. *New Phytol.* 184, 829–837. doi: 10.1111/j.1469-8137.2009.02839.x

Hewitt, E. J. (1966). *Sand and Water Culture Methods Used in the Study of Plant Nutrition*. Farnham Royal: Commonwealth Agricultural Bureaux.

Holopainen, J. K., and Gershenzon, J. (2010). Multiple stress factors and the emission of plant VOCs. *Trends Plant Sci.* 15, 176–184. doi: 10.1016/j.tplants.2010.01.006

Javot, H., Penmetsa, R. V., Terzaghi, N., Cook, D. R., and Harrison, M. J. (2007). A medicago truncatula phosphate transporter indispensable for the arbuscular mycorrhizal symbiosis. *Proc. Natl. Acad. Sci. U.S.A.* 104, 1720–1725. doi: 10.1073/pnas.0608136104

Kask, K., Kännaste, A., Talts, E., Copolovici, L., and Niinemets, Ü. (2016). How specialized volatiles respond to chronic and short-term physiological and shock heat stress in *Brassica nigra*. *Plant Cell Environ.* 39, 2027–2042. doi: 10.1111/pce.12775

Lenoir, I., Fontaine, J., and Sahraoui, L.-H. (2016). Arbuscular mycorrhizal fungal responses to abiotic stresses: a review. *Phytochemistry* 123, 4–15. doi: 10.1016/j.phytochem.2016.01.002

Liu, F., Xu, Y., Han, G., Wang, W., Li, X., and Cheng, B. (2018). Identification and functional characterization of a maize phosphate transporter induced by mycorrhiza formation. *Plant Cell Physiol.* 59, 1683–1694. doi: 10.1093/pcp/pcy094

Liu, F., Xu, Y. J., Jiang, H. H., Jiang, C. S., Du, Y. B., Gong C., et al. (2016). Systematic identification, evolution and expression analysis of the *Zea mays* PHT1 gene family reveals several new members involved in root colonization by arbuscular mycorrhizal fungi. *Int. J. Mol. Sci.* 17:E930. doi: 10.3390/ijms17060930

Loreto, F., and Schnitzler, J. P. (2010). Abiotic stresses and induced BVOCs. *Trends Plant Sci.* 15, 154–166. doi: 10.1016/j.tplants.2009.12.006

Nachappa, P., Culkin, C. T., Saya, P. M. II, Han, J., and Nalam, V. J. (2016). Water stress modulates soybean aphid performance, feeding behavior, and virus transmission in soybean. *Front. Plant Sci.* 7:552. doi: 10.3389/fpls.2016.00552

Nagy, R., Drissner, D., Amrhein, N., Jakobsen, I., and Bucher, M. (2009). Mycorrhizal phosphate uptake pathway in tomato is phosphorus-repressible and transcriptionally regulated. *New Phytol.* 181, 950–959. doi: 10.1111/j.1469-8137.2008.02721.x

Nagy, R., Karandashov, V., Chague, V., Kalinkevich, K., Tamasloukht, M., Xu, G., et al. (2005). The characterization of novel mycorrhiza-specific phosphate transporters from *Lycopersicon esculentum* and *Solanum tuberosum* uncovers functional redundancy in symbiotic phosphate transport in solanaceous species. *Plant J.* 42, 236–250. doi: 10.1111/j.1365-313X.2005.02364.x

Niederbacher, B., Winkler, J., and Schnitzler, J.-P. (2015). Volatile organic compounds as non-invasive markers for plant phenotyping. *J. Exp. Bot.* 66, 5403–5416. doi: 10.1093/jxb/erv219

Niinemets, Ü. (2010). Mild versus severe stress and BVOCs: thresholds: priming and consequences. *Trends Plant Sci.* 15, 145–153. doi: 10.1016/j.tplants.2009.11.008

Oksanen, J., Blanchet, F. G., Kindt, R., Legendre, P., Minchin, P. R., O'Hara, R. B., et al. (2013). *Vegan: Community Ecology Package. R Package Version. 2.0*.

Olowu, R. A., Adewuyi, G. O., Onipede, O. J., Lawal, O. A., and Sunday, O. M. (2015). Concentration of heavy metals in root, stem and leaves of *Acalypha indica* and *Panicum maximum jacq* from three major dumpsites in Ibadan Metropolis, South West Nigeria. *Am. J. Chem.* 5, 40–48. doi: 10.5923/j.chemistry.20150501.06

Osakabe, Y., Osakabe, K., Shinozaki, K., and Tran, L.-S. P. (2014). Response of plants to water stress. *Front. Plant Sci.* 5:86. doi: 10.3389/fpls.2014.00086

Pollastri, S., Savvides, A., Pesando, M., Lumini, E., Volpe, M. G., Ozudogru, E. A., et al. (2018). Impact of two arbuscular mycorrhizal fungi on *Arundo donax* L. response to salt stress. *Planta* 247, 573–585. doi: 10.1007/s00425-017-2808-3

Quiroga, G., Erice, G., Aroca, R., Chaumont, F., and Ruiz-Lozano, J. M. (2017). Enhanced drought stress tolerance by the arbuscular mycorrhizal symbiosis in a drought-sensitive maize cultivar is related to a broader and differential regulation of host plant aquaporins than in a drought-tolerant cultivar. *Front. Plant Sci.* 8:1056. doi: 10.3389/fpls.2017.01056

R Development Core Team (2012). *R: A Language and Environment for Statistical Computing*. Vienna: R Foundation for Statistical Computing. Available at: http://www.R-project.org/

Rasmussen, R. (2001). "Quantification on the lightcycler," in *Rapid Cycle Real-time PCR, Methods and Applications,* eds S. Meuer, C. Wittwer, and K. Nakagawara (Heidelberg: Springer Press).

Rivero, J., Álvarez, D., Flors, V., Azcón-Aguilar, C., and Pozo, M. J. (2018). Root metabolic plasticity underlies functional diversity in mycorrhiza-enhanced stress tolerance in tomato. *New Phytol.* doi: 10.1111/nph.15295

Ruiz-Lozano, J. M., Aroca, R., Zamarreño, A. M., Molina, S., Andreo-Jiménez, B., Porcel, R., et al. (2016). Arbuscular mycorrhizal symbiosis induces strigolactone biosynthesis under drought and improves drought tolerance in lettuce and tomato. *Plant Cell Environ.* 39, 441–452. doi: 10.1111/pce.12631

Sardans, J., and Peñuelas, J. (2004). Increasing drought decreases phosphorus availability in an evergreen Mediterranean forest. *Plant Soil* 267, 367–377. doi: 10.1007/s11104-005-0172-8

Sasso, R., Iodice, L., Digilio, M. C., Carretta, A., Ariati, L., and Guerrieri, E. (2007). Host-locating response by the aphid parasitoid *Aphidius ervi* to tomato plant volatiles, *J. Plant Interact.* 2, 175–183. doi: 10.1080/17429140701591951

Sasso, R., Iodice, L., Pickett, J. A., Woodcock, C. M., and Guerrieri, E. (2009). Behavioural and electrophysiological responses of *Aphidius ervi* (Hymenoptera: Braconidae) to tomato plant volatiles. *Chemoecology* 19, 195–201. doi: 10.1007/s00049-009-0023-9

Sawers, R. J. H., Svane, S. F., Quan, C., Grønlund, M., Wozniak, B., Gebreselassie, M.-N., et al. (2017). Phosphorus acquisition efficiency in arbuscular mycorrhizal maize is correlated with the abundance of root-external hyphae and the accumulation of transcripts encoding PHT1 phosphate transporters. *New Phytol.* 214, 632–643. doi: 10.1111/nph.14403

Smith, F. A., and Smith, S. E. (2011). What is the significance of the arbuscular mycorrhizal colonisation of many economically important crop plants? *Plant Soil* 348, 63–79. doi: 10.1007/s11104-011-0865-0

Smith, S. E., and Smith, F. A. (2012). Fresh perspectives on the roles of arbuscular mycorrhizal fungi in plant nutrition and growth. *Mycologia* 104, 1–13. doi: 10.3852/11-229

Sokal, R. R., and Rohlf, F. J. (1995). *Biometry: The Principles and Practice of Statistics in Biological Research*, 3rd Edn. New York, NY: W.H. Freeman and Co.

Subramanian, K. S., Santhanakrishnan, P., and Balasubramanian, P. (2006). Responses of field grown tomato plants to arbuscular mycorrhizal fungal colonization under varying intensities of drought stress. *Sci. Hortic.* 107, 245–253. doi: 10.1016/j.scienta.2005.07.006

Sun, T., Li, M., Shao, Y., Yu, L., and Ma, F. (2017). Comprehensive genomic identification and expression analysis of the phosphate transporter (PHT) gene family in Apple. *Front. Plant Sci.* 8:426. doi: 10.3389/fpls.2017.00426

Tamura, Y., Kobae, Y., Mizuno, T., and Hata, S. (2012). Identification and expression analysis of arbuscular mycorrhiza-inducible phosphate transporter genes of soybean. *Biosci. Biotech. Biochem.* 76, 309–313. doi: 10.1271/bbb.110684

Tian, H., Yuan, X., Duan, J., Li, W., Zhai, B., and Gao, Y. (2017). Influence of nutrient signals and carbon allocation on the expression of phosphate and nitrogen transporter genes in winter wheat (*Triticum aestivum* L.) roots colonized by arbuscular mycorrhizal fungi. *PLoS One* 12:e0172154. doi: 10.1371/journal.pone.0172154

Volpe, V., Dell'Aglio, E., Giovannetti, M., Ruberti, C., Costa, A., Genre, A., et al. (2013). An AM-induced, MYB-family gene of *Lotus japonicus* (LjMAMI) affects root growth in an AM-independent manner. *Plant J.* 73, 442–455. doi: 10.1111/tpj.12045

Volpe, V., Giovannetti, M., Sun, X. G., Fiorilli, V., and Bonfante, P. (2016). The phosphate transporters LjPT4 and MtPT4 mediate early root responses to phosphate status in non mycorrhizal roots. *Plant Cell Environ.* 39, 660–671. doi: 10.1111/pce.12659

Wang, D., Lv, S., Jiang, P., and Li, Y. (2017). Roles, regulation, and agricultural application of plant phosphate transporters. *Front. Plant Sci.* 8:817. doi: 10.3389/fpls.2017.00817

Wang, B., Yao, Z., Zhao, S., Guo, K., Sun, J., and Zhang, H. (2014). Arbuscular mycorrhizal fungal application to improve growth and tolerance of processing tomato (Lycopersicon esculentum Miller) under drought stress. *J. Food Agric. Environ.* 12, 452–457.

Wu, Y., Spollen, W. G., Sharp, R. E., Hetherington, P. R., and Fry, S. C. (1994). Root growth maintenance at low water potentials: increased activity of xyloglucan endotransglycosylase and its possible regulation by abscisic acid. *Plant Physiol.* 106, 607–615. doi: 10.1104/pp.106.2.607

Yang, S.-Y., Grønlund, M., Jakobsen, I., Grotemeyer, M. S., Rentsch, D., Miyao, A., et al. (2012). Nonredundant regulation of rice arbuscular mycorrhizal symbiosis by two members of the phosphate transporter1 gene family. *Plant Cell* 24, 4236–4251. doi: 10.1105/tpc.112.104901

Zhang, C., Meng, S., Li, M., and Zhao, Z. (2016). Genomic identification and expression analysis of the phosphate transporter gene family in poplar. *Front. Plant Sci.* 7:1398. doi: 10.3389/fpls.2016.01398

Zou, Y.-N., Wang, P., Liu, C.-Y., Ni, Q.-D., Zhang, D.-J., and Wu, Q.-S. (2017). Mycorrhizal trifoliate orange has greater root adaptation of morphology and phytohormones in response to drought stress. *Sci. Rep.* 7:41134. doi: 10.1038/srep41134

Conflict of Interest Statement: The authors declare that the research was conducted in the absence of any commercial or financial relationships that could be construed as a potential conflict of interest.

frontiers in Plant Science

REVIEW
published: 23 October 2018
doi: 10.3389/fpls.2018.01473

Plant Growth-Promoting Rhizobacteria: Context, Mechanisms of Action, and Roadmap to Commercialization of Biostimulants for Sustainable Agriculture

*Rachel Backer[1], J. Stefan Rokem[2], Gayathri Ilangumaran[1], John Lamont[1], Dana Praslickova[1], Emily Ricci[1], Sowmyalakshmi Subramanian[1] and Donald L. Smith[1]**

[1] Department of Plant Science, McGill University, Montreal, QC, Canada, [2] School of Medicine, Department of Microbiology and Molecular Genetics, Institute for Medical Research Israel-Canada, The Hebrew University of Jerusalem, Jerusalem, Israel

OPEN ACCESS

Edited by:
Giuseppe Colla, Università degli Studi della Tuscia, Italy

Reviewed by:
Raffaella Balestrini, Consiglio Nazionale delle Ricerche (CNR), Italy
Lori Hoagland, Purdue University, United States

***Correspondence:**
Donald L. Smith
donald.smith@mcgill.ca

Specialty section:
This article was submitted to Plant Microbe Interactions, a section of the journal Frontiers in Plant Science

Received: *30 May 2018*
Accepted: *20 September 2018*
Published: *23 October 2018*

Citation:
Backer R, Rokem JS, Ilangumaran G, Lamont J, Praslickova D, Ricci E, Subramanian S and Smith DL (2018) Plant Growth-Promoting Rhizobacteria: Context, Mechanisms of Action, and Roadmap to Commercialization of Biostimulants for Sustainable Agriculture. Front. Plant Sci. 9:1473. doi: 10.3389/fpls.2018.01473

Microbes of the phytomicrobiome are associated with every plant tissue and, in combination with the plant form the holobiont. Plants regulate the composition and activity of their associated bacterial community carefully. These microbes provide a wide range of services and benefits to the plant; in return, the plant provides the microbial community with reduced carbon and other metabolites. Soils are generally a moist environment, rich in reduced carbon which supports extensive soil microbial communities. The rhizomicrobiome is of great importance to agriculture owing to the rich diversity of root exudates and plant cell debris that attract diverse and unique patterns of microbial colonization. Microbes of the rhizomicrobiome play key roles in nutrient acquisition and assimilation, improved soil texture, secreting, and modulating extracellular molecules such as hormones, secondary metabolites, antibiotics, and various signal compounds, all leading to enhancement of plant growth. The microbes and compounds they secrete constitute valuable biostimulants and play pivotal roles in modulating plant stress responses. Research has demonstrated that inoculating plants with plant-growth promoting rhizobacteria (PGPR) or treating plants with microbe-to-plant signal compounds can be an effective strategy to stimulate crop growth. Furthermore, these strategies can improve crop tolerance for the abiotic stresses (e.g., drought, heat, and salinity) likely to become more frequent as climate change conditions continue to develop. This discovery has resulted in multifunctional PGPR-based formulations for commercial agriculture, to minimize the use of synthetic fertilizers and agrochemicals. This review is an update about the role of PGPR in agriculture, from their collection to commercialization as low-cost commercial agricultural inputs. First, we introduce the concept and role of the phytomicrobiome and the agricultural context underlying food security in the 21st century. Next, mechanisms of plant growth promotion by PGPR are discussed, including signal exchange between plant roots and PGPR and how these relationships modulate plant abiotic stress responses via

induced systemic resistance. On the application side, strategies are discussed to improve rhizosphere colonization by PGPR inoculants. The final sections of the paper describe the applications of PGPR in 21st century agriculture and the roadmap to commercialization of a PGPR-based technology.

Keywords: phytomicrobiome, holobiont, rhizosphere, PGPR, sustainable agriculture, climate change resilience, roadmap, deployment

INTRODUCTION

A plant growing under field conditions is not an individual; it is a complex community (Lundberg et al., 2012) with subtle and relatively constant partner relationships. A well-structured and regulated community of microorganisms is always associated with the plant (Turner et al., 2013; Chaparro et al., 2014; Lebeis, 2014; Bulgarelli et al., 2015; Smith et al., 2015b). This community is the phytomicrobiome (Smith et al., 2017); the phytomicrobiome plus the plant is the holobiont (Berg et al., 2016; Theis et al., 2016; Smith et al., 2017). Microbiome relationships exist with all multi-cellular organisms, and probably all eukaryotes. In fact, these probably predate the colonization of the land by plants (Berg et al., 2014). This microbial community has been associated with terrestrial plants since their earliest evolution, to assist early land plants faced with challenges such as access to nutrients, novel and often-stressful conditions and pathogens (Smith et al., 2015a).

There are elements (including bacteria and fungi) of the phytomicrobiome associated will all major plant structures (flowers, fruits, stems, leaves, and roots) (Berg et al., 2016). However, conditions vary substantially among these structures, leading to specialized microbial populations inhabiting each one. The microbial community associated with the roots (the rhizomicrobiome), is the most populous and elaborate of all those associated with higher plants. The best understood and characterized example is the nitrogen-fixing rhizobia associated with legumes (Gray and Smith, 2005). Many members of the phytomicrobiome cannot be cultured and it has only been since the advent of metagenomics (Hirsch and Mauchline, 2012) and related methods that we are able to assess how membership is changed by conditions, plant genotype (Delaplace et al., 2015; Poli et al., 2016; Wintermans et al., 2016) and plant development.

The plant exerts considerable control over the composition of the rhizomicrobiome (Zhang et al., 2017). It produces root exudates of various compositions (Chaparro et al., 2012; Trabelsi and Mhamdi, 2013), which can be more suitable as a source of reduced C, to some microbes than others. The plant also produces signal compounds that recruit specific species and regulate their genetic and biochemical activities (Nelson and Sadowsky, 2015; Massalha et al., 2017; Smith et al., 2017). In addition, the soil microbial community undertakes various aspects of self-regulation (Leach et al., 2017). The microbes can produce quorum sensing compounds to communicate when conditions warrant a collective physiological shift (Chauhan et al., 2015). Plants have evolved to respond to microbial quorum sensing compounds and to produce analogs, providing plants with another level of regulation over the rhizomicrobiome (Ortiz-Castro et al., 2009). Finally, it is now becoming apparent that there is some degree of hierarchy within the phytomicrobiome and that there are key members, termed "hub species" (Agler et al., 2016) or "core species" (Toju et al., 2018), whose activities are regulated by plants, and hub species in turn regulate broader activities within the phytomicrobiome. Most hub species have probably been part of the phytomicrobiome for a very long time, allowing for development of their central position (van der Heijden and Hartmann, 2016).

In the soil, there is a gradient of intimacy between plant roots and microbes extending away from the plant root: the degree of plant influence over the microbial community increases nearer the root surface (**Figure 1**). This zone is now generally referred to as the rhizosphere, however, the term was originally coined by Hiltner (1904) to describe the soil microorganisms around and inside roots. Now, microbes living on the root surface are said to inhabit the rhizoplane, and those living inside the root are said to be endophytes (Gray and Smith, 2005; Zhang et al., 2017). Mitochondria and plastids (including the chloroplasts) represent some of the oldest and most intimate, aspects of the phytomicrobiome. They evolved from plant-associated microbes into the permanent subcellular structures we see today.

Our current understanding of the phytomicrobiome has demonstrated two main aspects. First, we know shockingly little about it (Quiza et al., 2015). Second, the relationships we have studied between rhizomicrobiome members and plants have shown that there is a tremendous potential in exploiting this community of organisms to increase worldwide crop production (Barea, 2015; Nehra and Choudhary, 2015; Smith et al., 2015b). This review is an update regarding the role of plant-growth promoting rhizobacteria (PGPR) in agriculture, from their collection to commercialization as a low-cost commercial agricultural input. While also we recognize the value of PGPR as a tool for phytoremediation, however, this is beyond the scope of our review; excellent information on this topic can be found in other review articles.

AGRICULTURAL CONTEXT: A "FRESH" GREEN REVOLUTION IN THE FACE OF CLIMATE CHANGE

The Green Revolution of the 20th century enabled unprecedented gains in global food production. The Green Revolution was roughly comprised of two main advances; chemical inputs (pesticides, herbicides, and chemical fertilizers) and

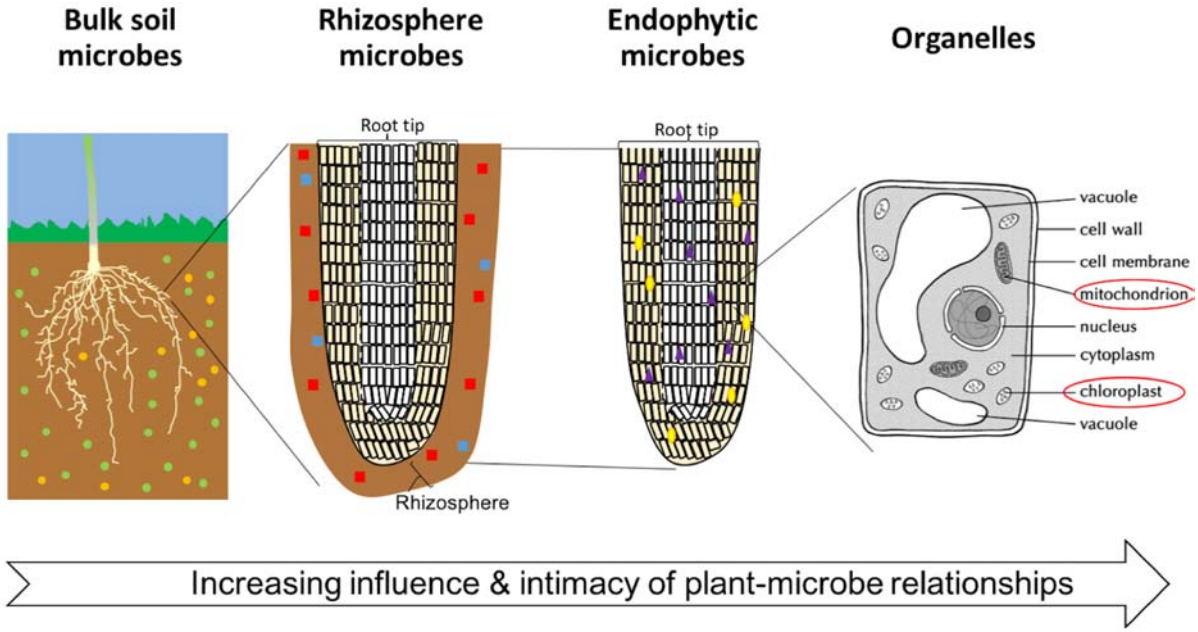


FIGURE 1 | The degree of intimacy and influence of the plant-microbe interactions. Microbes are represented by small colored (red, green, yellow, purple, and blue) shapes. Diversity and number of microbes is variable between soils, distance from plant roots, crop species, and plant tissue.

improved crop plants (through targeted breeding and advanced genetic manipulations). However, gains associated with fertilizer inputs carry high environmental costs. A new revolution in agricultural innovation will be needed to sustain the food, fiber, and fuel needs of a growing global population and a changing climate through the 21st century. A "Fresh" Green Revolution, perhaps the Bio-Revolution, needs to be based on fewer intensive inputs with reduced environmental impact. A Bio-Revolution could be based on 1) biological inputs through utilization of the phytomicrobiome (with inoculants, microbially produced compounds, etc.), and improved crops (by manipulation of the phytomicrobiome community structure) (Timmusk et al., 2017). The use of microbial based agricultural inputs has a long history, beginning with broad-scale rhizobial inoculation of legumes in the early 20th century (Desbrosses and Stougaard, 2011). More recently, strains of *Bacillus, Pseudomonas, Glomus,* and others have been commercialized. The use of bacterial taxa in plant production has been reviewed previously for *Bacillus* (Borriss, 2011), *Pseudomonas* (Santoyo et al., 2012; Sivasakthi et al., 2014), *Actinobacteria* (Shivlata and Satyanarayana, 2017), and *Lactobacillus* (Lamont et al., 2017). In addition, *Acetobacter, Azospirillum*, *Paenibacillus*, *Serratia*, *Burkholderia, Herbaspirillum,* and *Rhodococcus* have also been shown to enhance crop production (Babalola, 2010).

The effects of climate change are expected to impose more environmental stresses on crops worldwide (Pachauri et al., 2014). Moreover, as climate change progresses throughout the 21st century, significant areas of high-quality agricultural lands will likely be lost to rising seas, erosion, salinization, and desertification. This means that crop yields will need to be maintained, in spite of production on a smaller area of land, under more stressful conditions. The phytomicrobiome plays a critical role in the survival of the holobiont, particularly for plants growing in extreme environments. Some plants that live in hypersaline coastal environments or geothermal soils rely on endophytic fungi to survive (Rodriguez and Redman, 2008). Likewise, constitutive microbial communities of agave (Coleman-Derr et al., 2016) and cacti (Fonseca-García et al., 2016) likely aid in the survival of these plants in very dry habitats. The microbiomes of plants native to extreme environments may be rich sources of stress-ameliorating microbes.

PHYSIOLOGY OF PLANT-GROWTH PROMOTING RHIZOBACTERIA

Plant-microbe co-evolution has led to some of the bacteria becoming facultative intracellular endophytes (Bulgarelli et al., 2013). Among these free-living bacteria are PGPR that exert beneficial effects on plants through direct and indirect mechanisms. Beneficial rhizobacteria have been utilized to improve water and nutrient uptake, abiotic and biotic stress tolerance. Even though numerous soil bacteria have been reported to promote plant growth and development, the mode(s) of action by which the bacteria exhibit beneficial activities are often not well understood. The molecular basis of plant-bacteria interaction mechanisms responsible for the physiological changes are beginning to be discerned, mainly due to the emerging "omics" approaches.

Nutrient Acquisition by PGPR

Soils with dynamic microbial ecologies and high organic matter typically have lower fertilizer requirements than conventionally managed soils (Bender et al., 2016). For example, bulk microbial activity in soils is often considered when managing the application of organic nutrient sources. Phytomicrobiome research is beginning to reveal specific plant-microbe interactions that directly aid in plant nutrition (Beattie, 2015). Microbes that assist in plant nutrient acquisition (biofertilizers) act through a variety of mechanisms including augmenting surface area accessed by plant roots, nitrogen fixation, P-solubilization, siderophore production and HCN production (Pii et al., 2015). Therefore, manipulating microbial activity has great potential to provide crops with nutritional requirements.

The most extensively studied and exploited beneficial plant-bacteria relationship is the N-fixing symbiosis between rhizobia and legumes. In this relationship, legumes provide rhizobia with reduced C and a protected, anaerobic environment required for nitrogenase activity, while rhizobia provide the legumes with biologically available N. Within this symbiosis, both rhizobia and legume undergo significant transformations. The legume forms a new organ, the nodule, to house the rhizobia, and the rhizobia, in turn, changes from its free-living rod-shaped cell type to a branched, N-fixing bacteroid (Oke and Long, 1999). Rhizobial N-fixation contributes significant amounts of N to global agricultural systems, with estimates ranging from 20 to 22 Tg N per year (Herridge et al., 2008) up to 40 Tg N per year (Galloway et al., 2008). Rhizobial inoculants of leguminous crops are the earliest example of commercial microbial products in agriculture and still represent the most widely used agricultural inoculants (Bashan, 1998). However, genetic improvements in efficiency of the N-fixing symbiosis of rhizobia and crop plants have been elusive. The fixation of atmospheric nitrogen and conversion to ammonia is an energy demanding process, which means oxidative phosphorylation of carbon sources to generate ATP must be favored over glycogen synthesis within the bacterial cell, to increase nitrogen fixation. However, experiments with glycogen synthase deletion mutants of *Rhizobium tropici* have not survived in soil environments, despite increased dry matter and nodule number in inoculated bean plants (Marroquí et al., 2001).

Starting in the early 21st century, interest began to mount around the development of commercial inoculants of free-living N-fixing bacteria such as *Azoarcus* sp., *Burkholderia* sp., *Gluconacetobacter* sp., *Diazotrophicus* sp., *Herbaspirillum* sp., *Azotobacter* sp., *Bacillus polymyxa*, and especially *Azospirillum* sp. (Vessey, 2003). These free-living diazotrophs provide N to a much wider range of crop plants than rhizobia. Commercial inoculants of *Azospirillum*, produced by small and medium sized companies around the world, have been effective in increasing the yield of various cereal crops (Bashan and de-Bashan, 2015). Other bacteria that do not fix N have been shown to increase N uptake in plants, thus increasing nitrogen use efficiency (Adesemoye et al., 2008; Adesemoye and Kloepper, 2009), likely due to increased root growth, which allows plants to access more soil (Beattie, 2015).

Following Liebig's law of the minimum in mind, the next most limiting nutrient for crop plants after N is usually P. While most agricultural soils contain ample quantities of P, much of it is in non-soluble forms. To supplement indigenous soil P, crops are typically fertilized with rock phosphate mined from one of a few large deposits (up to 85% of the world's rock phosphate is estimated to be in Morocco and Western Sahara). Furthermore, phosphorus solubilizing microorganisms (PSMs) can help plants access the reservoir of non-labile phosphorus by releasing it from its recalcitrant forms. Inorganic P complexed with Ca, Fe, or Al can be solubilized by organic acids or H^+ ions excreted by PSMs. Similarly, phytase produced by PSMs can liberate reactive P from organic compounds. Production of HCN by PGPR was originally thought to promote plant growth by suppressing pathogens, however, this idea has recently been challenged by Rijavec and Lapanje (2016), who argued that HCN indirectly increases P availability by metal chelation and sequestration of these geochemical entities. PSMs produce organic acids to reduce metal toxicity by using these compounds to transform metal species to immobile forms or chelate them for mobility, to be carried into the plant tissues for further phyto-extraction possibilities (Ahemad, 2015). The PSM *Bacillus megaterium* has been commercialized as BioPhos (BioPower Lanka, Sri Lanka) and can reduce phosphate fertilizer requirements of plantation crops up to 75% (Mehnaz, 2016). Strains of P- solublizing *Pseudomonas striata*, *B. Polymyxa,* and *B. megaterium* have also been commercialized by AgriLife (India) (Mehnaz, 2016).

Other nutrient elements, such as Fe and Zn can limit crop yields. Like P, Fe can also be abundant in soils, but unavailable to plants. Many bacterial strains increase the availability of Fe through the production of organic acids or siderophores (Kloepper et al., 1980; Neilands, 1995; Ahmed and Holmstrom, 2014). Siderophores also act to control pathogenic microbes by depriving them Fe (Ahmed and Holmstrom, 2014; Saha et al., 2016). A commercial formulation of the Fe mobilizing bacteria, *Acidithiobacillus ferrooxidans* has been developed by AgriLife (India) (Mehnaz, 2016), although this genus apparently solubilizes Fe through organic acid production rather than with siderophores (Bhatti and Yawar, 2010). Several strains of Zn-mobilizing bacteria have been shown to increase Zn uptake, and thus increase yield in several crops, including rice (Tariq et al., 2007; Shakeel et al., 2015), wheat and soybean (Ramesh et al., 2014). While the mechanisms of Zn-mobilizers remain uncertain, they are likely similar to those of PSMs and Fe-mobilizers, namely the production of chelating agents and organic acids (Hafeez et al., 2013).

Signal Exchange Between Plant Roots and PGPR

Plant Hormones Produced by PGPR

Phytohormones are key players in regulating plant growth and development. They also function as molecular signals in response to environmental factors that otherwise limit plant growth or become lethal when uncontrolled (Fahad et al., 2015). Many rhizosphere bacteria are known to excrete hormones for root

uptake or manipulate hormone balance in the plants to boost growth and stress response.

Many PGPR can produce auxins (Omer et al., 2004; Gupta et al., 2015) to exert particularly strong effects on root growth (Jha and Saraf, 2015) and architecture (Vacheron et al., 2013). Indole-3-acetic acid (IAA) is the most widely studied auxin produced by PGPR. It is involved in plant-microbe interactions (e.g., Ahemad and Kibret, 2014; Afzal et al., 2015). The function of exogenous IAA is dependent on the endogenous IAA levels in plants. At optimal IAA concentrations in plants, application of bacterial IAA may have neutral, positive, or negative effects on plant growth (Spaepen and Vanderleyden, 2011). PGPR that produce auxins have been shown to elicit transcriptional changes in hormone, defense-related, and cell wall related genes (Spaepen et al., 2014), induce longer roots (Hong et al., 1991), increase root biomass and decrease stomata size and density (Llorente et al., 2016), and activate auxin response genes that enhance plant growth (Ruzzi and Aroca, 2015).

Many PGPR produce cytokinins and gibberellins (Gupta et al., 2015; Kumar et al., 2015) but the role of bacterially synthesized hormones in plants, and bacterial mechanism of synthesis, are not yet completely understood (Garcia de Salamone et al., 2001; Kang et al., 2009). Some strains of PGPR can promote relatively large amounts of gibberellins, leading to enhanced plant shoot growth (Jha and Saraf, 2015). Interactions of these hormones with auxins can alter root architecture (Vacheron et al., 2013). Production of cytokinins by PGPR can also lead to enhanced root exudate production by the plant (Ruzzi and Aroca, 2015) potentially increasing the presence of PGPR associated with the plant.

Ethylene is a gaseous hormone, active at extremely low concentrations (0.05 mL L^{-1}) and is a "stress hormone," as illustrated by its concentration spiking during various abiotic and biotic stresses. Accumulation of ethylene in response to stress may increase plant tolerance or exacerbate stress-response symptoms and senescence (Morgan and Drew, 1997). PGPR function has been studied under both stressed and unstressed conditions and often provides greater growth stimulation under stressful conditions, for instance, under drought stress (Rubin et al., 2017). Ethylene plays an important role for improving plant stress tolerance for some PGPR (Nadeem et al., 2014): PGPR secrete 1-aminocyclopropane-1-carboxylase (ACC) deaminase which reduces ethylene production in plants (Glick, 2014; Vejan et al., 2016). Many studies have shown enhanced stress tolerance in plants through inoculation with PGPR that produce ACC deaminase. This appears to occur since PGPR are able to keep ethylene levels from reaching levels sufficient to reduce plant growth (Ahemad and Kibret, 2014; Pérez-Montaño et al., 2014; Ruzzi and Aroca, 2015), as has been demonstrated with *Camelina sativa* (Heydarian et al., 2016).

Other Microbe-to-Plant Signal Molecules

A wide range of secondary metabolites and volatile organic compounds (VOCs) produced by bacteria can improve stress-tolerance and/or stimulate growth in plants. For example, polyamines play important physiological and protective roles in plants. *B. megaterium* BOFC15 secretes a polyamine, spermidine, and induces polyamine production in *Arabidopsis*, resulting in an increase in biomass, altered root architecture and elevated photosynthetic capacity. The inoculated plants exhibited higher drought tolerance and abscisic acid (ABA) content under PEG induced water-deficit stress (Zhou et al., 2016). A range of PGPR produce HCN, which can control the level of deleterious microbes in the rhizosphere (Kumar et al., 2015). VOC produced by PGPR stimulate plant growth, resulting in increased shoot biomass and improve plant stress resistance (Bailly and Weisskopf, 2012; Ruzzi and Aroca, 2015).

The microbes of the phytomicrobiome also affect each other's activities through signal compounds (Hagai et al., 2014; Massalha et al., 2017). These signals amount to hormones of the holobiont. For example, lumichrome and riboflavin can act as microbe-to-plant signal compounds able to stimulate plant growth. Both compounds can cause meaningful alterations in plant development; lumichrome can accelerate appearance of leaves (more rapid development) and leaf expansion (enhanced growth). In addition, it can increase plant height and overall leaf area, resulting in improved production of biomass. This is true over a wide range of plant types including both monocots and dicots (Dakora et al., 2015).

Microbe-to-plant signal compounds (e.g., lipo-chitooligosaccharides and thuricin 17) have been shown to increase plant growth for diverse species, particularly when plants are growing under stressful conditions (Subramanian and Smith, 2015; Subramanian et al., 2016b; Zipfel and Oldroyd, 2017). The receptor for the lipo-chitooligosaccharides is a LysM kinase for the legume-rhizobia symbioses; this receptor system seems to have evolved for pathogen detection almost Two billion years ago (Spaink, 2004; Gust et al., 2012; Carotenuto et al., 2017). The microbe-to-plant signal in the N_2-fixing *Frankia* symbiosis remains to be identified but appears not to be an LCO (Chabaud et al., 2016).

Root Exudates as Plant-to-Microbe Signals

Plants excrete considerable control over the microbes they associated with (Berendsen et al., 2012; Badri et al., 2013; Turner et al., 2013; Massalha et al., 2017); even of simple genotype differences within a plant species can have meaningful effects (Peiffer et al., 2013; Winston et al., 2014). Some of this control is the result of inter-organismal signals (Smith et al., 2017). Starting when the seed is imbibing and germinating, then when roots are growing and finally senescing, molecules are released from roots into the surrounding soil. These molecules support microbial growth and activity in the rhizosphere (Nelson, 2004a, 2017; Schiltz et al., 2015). Variation in root exudation (timing, amount, and/or constituents) provides a mechanism by which plants can manipulate composition and abundances of their root-associated microbiota (Bakker et al., 2012). Exudates are thought to consist mainly of sugars, amino acids, and organic acids that are present at high concentrations in the cytoplasm of the plant, but also include smaller amounts of complex secondary metabolites such as flavonoids, terpenes, and phenolic compounds that can attract specific microbes in the rhizosphere (Jones et al., 2004; Bais et al., 2006; Musilova et al., 2016). It has also been suggested that exudation of the signal molecules jasmonic

acid and salicylic acid into the rhizosphere can be involved in the interplay between roots and microbes during the initial events of colonization (Gutjahr and Paszkowski, 2009; Doornbos et al., 2011). Root exudation is genetically regulated and can thus shape distinct rhizobacterial communities for different plant genotypes, resulting in highly variable exudates among plant species, individual plant types within the same species, at different plant developmental stages, growth conditions, and biotic interactions (Gransee and Wittenmayer, 2000; Mougel et al., 2006; Broeckling et al., 2008; Houlden et al., 2008; Badri and Vivanco, 2009; Micallef et al., 2009; Badri et al., 2013; Kristin and Miranda, 2013).

PGPR IMPROVE PLANT GROWTH UNDER STRESSFUL GROWING CONDITIONS

The mechanisms that regulate stress tolerance in plants are intricate and complex, in part because plants are sessile organisms (Wani et al., 2016) which have no choice but to stand where they are and "take it." Improving stress tolerance in crop plants through conventional breeding is a long and capital-intensive process, while genetic engineering is associated with ethical and social acceptance issues. The role of beneficial microorganisms is gaining importance in stress management and the development of climate change resilient agriculture. Recent studies have exploited molecular techniques to understand the mode of action of the plant-microbe interactions resulting in induced stress tolerance.

Abiotic Stress Tolerance Associated With PGPR

Pseudomonas putida MTCC5279 ameliorated drought stress in chickpea (*Cicer arietinum*) plants by modulating membrane integrity, osmolyte accumulation (proline, glycine betaine) and ROS scavenging ability. Stress responses were positively modulated by the bacteria resulting in differential expression of genes involved in ethylene biosynthesis (ACO and ACS), salicylic acid (PR1), jasmonate (MYC2) transcription activation, SOD, CAT, APX, and GST (code for antioxidant enzymes), DREB1A (dehydration responsive element binding), NAC1 (transcription factors expressed under abiotic stress), LEA and DHN (dehydrins) (Tiwari et al., 2016). Application of thuricin 17 produced by *Bacillus thuringiensis* NEB17 to soybean (*Glycine max*) under water-deficit conditions resulted in modification of root structures and increased root and nodule biomass, root length, root ABA, and total nitrogen content (Prudent et al., 2015). Beneficial microbes also help plants cope with flooding stress. Rice (*Oryza sativa*) seedlings inoculated with an ACC deaminase producing strain of *Pseudomonas fluorescens* REN1 increased root elongation under constantly flooded conditions (Etesami et al., 2014).

Salt stress effects can be diminished by ACC deaminase. Pea plants inoculated with *Variovorax paradoxus* 5C-2, which produce ACC deaminase, had increased photosynthetic rate, electron transport, balanced ion homeostasis through increased K^+ flow to shoots and Na^+ deposition on roots, decreased stomatal resistance and xylem balance pressure and increased biomass under salt stress at 70 and 130 mM NaCl (Wang et al., 2016). For okra, PGPR producing ACC enhanced salt tolerance, increased antioxidant enzyme activities (SOD, APX, and CAT) and upregulated ROS pathway genes (CAT, APX, GR, and DHAR) (Habib et al., 2016). Maize seedlings inoculated with *Bacillus amyloliquefaciens* SQR9, had enhanced salt stress tolerance, including enhanced the chlorophyll content, compared with the control. Additional analysis showed that the mechanisms were related to enhanced total soluble sugar content leading to decreased cell destruction, improved peroxidase/catalase activity and glutathione content for scavenging ROS, and reduced Na^+ levels in the plant. These physiological manifestations were confirmed by measured upregulation of RBCS, RBCL, H^+ −PPase, HKT1, NHX1, NHX2, and NHX3 genes, as well as downregulation of NCED expression, as determined by qPCR (Chen et al., 2016). Wheat (*Triticum aestivum*) plants inoculated with the halotolerant *Dietzia natronolimnaea* showed upregulation of genes involved in the ABA-signaling cascade, salt overly sensitive (SOS) pathway, ion transporters, and antioxidant enzymes; stress tolerance is induced by modulation of complex network of gene families (Bharti et al., 2016).

Exposure to cold and/or heat reduce yield and, in worst case scenarios, result in crop failure (Cheng, 2014). A gibberellin-producing PGPR, *Serratia nematodiphila* increases pepper (*Capsicum annum*) growth under low temperature stress conditions. The inoculated plants contained more GA4 and ABA and less salicylate and jasmonate (Kang et al., 2015). Inoculation with *Burkholderia phytofirmans* PsJN modulated carbohydrate metabolism to reduce chilling damage to grapevine (*Vitis vinifera*) plantlets exposed to low temperature stress (Fernandez et al., 2012). Inoculation of tomato (*Solanum lycopersicum*) plants exposed to low temperatures with *Pseudomonas vancouverensis* OB155 and *P. frederiksbergensis* OS261 increased expression of cold acclimation genes and antioxidant activity in leaf tissues (Subramanian et al., 2015).

Biocontrol and Induced Systemic Resistance for Biotic Stress Tolerance

Bacillus amyloliquefaciens (SN13) is a biocontrol agent against *Rhizoctonia solani,* by prolonging tolerance through enhanced defense response in the plants. The colonized plants exhibit modulation of phytohormone signaling, sustained maintenance of elicitors, production of secondary metabolites and balance of reactive oxygen species and scavengers producing ROS scavengers (Srivastava et al., 2016). Cotton (*Gossypium hirsutum*) plants inoculated with *Bacillus* spp. exhibited increased gossypol and jasmonic acid secretion reducing larval feeding by *Spodoptera exigua*. Transcript levels of genes involved in synthesis of allelochemicals and jasmonates were higher in inoculated plants as was suppression of the pest (Zebelo et al., 2016). *Enterobacter asburiae* BQ9 induced resistance against tomato yellow leaf curl virus by increasing the expression of defense-related genes and antioxidant enzymes, including

phenylalanine ammonia lyase, peroxidase, catalase, and superoxide dismutase (Li et al., 2016). Soil inoculation with *Peanibacillus lentimorbus* B-30488 decreased cucumber mosaic virus RNA accumulation in *Nicotiana tabacum* cv. White burley leaves by 91%. This was associated with an increase in stress and pathogenesis-related gene expression and antioxidant enzyme activity suggesting induced resistance against the virus. PGPR colonization resulted in improved tissue heath and physiology of plants, which produced more flowers and seeds (Kumar et al., 2016). The bacteria also produce ACC deaminase and induce tolerance against southern blight disease in tomato caused by *Scelerotium rolfsii*. The inoculated plants showed modulation of the ethylene pathway and antioxidant enzyme activities; systemic tolerance was corroborated by pathogen related gene expression analysis (Dixit et al., 2016). Acyl-homoserine lactones (AHL)-producing *Serratia liquefaciens* MG1 and *P. putida* IsoF elicited induced systemic resistance (ISR) in tomato (*S. lycopersicum*) against *Alternaria alternate* whereas AHL-null mutant strains of both PGPR resulted in reduced ISR (Schuhegger et al., 2006). Root exudates have been found to contain chemicals that mimic AHL signals, stimulating beneficial rhizosphere associations while inhibiting pathogenic bacteria (Teplitski et al., 2000).

Besides functioning as biocontrol agents, PGPR protect plants against pathogens by eliciting biochemical and molecular defense responses within the plant (Lugtenberg and Kamilova, 2009). PGPR can trigger ISR in plants, which activates pathogenesis-related genes, mediated by phytohormone signaling pathways and defense regulatory proteins to prime plants against future pathogen attack (Pieterse et al., 2014). Bacterial signal compounds and microbe-associated molecular triggers, such as chitin oligomers, have been shown to modulate ISR induction in plants. Pathogen cell-surface factors such as flagellins and *O*-antigen of lipopolysaccharides elicit ISR, whereas analogs of salicylic acid and jasmonic acid trigger ethylene to elicit NPR1 mediated systemic acquired resistance (SAR) in plants (Ping and Boland, 2004).

STRATEGIES FOR IMPROVING RHIZOSPHERE COLONIZATION BY PGPR INOCULANTS

Under field conditions, other external factors come into play and the ability of soil bacteria to elicit positive effects on plant growth can be impaired and so that the effects of applying specific PGPM can be variable (Nelson, 2004b). The plant rhizosphere is colonized by microorganisms from the soil and the seed. The determinants of soil microorganisms are based on properties such as C and N availability, organic matter content, water availability and pH (Bossio et al., 1998; Drenovsky et al., 2004; Garcia-Pausas and Paterson, 2011) as well as biogeographic patterns including soil type and seasonality (Kristin and Miranda, 2013). Hence it is necessary to develop strategies for effective inoculation methods, so that bacteria of interest gain advantage in colonization efficiency over others. Product quality, compatibility, and stability determine effective colonization and consistent performance of the inoculum under field conditions (Lee et al., 2016).

Biofilm Versus Planktonic Inoculum

Plant-associated biofilms have been shown to establish themselves on various parts of plants such as leaves, roots, seeds and internal vasculature (Ramey et al., 2004; Ude et al., 2006; Danhorn and Fuqua, 2007; Eberl et al., 2007). The ability to form biofilms not only enhances bacterial survival but also enhances plant growth through the various PGPR-associated mechanisms described in the previous section, often to a greater extent than their planktonic cell counterparts (Ricci, 2015). Another advantage of biofilms over planktonic cells is their higher resistance to antibiotics, leading to improved chance of survival in a competitive soil environment (Mah et al., 2003). This is an important consideration when applying microbial inoculants to soils where microbes face intense competition and may not be as well adapted to challenging conditions as indigenous soil microbes (Anderl et al., 2000; Mah and O'Toole, 2001; Whiteley et al., 2001; Donlan, 2002; Walters et al., 2003; Resch et al., 2005; Zhang and Mah, 2008; Beaudoin et al., 2012). An alternative mechanism by which biofilms enhance plant growth is through biocontrol of disease organisms (Innerebner et al., 2011), such as competitive colonization of the rhizosphere and the production of antimicrobial compounds (Bais et al., 2004; Lugtenberg and Kamilova, 2009; Chen et al., 2013).

The literature contains several examples of the PGPR activity of biofilms. Single and dual-species biofilms produced from *Pseudomonas, Trichoderma, Bradyrhizobium,* and *Penicillium* showed greater ammonia production, IAA production, phosphate solubilization, siderophore production, and/or nitrogenase activity than the planktonic inocula (Bais et al., 2004; Jayasinghearachchi and Seneviratne, 2004; Triveni et al., 2012; Mohd and Ahmad, 2014). Furthermore, when the biofilms were used to inoculate seeds, cotton seed germination, wheat root and shoot length, soybean dry weights and nitrogen accumulation, and maize seed germination and root length were increased compared to plants inoculated with planktonic cells (Mohd and Ahmad, 2014).

Using Biochar to Promote Microbial Growth and Survival in Soil

Biochar has received much attention in the scientific literature over the last decade, as a soil amendment due to its ability to improve soil fertility and increase crop yields. Biochar can change soil fertility parameters that influence microbial survival in soil, including pH, organic matter content, cation exchange capacity and nutrient retention, water retention and oxygen tension, bulk density and provide niche spaces for microbes, thus preventing grazing by fungal predators (Major, 2009; Clough and Condron, 2010; Gaskin et al., 2010; Singh et al., 2010; Van Zwieten et al., 2010; Kameyama et al., 2012; Jaafar, 2014; Ye et al., 2016; Backer et al., 2017; Jenkins et al., 2017). Recent research has also investigated the use of biochar as a carrier material for microbial inoculants, applied as seed-coatings, constituting a sustainable alternative to peat-based inoculants,

and promoting early colonization of the rhizosphere with beneficial microorganisms (Rondon et al., 2007; Budania and Yadav, 2014; Adam et al., 2016; Deb et al., 2016; Egamberdieva et al., 2016; Głodowska et al., 2016; Kim et al., 2016; Shanta et al., 2016; Siddiqui et al., 2016; Sun et al., 2016; Traxler et al., 2016; Nadeem et al., 2017; Vecstaudza et al., 2017). It is important to note, however, that not all biochar materials are the same; biochar production conditions and feedstock materials have a large influence on the biological, chemical and physical properties of the final biochar material and while many provide desirable effects on soil fertility, some can be toxic to microbes and/or plants (Nguyen et al., 2017; Wang et al., 2017).

Challenges Moving From the Lab to the Field

While the technology of bio-inoculants holds a promising future, some major bottle necks have to be addressed to increase their efficacy. The use of PGPRs as inoculants is centuries old; the use of these inoculants have been largely focussed on legumes and cereals (Sessitsch and Mitter, 2015). Development of new PGPR inocula is based on laboratory screening assays that rely on specific PGPR mechanisms, namely nitrogen fixation, ACC deaminase activity, auxin synthesis and calcium phosphate solubilization. However, screening of pure culture isolates for those with PGPR functions does not always result in isolates that promote plant growth under field conditions. At the same time, those which have minimal *in vitro* growth promoting functions may have alternate mechanisms to promote plant growth. Since these mechanisms are less well-understood, they are difficult to screen for under laboratory conditions. As a result, beneficial strains that employ these mechanisms are discarded based on poor performance on classical *in vitro* PGPR screening methods (Cardinale et al., 2015).

Developing inocula containing highly effective microbes with a long shelf-life and high rhizosphere colonization rate poses a major challenge for commercialization. PGPR are often used to inoculate plant material without an appropriate carrier or in quantities that do not allow for efficient rhizosphere colonization under field conditions, due to competition with resident soil micro- and macro-fauna. In addition, soils growing high value crops are often fumigated with broad spectrum biocidal fumigants that alter the bio-community structure of the soil. Long-term fumigation affects soil microbes and their interactions that help plants with nutrient acquisition and mobilization, thereby affecting soil health (Dangi et al., 2017). This may also pose a challenge to rhizosphere colonization by PGPR inocula.

Plant breeding has been instrumental in the success of Green Revolution. However, in the context of bio-inoculants, very little has been done to integrate microbiome-based plant breeding to achieve a heritable PGPR community that enhances crop productivity (Mitter et al., 2013; Trivedi et al., 2017). The Green Revolution also has introduced inorganic fertilizers, pesticides, and herbicides into soils leading to extensive damage in the form of contaminants. Combining bioremediation with plant growth promotion would be a beneficial approach in addressing this global agriculture problem. Designing microbial consortia to address various aspects of bioremediation and plant growth potential is an essential aspect to this approach (Macouzet, 2016; Baez-Rogelio et al., 2017). Synthesis of bio-inoculants for specific soil conditions, to overcome environmental constraints, and training farmers and associated staff to efficiently apply them to crop plants is very important element in the development and deployment of more beneficial inocula (Bashan, 2016; Parnell et al., 2016; Itelima et al., 2018).

APPLICATIONS

Bacteria with multiple benefits can be advantageous in commercial agriculture and are relevant to the bio-economy. Many plants of economic significance are grown in monoculture and require amendments for optimal growth and yield, as well as protection against disease organisms (Vejan et al., 2016; Andreote and Pereira, 2017).

Increasing Yield and Decreasing Fertilizer Inputs

Utilization of bacterial consortia has inconsistent effects on crop yield (Wu et al., 2009). The mixing of a bacterium (*B. amyloliquefaciens*) with a fungus (*Trichoderma virens*) improves yields of corn and tomato, among other crops (Akladious and Abbas, 2012; Molla et al., 2012) and is available in the market place. The company Excalibre-SA (ABM) combines *Trichoderma* with *Bradyrhizobium* for improved growth of soybean while BioGrow Endo (Mycorrhizal Applications) combines arbuscular mycorrhizal fungi and *Trichoderma* for improved growth and treatment of pathogens present in the soil; both of which are commercially available.

Inoculation with N-fixing bacteria (*Azospirillum* and *Azobacter*) allowed half-rate N-fertilizer application and increased sesame seed yield and oil quality (Shakeri et al., 2016). Similar effects were shown for *Azospirillum vinelandii* inoculated *Brassica carinata* cv. *Peela raya* (Nosheen et al., 2016a,b). A consortium of bacteria (*Bacillus cereus* PX35, *Bacillus subtilis* SM21, and *Serrati asp* XY2) reduced the incidence of root knot nematode (*Meloidogyne incognito*) in tomato, increased fruit yield (31.5 to 39%) and quality (soluble sugars, vitamin C, and titratable acids) (Niu et al., 2016).

Advanced biofuels are derived from non-food biomass (Ajjawi et al., 2017), often lignocellulosic material, to minimize any competition with food production; the long-term goal is provision of renewable fuels, along with high value bio-products, to reduce the atmospheric CO_2 emissions associated with fossil fuels (Rokem and Greenblatt, 2015). Conversion of lignocellulosic material to fuel needs to become easier and less expensive to make this fuel economically competitive (Kuhad et al., 2011); in addition, there needs to be improved biomass availability from purpose-grown biomass crops (e.g., *Miscanthus,* switchgrass, and *Sorghum bicolour*) (Carpita and McCann, 2008; Lynd et al., 2008; Margaritopoulou et al., 2016; McCalmont et al., 2017). The growth and productivity of purpose grown biofuel crops can be improved through inoculation with PGPR (Smith et al., 2015a) as has been demonstrated for switchgrass

(Ker et al., 2012, 2014; Shanta et al., 2016; Arunachalam et al., 2017). Marginal and contaminated lands can be used to grow biofuel crops in order to avoid conflicts around food versus energy crops. With the use of PGPR that contain natural potential to cope with soil contaminants, the biofuel crops could be used efficiently for phytoremediation and also to reduce high levels of agrochemicals residues in agriculture lands (Weyens et al., 2009b; Evangelou and Deram, 2014).

Improving Disease Control and Reducing the Use of Agrochemicals

Biologicals are an alternative method for combating plant pathogens (Harman, 2000), and there are commercially available examples (Velivelli et al., 2014). Beneficial rhizobacteria may secrete antibiotics and other compounds antagonistic to plant pathogens. Production of antibiotics is one of the more common biocontrol mechanisms (Fravel, 1988; Doumbou et al., 2001; Compant et al., 2005). There are commercially available examples of biocontrol agents (Velivelli et al., 2014).

Pathogens often develop resistance to the antibiotics and other mechanisms of biocontrol, so that they cannot be fully controlled in the long-term. A holistic approach with multiple controlling methods is probably better than excessive dependency on a single solution when confronting pathogens. Over the long term, pathogen-antagonistic bacteria will also evolve their mode of action to counteract the pathogens. PGPR also produce antibiotics such as lipopeptides, polyketides and antifungal metabolites that suppress pathogens (Prashar et al., 2013).

ROADMAP TO COMMERCIALIZATION

Bioformulations of the products for plant growth promotion, soil fertility and suppression of phytopathogens offer green alternatives to conventional agrochemicals (Arora et al., 2016). Agricultural products can be developed on the basis of live single- or multi-species inoculum or based on isolated signal molecules. In the case of signal compounds, one can use microbe-to-plant signals, for direct effects on the plants, or even plant-to-microbe signals to trigger enhanced production of the microbe-to-plant signals in the soil environment, assuming the presence of the microbe in the soil. One could also use plant-to-microbe signals to control the composition of the phytomicrobiome in ways that are beneficial to the crop plants.

The development of PGPR-based inoculants is not strictly defined but generally includes the following steps:

(1) Isolation of the bacteria from roots or other plant tissues.
(2) Laboratory and controlled growth environment screening.
(3) Field screening for a range of crops, geographic locations, planting dates and soil types.
(4) Evaluation of the possible combinations of strains and/or signals.
(5) Consideration of the management practices (e.g., agrochemical use and rotation)
(6) Refinement of the product.
(7) Experiments confirming absence eco-toxicological effects.
(8) Product delivery formulation – e.g., peat, granular, liquid or wettable powder.
(9) Registration and regulatory approval of the product.
(10) Product available on the market.

Live PGPR Inoculum

For the development of a single-strain inoculum, one begins by isolating microbes from plants. This is achieved by extensive sampling of plants from a range of habitats (agricultural, dry, wet, cold, hot, and saline). Currently efforts are more focused on the rhizomicrobiome as it has the greatest microbial diversity. Once the cultivable strains have been isolated, they can be screened for ability to enhance germination of *Arabidopsis*, or crop plants. Promising isolates can then be screened for ability to accelerate emergence and early plant growth, under controlled environment conditions. Germination and early plant growth experimentation should be conducted under both optimal and stressful plant-growth conditions. In general, the easiest stress to apply uniformly is salt stress; salt stress responses are generally representative of responses expected for other stresses (Subramanian et al., 2016a,b). However, if a signal molecule responsible for effects on plant growth is a protein, saline conditions may denature it, rendering it ineffective; this is why experiments should also be conducted under optimal and other stressful conditions, time and resources permitting. The most promising PGPR can then be evaluated under the more complex and demanding conditions of the field, to select the top-performing strains for commercialization.

When screening for strains that control diseases (Weyens et al., 2009a; Wagner et al., 2014) Petri plate assays can be used to test for biocontrol activity against common plant pathogens. The disease strain is inoculated onto potato dextrose agar (PDA), and the PGPR strain is inoculated on a disk of filter paper to determine an inhibition or kill zone around the disk (Ilangumaran and Smith, 2017; Ilangumaran et al., 2017; Takishita et al., 2018). Results can be validated *in planta*, under controlled conditions and eventually under field conditions.

It is clear that some strains will be overlooked with this approach. Not all PGPR strains will be cultivable. In addition, there could be strains that do not show promising results at early stages (e.g., do not affect germination) but would enhance subsequent growth. However, given the large number of strains to evaluate at this stage, we must accept this risk and consider revisiting the situation once initial-stage screening is complete.

In the case of consortia, managing the strains so that they are in consistent proportions within the resulting product can be a challenge; combining the strains near or at the end of their growth cycles may result in the most reliable outcomes. However, consortia, through interactions among the strains, may well offer advantages over single strain-based inoculum.

Signal Compound-Based Products

For strains showing promise, effective signal compounds, potentially biostimulants, can be isolated and developed into products. To do this, PGPR strains are grown in broth cultures and then the cells are removed through a combination of centrifugation and filtration (Gray and Smith, 2005; Gray et al., 2006). The

supernatant can then be evaluated for the ability to promote seed germination and early plant growth, as described in the "Live PGPR inoculum" section. If the liquid promotes growth, then it can be concentrated and subjected to HPLC for fractionation. Fractions corresponding to peaks are collected and their ability to promote plant growth under controlled conditions, or biocontrol activity against a pathogen, using the Petri plate methods described in "Live PGPR inoculum" section. Once a given peak has demonstrated activity, the compound is isolated, purified and subjected to mass spectrometry and, possibly other chemical analyses, to determine its identity.

At this time, there are signal-based products on the market that use microbe-to-plant signals. In some cases, the signal molecule is produced on an industrial scale by cultivation of PGPR in the presence of a plant-to-microbe signal molecule which triggers the production of the microbe-to-plant signal molecule. For example, the production of LCOs by rhizobia can be triggered by addition of appropriate plant-to-microbe signals, generally isoflavonoids (Smith et al., 2015b), although in some cases jasmonates can also be used (Mabood et al., 2014). The addition of isoflavonoids to trigger LCO production has been developed as a technology and is now widely applied as a growth enhancement for a broad range of crops (Smith et al., 2017). Thuricin 17, a small protein produced by *B. thuringiensis* NEB17, and LCOs can both be extremely effective in mitigating the effects of abiotic stresses on a wide range of crop plants (Subramanian et al., 2016a,b). Thuricin 17 is in the early stages of being commercialized.

Product Formulation, Registration and Intellectual Property

To generate PGPR- or signal compound-based products, formulations must be developed that allow for even distribution in the field. For example, the legume inoculant industry has focused on solid carriers, the most common of which is sterilized peat (Bashan et al., 2014), which is inoculated with cells, and adhered to seeds using sticking agent at the time of sowing. Due to concerns about sustainable sourcing of peat, alternative solid carriers such as alginate (Bashan, 2016) have been investigated. Recently, biochar has been shown to be a high potential alternative because its porosity and nutrient content can be altered according to source material and production conditions (Głodowska et al., 2016).

Alternatively, liquid inoculants can be sprayed onto seeds prior to sowing or dripped into the seed furrow at the time of sowing. Signal molecules are probably best applied as liquid sprays, although slow release solid formulations could also be investigated. The ones commercialized so far have been effective at very low concentrations, so the actual mass or volume of the signals *per se* is extremely low. Storage and product lifespan are important considerations that need to be determined for a given product, to ensure microbial survival and/or bioactivity of the strain or compound of interest.

Another consideration is acute versus chronic application of PGPR or signal molecules. Acute application occurs just once or a limited number of times during a growing season, on the seed or at a target stage of crop development, or in response to environmental conditions, such as onset of drought. In the case of chronic application, the product could be applied at regularly timed sprays or as a slow-release seed treatment.

As the product nears the marketplace, it is necessary to have approval for registration. In Canada, this often requires safety and efficacy data; the product must also meet other specific regulatory requirements. However, when the technology is very novel, it may not fit into pre-existing regulatory categories and therefore require the regulatory agency to conduct consultations. Important considerations include manufacturing practices and documentation of efficacy and safety from a third party.

Currently, the regulatory procedures for registration and commercialization of biostimulants are complex. The main reason for the absence of a specific harmonized framework for European Union, United States, and Canada, is that there is no standard legal or regulatory definition for plant biostimulants. Du Jardin (2015) proposed the following definition: "A plant biostimulant is any substance or microorganism applied to plants with the aim to enhance nutrition efficiency, abiotic stress tolerance and/or crop quality traits, regardless of its nutrients content." This definition could be amended to include: By extension, plant biostimulants also designate commercial products containing mixtures of such substances and/or microorganisms.

The biostimulants currently available in Europe, are registered via two routes: (1) the European pesticides law which combines supranational and national provisions for introducing plant protection products on the national markets or (2) following the national regulations on fertilizers specific to each European state. In the United States, federal agencies (EPA and USDA) regulate registration of biostimulant products. Every state has its own set of compliance programs for their registration, which follow state-specific standards, fees and other mandates (Du Jardin, 2015). Presently every product submitted for registration in Canada, is considered as a unique product; therefore, every biostimulant is commercialized via its own pathway. When the product is sold it will be under a label with specific claims. If the claims are around enhanced nutrient uptake and other fertility aspects the product may be grouped with fertilizers and approval may be more straightforward. If the product is a biocontrol agent (Berendsen et al., 2012; Gu et al., 2016) with claims related to -cidal activity there will be additional scrutiny and time involved. It can be wise to claim fewer properties at the early stages of licensing to move more quickly to market, however, this may constrain the ability to claim further benefits after licensing. In terms of efficacy testing, if this is required, it may be good to have, at least in the later stages, on-farm testing, as this causes the grower community to be more engaged, which enhances acceptance and edges toward marketing.

Of course, underlying all stages of product development is the matter of intellectual property. One can no longer patent life forms or naturally occurring compounds, but formulations and uses can be patented (Matthews and Cuchiara, 2014). Thus, when a novel technology is possible, a patent search must be conducted. If there is freedom to operate (FTO) then a patent application can be filed; if enough supporting data is available,

the full application can occur immediately. If time is required to produce supporting data, an application for a 1-year provisional patent can be submitted and followed by a full and formal patent application.

Private-Public Partnerships for Increased Knowledge and Improved Training

Every step in the process from microbe isolation to licensing is laborious, expensive and requires time. Collaboration between industrial, academic and government research should become an important part of the product development process. Biotechnology organizations, for example, Genentech in South San Francisco, California encouraged their researchers to conduct side scientific projects and share their outcomes in publications. Universities are now pursuing commercialization of their innovation discoveries. Today, associations among companies and the scholarly world are common (Tachibana, 2013). As the sector develops there will be a need to train more experts in the area, through university research activities, often in collaboration with industry, as this brings the commercialization perspective to the research activities and imparts it to the trainee.

CONCLUSION

The relationships between plants and the phytomicrobiome are ancient and represent the result of a very long coevolution. Evolution is pragmatic, random and relentless, and we should expect to discover many additional and sometimes surprising relationships that are beneficial to crops, and therefore global food production. It is clear that members of the phytomicrobiome offer huge potential in terms of new and more sustainable crop management practices, however, it is also clear that we understand only a tiny amount of this potential and a very great deal remains to be done.

Probably the easiest area for exploitation at the outset will be around single strains or consortia with small numbers of members and/or the signal compounds they produce. These could be focused on stimulation of plant growth, particularly under adverse conditions, such heat and drought stress, which are becoming increasingly prevalent as climate change progresses. Another set of products could be focused on plant disease control. We have examined the steps necessary to develop these technologies into products and have them approved for sale through the regulatory process.

Finally, one should take care to have "public license." At this point the public perception of "bio" is not overly well formed, but generally positive. At the same time, there is public concern around the use of "chemicals" and biologicals are seen as a positive alternative, in the form of "plant probiotics." It is our duty to try to anticipate any problems with phytomicrobiome technologies and to forestall their development, while projecting the benefits to the public. These technologies should be compliant with organic crop production practices and it would be useful to have them registered as such. The phytomicrobiome offers enormous potential for agricultural benefit, in terms of global food security, crop production sustainability and making agricultural systems climate change resilient. We need to ensure that this is approached in a systematic, thorough and broadly considered manner.

AUTHOR CONTRIBUTIONS

Each author contributed by generating drafts of specific sections of the manuscript and then participating in repeated editing of the manuscript as it moved toward its final form.

FUNDING

Generation of this review was supported by BioFuelNet Canada (funded by the Canadian Networks of Centers of Excellence).

ACKNOWLEDGMENTS

We acknowledge the Hebrew University of Jerusalem, which made Dr. Rokem's time available for this work.

REFERENCES

Adam, E., Groenenboom, A. E., Kurm, V., Rajewska, M., Schmidt, R., Tyc, O., et al. (2016). Controlling the microbiome: microhabitat adjustments for successful biocontrol strategies in soil and human gut. *Front. Microbiol.* 7:1079. doi: 10.3389/fmicb.2016.01079

Adesemoye, A., Torbert, H., and Kloepper, J. (2008). Enhanced plant nutrient use efficiency with PGPR and AMF in an integrated nutrient management system. *Can. J. Microbiol.* 54, 876–886. doi: 10.1139/w08-081

Adesemoye, A. O., and Kloepper, J. W. (2009). Plant–microbes interactions in enhanced fertilizer-use efficiency. *Appl. Microbiol. Biotechnol.* 85, 1–12. doi: 10.1007/s00253-009-2196-0

Afzal, I., Shinwari, Z. K., and Iqrar, I. (2015). Selective isolation and characterization of agriculturally beneficial endophytic bacteria from wild hemp using canola. *Pak. J. Bot.* 47, 1999–2008.

Agler, M. T., Ruhe, J., Kroll, S., Morhenn, C., Kim, S. T., Weigel, D., et al. (2016). Microbial hub taxa link host and abiotic factors to plant microbiome variation. *PLoS Biol.* 14:e1002352. doi: 10.1371/journal.pbio.1002352

Ahemad, M. (2015). Phosphate-solubilizing bacteria-assisted phytoremediation of metalliferous soils: a review. *3 Biotech* 5, 111–121. doi: 10.1007/s13205-014-0206-0

Ahemad, M., and Kibret, M. (2014). Mechanisms and applications of plant growth promoting rhizobacteria: current perspective. *J. King Saud Univ. Sci.* 26, 1–20. doi: 10.1016/j.jksus.2013.05.001

Ahmed, E., and Holmstrom, S. J. (2014). Siderophores in environmental research: roles and applications. *Microb. Biotechnol.* 7, 196–208. doi: 10.1111/1751-7915.12117

Ajjawi, I., Verruto, J., Aqui, M., Soriaga, L. B., Coppersmith, J., Kwok, K., et al. (2017). Lipid production in *Nannochloropsis gaditana* is doubled by decreasing expression of a single transcriptional regulator. *Nat. Biotechnol.* 35, 647–652. doi: 10.1038/nbt.3865

Akladious, S. A., and Abbas, S. M. (2012). Application of Trichoderma harziunum T22 as a biofertilizer supporting maize growth. *Afr. J. Biotechnol.* 11, 8672–8683.

Anderl, J. N., Franklin, M. J., and Stewart, P. S. (2000). Role of antibiotic penetration limitation in *Klebsiella pneumoniae* biofilm resistance to ampicillin and ciprofloxacin. *Antimicrob. Agents Chemother.* 44, 1818–1824.

Andreote, F. D., and Pereira, E. S. M. C. (2017). Microbial communities associated with plants: learning from nature to apply it in agriculture. *Curr. Opin. Microbiol.* 37, 29–34. doi: 10.1016/j.mib.2017.03.011

Arora, N. K., Mehnaz, S., and Balestrini, R. (2016). *Bioformulations: For Sustainable Agriculture*. Berlin: Springer. doi: 10.1016/j.mib.2017.03.011

Arunachalam, S., Schwinghamer, T., Dutilleul, P., and Smith, D. L. (2017). Multi-year effects of biochar, lipo-chitooligosaccharide, thuricin 17, and experimental bio-fertilizer for switchgrass. *Agron. J.* 110, 77–84. doi: 10.2134/agronj2017.05.0278

Babalola, O. O. (2010). Beneficial bacteria of agricultural importance. *Biotechnol. Lett.* 32, 1559–1570. doi: 10.1007/s10529-010-0347-0

Backer, R. G. M., Saeed, W., Seguin, P., and Smith, D. L. (2017). Root traits and nitrogen fertilizer recovery efficiency of corn grown in biochar-amended soil under greenhouse conditions. *Plant Soil* 415, 465–477. doi: 10.1007/s10529-010-0347-0

Badri, D. V., Chaparro, J. M., Zhang, R., Shen, Q., and Vivanco, J. M. (2013). Application of natural blends of phytochemicals derived from the root exudates of Arabidopsis to the soil reveal that phenolic-related compounds predominantly modulate the soil microbiome. *J. Biol. Chem.* 288, 4502–4512. doi: 10.1074/jbc.M112.433300

Badri, D. V., and Vivanco, J. M. (2009). Regulation and function of root exudates. *Plant Cell Environ.* 32, 666–681. doi: 10.1111/j.1365-3040.2008.01926.x

Baez-Rogelio, A., Morales-García, Y. E., Quintero-Hernández, V., and Muñoz-Rojas, J. (2017). Next generation of microbial inoculants for agriculture and bioremediation. *Microb. Biotechnol.* 10, 19–21. doi: 10.1111/1751-7915.12448

Bailly, A., and Weisskopf, L. (2012). The modulating effect of bacterial volatiles on plant growth: current knowledge and future challenges. *Plant Signal. Behav.* 7, 79–85. doi: 10.4161/psb.7.1.18418

Bais, H. P., Fall, R., and Vivanco, J. M. (2004). Biocontrol of *Bacillus subtilis* against infection of Arabidopsis roots by *Pseudomonas* syringae is facilitated by biofilm formation and surfactin production. *Plant Physiol.* 134, 307–319. doi: 10.4161/psb.7.1.18418

Bais, H. P., Weir, T. L., Perry, L. G., Gilroy, S., and Vivanco, J. M. (2006). The role of root exudates in rhizosphere interactions with plants and other organisms. *Annu. Rev. Plant Biol.* 57, 233–266. doi: 10.1104/pp.103.028712

Bakker, M. G., Manter, D. K., Sheflin, A. M., Weir, T. L., and Vivanco, J. M. (2012). Harnessing the rhizosphere microbiome through plant breeding and agricultural management. *Plant Soil* 360, 1–13. doi: 10.1146/annurev.arplant.57.032905.105159

Barea, J. M. (2015). Future challenges and perspectives for applying microbial biotechnology in sustainable agriculture based on a better understanding of plant-microbiome interactions. *J. Soil Sci. Plant Nutr.* 15, 261–282. doi: 10.1007/s11104-012-1361-x

Bashan, N. (2016). Inoculant formulations are essential for successful inoculation with plant growth-promoting bacteria and business opportunities. *Indian Phytopathol.* 69, 739–743.

Bashan, Y. (1998). Inoculants of plant growth-promoting bacteria for use in agriculture. *Biotechnol. Adv.* 16, 729–770. doi: 10.1016/S0734-9750(98)00003-2

Bashan, Y., and de-Bashan, L. E. (2015). "Inoculant preparation and formulations for azospirillum spp," in *Handbook for Azospirillum*, eds F. D. Cassán, Y. Okon, and C. M. Creus (Berlin: Springer), 469–485.

Bashan, Y., De-Bashan, L. E., Prabhu, S., and Hernandez, J.-P. (2014). Advances in plant growth-promoting bacterial inoculant technology: formulations and practical perspectives (1998–2013). *Plant Soil* 378, 1–33. doi: 10.1007/s11104-013-1956-x

Beattie, G. A. (2015). Microbiomes: curating communities from plants. *Nature* 528, 340–341. doi: 10.1038/nature16319

Beaudoin, T., Zhang, L., Hinz, A. J., Parr, C. J., and Mah, T. F. (2012). The biofilm-specific antibiotic resistance gene ndvB is important for expression of ethanol oxidation genes in *Pseudomonas aeruginosa* biofilms. *J. Bacteriol.* 194, 3128–3136. doi: 10.1128/JB.06178-11

Bender, S. F., Wagg, C., and Van Der Heijden, M. G. (2016). An underground revolution: biodiversity and soil ecological engineering for agricultural sustainability. *Trends Ecol. Evol.* 31, 440–452. doi: 10.1016/j.tree.2016.02.016

Berendsen, R. L., Pieterse, C. M., and Bakker, P. A. (2012). The rhizosphere microbiome and plant health. *Trends Plant Sci.* 17, 478–486. doi: 10.1016/j.tplants.2012.04.001

Berg, G., Grube, M., Schloter, M., and Smalla, K. (2014). Unraveling the plant microbiome: looking back and future perspectives. *Front. Microbiol.* 5:148. doi: 10.3389/fmicb.2014.00148

Berg, G., Rybakova, D., Grube, M., and Koberl, M. (2016). The plant microbiome explored: implications for experimental botany. *J. Exp. Bot.* 67, 995–1002. doi: 10.1093/jxb/erv466

Bharti, N., Pandey, S. S., Barnawal, D., Patel, V. K., and Kalra, A. (2016). Plant growth promoting rhizobacteria *Dietzia natronolimnaea* modulates the expression of stress responsive genes providing protection of wheat from salinity stress. *Sci. Rep.* 6:34768. doi: 10.1038/srep34768

Bhatti, T. M., and Yawar, W. (2010). Bacterial solubilization of phosphorus from phosphate rock containing sulfur-mud. *Hydrometallurgy* 103, 54–59. doi: 10.1016/j.hydromet.2010.02.019

Borriss, R. (2011). "Use of plant-associated Bacillus strains as biofertilizers and biocontrol agents in agriculture," in *Bacteria in Agrobiology: Plant Growth Responses*, ed. D. K. Maheshwari (Berlin: Springer), 41–76.

Bossio, D. A., Scow, K. M., Gunapala, N., and Graham, K. J. (1998). Determinants of soil microbial communities: effects of agricultural management, season, and soil type on phospholipid fatty acid profiles. *Microb. Ecol.* 36, 1–12. doi: 10.1007/s002489900087

Broeckling, C. D., Broz, A. K., Bergelson, J., Manter, D. K., and Vivanco, J. M. (2008). Root exudates regulate soil fungal community composition and diversity. *Appl. Environ. Microbiol.* 74, 738–744. doi: 10.1128/AEM.02188-07

Budania, K., and Yadav, J. (2014). Effects of PGPR blended biochar and different levels of phosphorus on yield and nutrient uptake by chickpea. *Ann. Agric. Bio Res.* 19, 408–412.

Bulgarelli, D., Garrido-Oter, R., Munch, P. C., Weiman, A., Droge, J., Pan, Y., et al. (2015). Structure and function of the bacterial root microbiota in wild and domesticated barley. *Cell Host Microbe* 17, 392–403. doi: 10.1016/j.chom.2015.01.011

Bulgarelli, D., Schlaeppi, K., Spaepen, S., Ver Loren Van Themaat, E., and Schulze-Lefert, P. (2013). Structure and functions of the bacterial microbiota of plants. *Annu. Rev. Plant Biol.* 64, 807–838. doi: 10.1146/annurev-arplant-050312-120106

Cardinale, M., Ratering, S., Suarez, C., Zapata Montoya, A. M., Geissler-Plaum, R., and Schnell, S. (2015). Paradox of plant growth promotion potential of rhizobacteria and their actual promotion effect on growth of barley (Hordeum vulgare L.) under salt stress. *Microbiol. Res.* 181, 22–32. doi: 10.1016/j.micres.2015.08.002

Carotenuto, G., Chabaud, M., Miyata, K., Capozzi, M., Takeda, N., Kaku, H., et al. (2017). The rice LysM receptor-like kinase OsCERK1 is required for the perception of short-chain chitin oligomers in arbuscular mycorrhizal signaling. *New Phytol.* 214, 1440–1446. doi: 10.1111/nph.14539

Carpita, N. C., and McCann, M. C. (2008). Maize and sorghum: genetic resources for bioenergy grasses. *Trends Plant Sci.* 13, 415–420. doi: 10.1016/j.tplants.2008.06.002

Chabaud, M., Gherbi, H., Pirolles, E., Vaissayre, V., Fournier, J., Moukouanga, D., et al. (2016). Chitinase-resistant hydrophilic symbiotic factors secreted by Frankia activate both Ca2 + spiking and NIN gene expression in the actinorhizal plant *Casuarina glauca*. *New Phytol.* 209, 86–93. doi: 10.1111/nph.13732

Chaparro, J. M., Badri, D. V., and Vivanco, J. M. (2014). Rhizosphere microbiome assemblage is affected by plant development. *ISME J.* 8:790. doi: 10.1038/ismej.2013.196

Chaparro, J. M., Sheflin, A. M., Manter, D. K., and Vivanco, J. M. (2012). Manipulating the soil microbiome to increase soil health and plant fertility. *Biol. Fertil. Soils* 48, 489–499. doi: 10.1007/s00374-012-0691-4

Chauhan, H., Bagyaraj, D., Selvakumar, G., and Sundaram, S. (2015). Novel plant growth promoting rhizobacteria—prospects and potential. *Appl. Soil Ecol.* 95, 38–53. doi: 10.1016/j.apsoil.2015.05.011

Chen, L., Liu, Y. P., Wu, G. W., Njeri, K. V., Shen, Q. R., Zhang, N., et al. (2016). Induced maize salt tolerance by rhizosphere inoculation of Bacillus amyloliquefaciens SQR9. *Physiol. Plant.* 158, 34–44. doi: 10.1111/ppl.12441

Chen, Y., Yan, F., Chai, Y., Liu, H., Kolter, R., Losick, R., et al. (2013). Biocontrol of tomato wilt disease by *Bacillus subtilis* isolates from natural environments depends on conserved genes mediating biofilm formation. *Environ. Microbiol.* 15, 848–864. doi: 10.1111/j.1462-2920.2012.02860.x

Cheng, Z.-M. (2014). *Introduction to the Special Issue: Stress Biology of Specialty Crops*. Didcot: Taylor & Francis.

Clough, T. J., and Condron, L. M. (2010). Biochar and the nitrogen cycle: introduction. *J. Environ. Qual.* 39, 1218–1223. doi: 10.2134/jeq2010.0204

Coleman-Derr, D., Desgarennes, D., Fonseca-Garcia, C., Gross, S., Clingenpeel, S., Woyke, T., et al. (2016). Plant compartment and biogeography affect microbiome composition in cultivated and native Agave species. *New Phytol.* 209, 798–811. doi: 10.1111/nph.13697

Compant, S., Duffy, B., Nowak, J., Clément, C., and Barka, E. A. (2005). Use of plant growth-promoting bacteria for biocontrol of plant diseases: principles, mechanisms of action, and future prospects. *Appl. Environ. Microbiol.* 71, 4951–4959. doi: 10.1128/AEM.71.9.4951-4959.2005

Dakora, F., Matiru, V., and Kanu, A. (2015). Rhizosphere ecology of lumichrome and riboflavin, two bacterial signal molecules eliciting developmental changes in plants. *Front. Plant Sci.* 6:700. doi: 10.3389/fpls.2015.00700

Dangi, S., Tirado-Corbalá, R., Gerik, J., and Hanson, B. (2017). Effect of long-term continuous fumigation on soil microbial communities. *Agronomy* 7:37. doi: 10.3390/agronomy7020037

Danhorn, T., and Fuqua, C. (2007). Biofilm formation by plant-associated bacteria. *Annu. Rev. Microbiol.* 61, 401–422. doi: 10.1146/annurev.micro.61.080706.093316

Deb, D., Kloft, M., Lassig, J., and Walsh, S. (2016). Variable effects of biochar and P solubilizing microbes on crop productivity in different soil conditions. *Agroecol. Sustain. Food Syst.* 40, 145–168. doi: 10.1080/21683565.2015.1118001

Delaplace, P., Delory, B. M., Baudson, C., Mendaluk-Saunier, De Cazenave, M., Spaepen, S., et al. (2015). Influence of rhizobacterial volatiles on the root system architecture and the production and allocation of biomass in the model grass *Brachypodium distachyon* (L.) P. Beauv. *BMC Plant Biol.* 15:195. doi: 10.1186/s12870-015-0585-3

Desbrosses, G. J., and Stougaard, J. (2011). Root nodulation: a paradigm for how plant-microbe symbiosis influences host developmental pathways. *Cell Host Microbe* 10, 348–358. doi: 10.1016/j.chom.2011.09.005

Dixit, R., Agrawal, L., Gupta, S., Kumar, M., Yadav, S., Chauhan, P. S., et al. (2016). Southern blight disease of tomato control by 1-aminocyclopropane-1-carboxylate (ACC) deaminase producing *Paenibacillus lentimorbus* B-30488. *Plant Signal. Behav.* 11:e1113363. doi: 10.1080/15592324.2015.1113363

Donlan, R. M. (2002). Biofilms: microbial life on surfaces. *Emerg. Infect. Dis.* 8, 881–890. doi: 10.3201/eid0809.020063

Doornbos, R. F., Geraats, B. P., Kuramae, E. E., Van Loon, L. C., and Bakker, P. A. (2011). Effects of jasmonic acid, ethylene, and salicylic acid signaling on the rhizosphere bacterial community of *Arabidopsis thaliana*. *Mol. Plant Microbe Interact.* 24, 395–407. doi: 10.1094/MPMI-05-10-0115

Doumbou, C. L., Salove, M. K. H., Crawford, D. L., and Beaulieu, C. (2001). Actinomycetes, promising tools to control plant diseases and to promote plant growth. *Phytoprotection* 82, 85–102. doi: 10.7202/706219ar

Drenovsky, R. E., Vo, D., Graham, K. J., and Scow, K. M. (2004). Soil water content and organic carbon availability are major determinants of soil microbial community composition. *Microb. Ecol.* 48, 424–430. doi: 10.1007/s00248-003-1063-2

Du Jardin, P. (2015). Plant biostimulants: definition, concept, main categories and regulation. *Sci. Hortic.* 196, 3–14. doi: 10.1016/j.scienta.2015.09.021

Eberl, L., von Bodman, S. B., and Fuqua, C. (2007). Biofilms on plant surfaces. *Biofilm Mode Life Mech. Adapt.* 214–233.

Egamberdieva, D., Wirth, S., Behrendt, U., Abd Allah, E. F., and Berg, G. (2016). Biochar treatment resulted in a combined effect on soybean growth promotion and a shift in plant growth promoting rhizobacteria. *Front. Microbiol.* 7:209. doi: 10.3389/fmicb.2016.00209

Etesami, H., Mirseyed Hosseini, H., and Alikhani, H. A. (2014). Bacterial biosynthesis of 1-aminocyclopropane-1-caboxylate (ACC) deaminase, a useful trait to elongation and endophytic colonization of the roots of rice under constant flooded conditions. *Physiol. Mol. Biol. Plants* 20, 425–434. doi: 10.1007/s12298-014-0251-5

Evangelou, M. W., and Deram, A. (2014). Phytomanagement: a realistic approach to soil remediating phytotechnologies with new challenges for plant science. *Int. J. Plant. Biol. Res.* 2:1023.

Fahad, S., Hussain, S., Bano, A., Saud, S., Hassan, S., Shan, D., et al. (2015). Potential role of phytohormones and plant growth-promoting rhizobacteria in abiotic stresses: consequences for changing environment. *Environ. Sci. Pollut. Res.* 22, 4907–4921. doi: 10.1007/s11356-014-3754-2

Fernandez, O., Theocharis, A., Bordiec, S., Feil, R., Jacquens, L., Clement, C., et al. (2012). *Burkholderia phytofirmans* PsJN acclimates grapevine to cold by modulating carbohydrate metabolism. *Mol. Plant Microbe Interact.* 25, 496–504. doi: 10.1094/MPMI-09-11-0245

Fonseca-García, C., Coleman-Derr, D., Garrido, E., Visel, A., Tringe, S. G., and Partida-Martínez, L. P. (2016). The cacti microbiome: interplay between habitat-filtering and host-specificity. *Front. Microbiol.* 7:150. doi: 10.3389/fmicb.2016.00150

Fravel, D. R. (1988). Role of antibiosis in the biocontrol of plant-diseases. *Annu. Rev. Phytopathol.* 26, 75–91. doi: 10.1146/annurev.py.26.090188.000451

Galloway, J. N., Townsend, A. R., Erisman, J. W., Bekunda, M., Cai, Z., Freney, J. R., et al. (2008). Transformation of the nitrogen cycle: recent trends, questions, and potential solutions. *Science* 320, 889-892. doi: 10.1126/science.1136674

Garcia de Salamone, I. E., Hynes, R. K., and Nelson, L. M. (2001). Cytokinin production by plant growth promoting rhizobacteria and selected mutants. *Can. J. Microbiol.* 47, 404–411. doi: 10.1139/w01-029

Garcia-Pausas, J., and Paterson, E. (2011). Microbial community abundance and structure are determinants of soil organic matter mineralisation in the presence of labile carbon. *Soil Biol. Biochem.* 43, 1705–1713. doi: 10.1016/j.soilbio.2011.04.016

Gaskin, J. W., Speir, R. A., Harris, K., Das, K. C., Lee, R. D., Morris, L. A., et al. (2010). Effect of peanut hull and pine chip biochar on soil nutrients, corn nutrient status, and yield. *Agron. J.* 102, 623–633. doi: 10.2134/agronj2009.0083

Glick, B. R. (2014). Bacteria with ACC deaminase can promote plant growth and help to feed the world. *Microbiol. Res.* 169, 30–39. doi: 10.1016/j.micres.2013.09.009

Głodowska, M., Husk, B., Schwinghamer, T., and Smith, D. (2016). Biochar is a growth-promoting alternative to peat moss for the inoculation of corn with a pseudomonad. *Agron. Sustain. Dev.* 36, 1–10. doi: 10.1007/s13593-016-0356-z

Gransee, A., and Wittenmayer, L. (2000). Qualitative and quantitative analysis of water-soluble root exudates in relation to plant species and development. *J. Plant Nutr. Soil Sci.* 163, 381–385. doi: 10.1002/1522-2624(200008)163:4<381::AID-JPLN381>3.0.CO;2-7

Gray, E. J., Di Falco, M., Souleimanov, A., and Smith, D. L. (2006). Proteomic analysis of the bacteriocin thuricin 17 produced by *Bacillus thuringiensis* NEB17. *FEMS Microbiol. Lett.* 255, 27–32. doi: 10.1111/j.1574-6968.2005.00054.x

Gray, E. J., and Smith, D. L. (2005). Intracellular and extracellular PGPR: commonalities and distinctions in the plant-bacterium signaling processes. *Soil Biol. Biochem.* 37, 395–412. doi: 10.1016/j.soilbio.2004.08.030

Gu, Y., Wei, Z., Wang, X. Q., Friman, V. P., Huang, J. F., Wang, X. F., et al. (2016). Pathogen invasion indirectly changes the composition of soil microbiome via shifts in root exudation profile. *Biol. Fertil. Soils* 52, 997–1005. doi: 10.1007/s00374-016-1136-2

Gupta, G., Parihar, S. S., Ahirwar, N. K., Snehi, S. K., and Singh, V. (2015). Plant growth promoting rhizobacteria (PGPR): current and future prospects for development of sustainable agriculture. *J. Microb. Biochem. Technol.* 7, 096–102.

Gust, A. A., Willmann, R., Desaki, Y., Grabherr, H. M., and Nürnberger, T. (2012). Plant LysM proteins: modules mediating symbiosis and immunity. *Trends Plant Sci.* 17, 495–502. doi: 10.1016/j.tplants.2012.04.003

Gutjahr, C., and Paszkowski, U. (2009). Weights in the balance: jasmonic acid and salicylic acid signaling in root-biotroph interactions. *Mol. Plant Microbe Interact.* 22, 763–772. doi: 10.1094/MPMI-22-7-0763

Habib, S. H., Kausar, H., and Saud, H. M. (2016). Plant growth-promoting rhizobacteria enhance salinity stress tolerance in okra through ROS-scavenging enzymes. *Biomed. Res. Int.* 2016:6284547. doi: 10.1155/2016/6284547

Hafeez, F. Y., Abaid-Ullah, M., and Hassan, M. N. (2013). "Plant growth-promoting rhizobacteria as zinc mobilizers: a promising approach for cereals biofortification," in *Bacteria in Agrobiology: Crop Productivity*, eds D. K. Maheshwari, M. Saraf, and A. Aeron (Berlin: Springer), 217–235.

Hagai, E., Dvora, R., Havkin-Blank, T., Zelinger, E., Porat, Z., Schulz, S., et al. (2014). Surface-motility induction, attraction and hitchhiking between bacterial

species promote dispersal on solid surfaces. *ISME J.* 8, 1147–1151. doi: 10.1038/ismej.2013.218

Harman, G. E. (2000). Myths and dogmas of biocontrol changes in perceptions derived from research on *Trichoderma harzinum* T-22. *Plant Dis.* 84, 377–393. doi: 10.1094/PDIS.2000.84.4.377

Herridge, D. F., Peoples, M. B., and Boddey, R. M. (2008). Global inputs of biological nitrogen fixation in agricultural systems. *Plant Soil* 311, 1–18. doi: 10.1007/s11104-008-9668-3

Heydarian, Z., Yu, M., Gruber, M., Glick, B. R., Zhou, R., and Hegedus, D. D. (2016). Inoculation of soil with plant growth promoting bacteria producing 1-aminocyclopropane-1-carboxylate deaminase or expression of the corresponding acds gene in transgenic plants increases salinity tolerance in camelina sativa. *Front. Microbiol.* 7:1966. doi: 10.3389/fmicb.2016.01966

Hiltner, L. (1904). Über neuere erfahrungen und probleme auf dem debiete der bo denbakteriologie und unter besonderer berucksichtigung der grundund und brache. *Zbl. Bakteriol.* 2, 14–25.

Hirsch, P. R., and Mauchline, T. H. (2012). Who's who in the plant root microbiome? *Nat. Biotechnol.* 30, 961–962. doi: 10.1038/nbt.2387

Hong, Y. W., Glick, B. R., and Pasternak, J. J. (1991). Plant microbial interaction under gnotobiotic conditions - a scanning electron-microscope study. *Curr. Microbiol.* 23, 111–114. doi: 10.1007/BF02092259

Houlden, A., Timms-Wilson, T. M., Day, M. J., and Bailey, M. J. (2008). Influence of plant developmental stage on microbial community structure and activity in the rhizosphere of three field crops. *FEMS Microbiol. Ecol.* 65, 193–201. doi: 10.1111/j.1574-6941.2008.00535.x

Ilangumaran, G., Lamont, J. R., and Smith, D. L. (2017). "The role of the phytomicrobiome in maintaining biofuel crop production in a changing climate," in *Microbes for Climate Resilient Agriculture*, eds P. L. Kashyap, A. K. Srivastava, S. P. Tiwari, and S. Kumar (Hoboken, NJ: John Wiley & Sons), 1–24.

Ilangumaran, G., and Smith, D. L. (2017). Plant growth promoting rhizobacteria in amelioration of salinity stress: a systems biology perspective. *Front. Plant Sci.* 8:1768. doi: 10.3389/fpls.2017.01768

Innerebner, G., Knief, C., and Vorholt, J. A. (2011). Protection of *Arabidopsis thaliana* against leaf-pathogenic *Pseudomonas* syringae by Sphingomonas strains in a controlled model system. *Appl. Environ. Microbiol.* 77, 3202–3210. doi: 10.1128/AEM.00133-11

Itelima, J. U., Bang, W. J., Onyimba, I. A., and Oj, E. (2018). A review: biofertilizer; a key player in enhancing soil fertility and crop productivity. *J. Microbiol. Biotechnol. Rep.* 2, 22–28.

Jaafar, N. M. (2014). "Biochar as a habitat for arbuscular mycorrhizal fungi," in *Mycorrhizal Fungi: Use in Sustainable Agriculture and Land Restoration*, eds Z. M. Solaiman, L. K. Abbott, and A. Varma (Berlin: Springer), 297–311.

Jayasinghearachchi, H. S., and Seneviratne, G. (2004). A bradyrhizobial-*Penicillium* spp. biofilm with nitrogenase activity improves N-2 fixing symbiosis of soybean. *Biol. Fertil. Soils* 40, 432–434. doi: 10.1007/s00374-004-0796-5

Jenkins, J. R., Viger, M., Arnold, E. C., Harris, Z. M., Ventura, M., Miglietta, F., et al. (2017). Biochar alters the soil microbiome and soil function: results of next-generation amplicon sequencing across Europe. *Glob. Chang. Biol. Bioenergy* 9, 591–612. doi: 10.1111/gcbb.12371

Jha, C. K., and Saraf, M. (2015). Plant growth promoting rhizobacteria (PGPR): a review. *E3 J. Agric. Res. Dev.* 5, 108–119.

Jones, D. L., Hodge, A., and Kuzyakov, Y. (2004). Plant and mycorrhizal regulation of rhizodeposition. *New Phytol.* 163, 459–480. doi: 10.1111/j.1469-8137.2004.01130.x

Kameyama, K., Miyamoto, T., Shiono, T., and Shinogi, Y. (2012). Influence of sugarcane bagasse-derived biochar application on nitrate leaching in calcaric dark red soil. *J. Environ. Qual.* 41, 1131–1137. doi: 10.2134/jeq2010.0453

Kang, S. M., Joo, G. J., Hamayun, M., Na, C. I., Shin, D. H., Kim, H. Y., et al. (2009). Gibberellin production and phosphate solubilization by newly isolated strain of *Acinetobacter calcoaceticus* and its effect on plant growth. *Biotechnol. Lett.* 31, 277–281. doi: 10.1007/s10529-008-9867-2

Kang, S. M., Khan, A. L., Waqas, M., You, Y. H., Hamayun, M., Joo, G. J., et al. (2015). Gibberellin-producing *Serratia nematodiphila* PEJ1011 ameliorates low temperature stress in *Capsicum annuum* L. *Eur. J. Soil Biol.* 68, 85–93. doi: 10.1016/j.ejsobi.2015.02.005

Ker, K., Seguin, P., Driscoll, B. T., Fyles, J. W., and Smith, D. L. (2012). Switchgrass establishment and seeding year production can be improved by inoculation with rhizosphere endophytes. *Biomass Bioenergy* 47, 295–301. doi: 10.1016/j.biombioe.2012.09.031

Ker, K., Seguin, P., Driscoll, B. T., Fyles, J. W., and Smith, D. L. (2014). Evidence for enhanced N availability during switchgrass establishment and seeding year production following inoculation with rhizosphere endophytes. *Arch. Agron. Soil Sci.* 60, 1553–1563. doi: 10.1080/03650340.2014.898840

Kim, H. S., Dolinar, K. D., Jarand, M. L., Traxler, V., Thompson, T. A., and Malyala, R. (2016). Methods for application of biochar. U.S. Provisional Patent Application Series No. 62/290,026. Denver, CO: Cool Planet Energy Systems, Inc.

Kloepper, J. W., Leong, J., Teintze, M., and Schroth, M. N. (1980). Enhanced plant growth by siderophores produced by plant growth-promoting rhizobacteria. *Nature* 286, 885–886. doi: 10.1038/286885a0

Kristin, A., and Miranda, H. (2013). The root microbiota—a fingerprint in the soil? *Plant Soil* 370, 671–686. doi: 10.1007/s11104-013-1647-7

Kuhad, R. C., Gupta, R., and Singh, A. (2011). Microbial cellulases and their industrial applications. *Enzyme Res.* 2011:280696. doi: 10.4061/2011/280696

Kumar, A., Bahadur, I., Maurya, B., Raghuwanshi, R., Meena, V., Singh, D., et al. (2015). Does a plant growth promoting rhizobacteria enhance agricultural sustainability. *J. Pure Appl. Microbiol.* 9, 715–724.

Kumar, S., Chauhan, P. S., Agrawal, L., Raj, R., Srivastava, A., Gupta, S., et al. (2016). *Paenibacillus lentimorbus* inoculation enhances tobacco growth and extenuates the virulence of cucumber mosaic virus. *PLoS One* 11:e0149980. doi: 10.1371/journal.pone.0149980

Lamont, J. R., Wilkins, O., Bywater-Ekegard, M., and Smith, D. L. (2017). From yogurt to yield: potential applications of lactic acid bacteria in plant production. *Soil Biol. Biochem.* 111, 1–9. doi: 10.1016/j.soilbio.2017.03.015

Leach, J. E., Triplett, L. R., Argueso, C. T., and Trivedi, P. (2017). Communication in the phytobiome. *Cell* 169, 587–596. doi: 10.1016/j.cell.2017.04.025

Lebeis, S. L. (2014). The potential for give and take in plant-microbiome relationships. *Front. Plant Sci.* 5:287. doi: 10.3389/fpls.2014.00287

Lee, S. K., Lur, H. S., Lo, K. J., Cheng, K. C., Chuang, C. C., Tang, S. J., et al. (2016). Evaluation of the effects of different liquid inoculant formulations on the survival and plant-growth-promoting efficiency of *Rhodopseudomonas palustris* strain PS3. *Appl. Microbiol. Biotechnol.* 100, 7977–7987. doi: 10.1007/s00253-016-7582-9

Li, H., Ding, X., Wang, C., Ke, H., Wu, Z., Wang, Y., et al. (2016). Control of tomato yellow leaf curl virus disease by *Enterobacter* asburiaeBQ9 as a result of priming plant resistance in tomatoes. *Turk. J. Biol.* 40, 150–159. doi: 10.3906/biy-1502-12

Llorente, B. E., Alasia, M. A., and Larraburu, E. E. (2016). Biofertilization with *Azospirillum brasilense* improves in vitro culture of *Handroanthus ochraceus*, a forestry, ornamental and medicinal plant. *N. Biotechnol.* 33, 32–40. doi: 10.1016/j.nbt.2015.07.006

Lugtenberg, B., and Kamilova, F. (2009). Plant-growth-promoting rhizobacteria. *Annu. Rev. Microbiol.* 63, 541–556. doi: 10.1146/annurev.micro.62.081307.162918

Lundberg, D. S., Lebeis, S. L., Paredes, S. H., Yourstone, S., Gehring, J., Malfatti, S., et al. (2012). Defining the core *Arabidopsis thaliana* root microbiome. *Nature* 488, 86–90. doi: 10.1038/nature11237

Lynd, L. R., Laser, M. S., Brandsby, D., Dale, B. E., Davison, B., Hamilton, R., et al. (2008). How biotech can transform biofuels. *Nat. Biotechnol.* 26, 169–172. doi: 10.1038/nbt0208-169

Mabood, F., Zhou, X., and Smith, D. L. (2014). Microbial signaling and plant growth promotion. *Can. J. Plant Sci.* 94, 1051–1063. doi: 10.4141/cjps2013-148

Macouzet, M. (2016). Critical aspects in the conception and production of microbial based plant biostimulants (MBPB). *Probiotic Intelligentsia* 5, 29–38.

Mah, T. F., and O'Toole, G. A. (2001). Mechanisms of biofilm resistance to antimicrobial agents. *Trends Microbiol.* 9, 34–39. doi: 10.1016/S0966-842X(00)01913-2

Mah, T. F., Pitts, B., Pellock, B., Walker, G. C., Stewart, P. S., and O'toole, G. A. (2003). A genetic basis for *Pseudomonas aeruginosa* biofilm antibiotic resistance. *Nature* 426, 306–310. doi: 10.1038/nature02122

Major, J. (2009). *Biochar Application to a Colombian Savanna Oxisol: Fate and Effect on Soil Fertility, Crop Production, Nutrient Leaching and Soil Hydrology*. Ithaca, NY: Cornell University.

Margaritopoulou, T., Roka, L., Alexopoulou, E., Christou, M., Rigas, S., Haralampidis, K., et al. (2016). Biotechnology towards energy crops. *Mol. Biotechnol.* 58, 149–158. doi: 10.1007/s12033-016-9913-6

Marroquí, S., Zorreguieta, A., Santamarí, A. C., Temprano, F., Soberón, M., Megías, M., et al. (2001). Enhanced symbiotic performance by *Rhizobium tropici*

glycogen synthase mutants. *J. Bacteriol.* 183, 854–864. doi: 10.1128/JB.183.3.854-864.2001

Massalha, H., Korenblum, E., Tholl, D., and Aharoni, A. (2017). Small molecules below-ground: the role of specialized metabolites in the rhizosphere. *Plant J.* 90, 788–807. doi: 10.1111/tpj.13543

Matthews, K. R., and Cuchiara, M. L. (2014). Gene patents, patenting life and the impact of court rulings on US stem cell patents and research. *Regen. Med.* 9, 191–200. doi: 10.2217/rme.13.93

McCalmont, J. P., Hastings, A., Mcnamara, N. P., Richter, G. M., Robson, P., Donnison, I. S., et al. (2017). Environmental costs and benefits of growing *Miscanthus* for bioenergy in the UK. *Glob. Chang. Biol. Bioenergy* 9, 489–507. doi: 10.1111/gcbb.12294

Mehnaz, S. (2016). "An overview of globally available bioformulations," in *Bioformulations: For Sustainable Agriculture*, eds N. K. Arora, S. Mehnaz, and R. Balestrini (Berlin: Springer), 267–281.

Micallef, S. A., Channer, S., Shiaris, M. P., and Colon-Carmona, A. (2009). Plant age and genotype impact the progression of bacterial community succession in the Arabidopsis rhizosphere. *Plant Signal. Behav.* 4, 777–780. doi: 10.1093/jxb/erp053

Mitter, B., Brader, G., Afzal, M., Compant, S., Naveed, M., Trognitz, F., et al. (2013). Advances in elucidating beneficial interactions between plants, soil, and bacteria. *Adv. Agron.* 121, 381–445. doi: 10.1016/B978-0-12-407685-3.00007-4

Mohd, M. A., and Ahmad, I. (2014). "A novel strain of *Pseudomonas fluorescens* WS1 Forms biofilm on root surface and enhances growth of wheat plant," in *2nd International Conference on Agricultural & Horticultural Sciences*, Hyderabad.

Molla, A. H., Haque, M. M., Haque, M. A., and Ilias, G. (2012). Trichoderma-enriched biofertilizer enhances production and nutritional quality of tomato (*Lycopersicon esculentum* mill.) and minimizes NPK fertilizer use. *Agric. Res.* 1, 265–272. doi: 10.1007/s40003-012-0025-7

Morgan, P. W., and Drew, M. C. (1997). Ethylene and plant responses to stress. *Physiol. Plant.* 100, 620–630. doi: 10.1111/j.1399-3054.1997.tb03068.x

Mougel, C., Offre, P., Ranjard, L., Corberand, T., Gamalero, E., Robin, C., et al. (2006). Dynamic of the genetic structure of bacterial and fungal communities at different developmental stages of *Medicago truncatula* Gaertn. cv. Jemalong line J5. *New Phytol.* 170, 165–175. doi: 10.1111/j.1469-8137.2006.01650.x

Musilova, L., Ridl, J., Polivkova, M., Macek, T., and Uhlik, O. (2016). Effects of secondary plant metabolites on microbial populations: changes in community structure and metabolic activity in contaminated environments. *Int. J. Mol. Sci.* 17:1205. doi: 10.3390/ijms17081205

Nadeem, S. M., Ahmad, M., Zahir, Z. A., Javaid, A., and Ashraf, M. (2014). The role of mycorrhizae and plant growth promoting rhizobacteria (PGPR) in improving crop productivity under stressful environments. *Biotechnol. Adv.* 32, 429–448. doi: 10.1016/j.biotechadv.2013.12.005

Nadeem, S. M., Imran, M., Naveed, M., Khan, M. Y., Ahmad, M., Zahir, Z. A., et al. (2017). Synergistic use of biochar, compost and plant growth-promoting rhizobacteria for enhancing cucumber growth under water deficit conditions. *J. Sci. Food Agric.* 97, 5139–5145. doi: 10.1002/jsfa.8393

Nehra, V., and Choudhary, M. (2015). A review on plant growth promoting rhizobacteria acting as bioinoculants and their biological approach towards the production of sustainable agriculture. *J. Appl. Nat. Sci.* 7, 540–556. doi: 10.31018/jans.v7i1.642

Neilands, J. B. (1995). Siderophores: structure and function of microbial iron transport compounds. *J. Biol. Chem.* 270, 26723–26726. doi: 10.1074/jbc.270.45.26723

Nelson, E. B. (2004a). Microbial dynamics and interactions in the spermosphere. *Annu. Rev. Phytopathol.* 42, 271–309. doi: 10.1146/annurev.phyto.42.121603.131041

Nelson, E. B. (2017). The seed microbiome: origins, interactions, and impacts. *Plant Soil* 422, 7–34. doi: 10.1007/s11104-017-3289-7

Nelson, L. M. (2004b). Plant growth promoting rhizobacteria (PGPR): prospects for new inoculants. *Crop Manag.* 3, 301–305. doi: 10.1094/cm-2004-0301-05-rv

Nelson, M. S., and Sadowsky, M. J. (2015). Secretion systems and signal exchange between nitrogen-fixing rhizobia and legumes. *Front. Plant Sci.* 6:491. doi: 10.3389/fpls.2015.00491

Nguyen, T. T. N., Xu, C.-Y., Tahmasbian, I., Che, R., Xu, Z., Zhou, X., et al. (2017). Effects of biochar on soil available inorganic nitrogen: a review and meta-analysis. *Geoderma* 288, 79–96. doi: 10.1016/j.geoderma.2016.11.004

Niu, D.-D., Zheng, Y., Zheng, L., Jiang, C.-H., Zhou, D.-M., and Guo, J.-H. (2016). Application of PSX biocontrol preparation confers root-knot nematode management and increased fruit quality in tomato under field conditions. *Biocontrol Sci. Technol.* 26, 174–180. doi: 10.1080/09583157.2015.1085489

Nosheen, A., Bano, A., and Ullah, F. (2016a). Bioinoculants: a sustainable approach to maximize the yield of Ethiopian mustard (*Brassica carinata* L.) under low input of chemical fertilizers. *Toxicol. Ind. Health* 32, 270–277. doi: 10.1177/0748233713498453

Nosheen, A., Bano, A., Yasmin, H., Keyani, R., Habib, R., Shah, S. T., et al. (2016b). Protein quantity and quality of safflower seed improved by NP fertilizer and Rhizobacteria (*Azospirillum* and *Azotobacter* spp.). *Front. Plant Sci.* 7:104. doi: 10.3389/fpls.2016.00104

Oke, V., and Long, S. R. (1999). Bacteroid formation in the *Rhizobium*–legume symbiosis. *Curr. Opin. Microbiol.* 2, 641–646. doi: 10.1016/S1369-5274(99)00035-1

Omer, Z. S., Tombolini, R., Broberg, A., and Gerhardson, B. (2004). Indole-3-acetic acid production by pink-pigmented facultative methylotrophic bacteria. *Plant Growth Regul.* 43, 93–96. doi: 10.1023/B:GROW.0000038360.09079.ad

Ortiz-Castro, R., Contreras-Cornejo, H. A., Macias-Rodriguez, L., and Lopez-Bucio, J. (2009). The role of microbial signals in plant growth and development. *Plant Signal. Behav.* 4, 701–712. doi: 10.4161/psb.4.8.9047

Pachauri, R. K., Allen, M. R., Barros, V. R., Broome, J., Cramer, W., Christ, R., et al. (2014). "Climate change 2014: synthesis report," in *Contribution of Working Groups I, II and III to the Fifth Assessment Report of the Intergovernmental Panel on Climate Change*, eds R. Pachauri and L. Meyer (Geneva: IPCC).

Parnell, J. J., Berka, R., Young, H. A., Sturino, J. M., Kang, Y., Barnhart, D. M., et al. (2016). From the lab to the farm: an industrial perspective of plant beneficial microorganisms. *Front. Plant Sci.* 7:1110. doi: 10.3389/fpls.2016.01110

Peiffer, J. A., Spor, A., Koren, O., Jin, Z., Tringe, S. G., Dangl, J. L., et al. (2013). Diversity and heritability of the maize rhizosphere microbiome under field conditions. *Proc. Natl. Acad. Sci. U.S.A.* 110, 6548–6553. doi: 10.1073/pnas.1302837110

Pérez-Montaño, F., Alías-Villegas, C., Bellogín, R., Del Cerro, P., Espuny, M., Jiménez-Guerrero, I., et al. (2014). Plant growth promotion in cereal and leguminous agricultural important plants: from microorganism capacities to crop production. *Microbiol. Res.* 169, 325–336. doi: 10.1016/j.micres.2013.09.011

Pieterse, C. M., Zamioudis, C., Berendsen, R. L., Weller, D. M., Van Wees, S. C., and Bakker, P. A. (2014). Induced systemic resistance by beneficial microbes. *Annu. Rev. Phytopathol.* 52, 347–375. doi: 10.1146/annurev-phyto-082712-102340

Pii, Y., Mimmo, T., Tomasi, N., Terzano, R., Cesco, S., and Crecchio, C. (2015). Microbial interactions in the rhizosphere: beneficial influences of plant growth-promoting rhizobacteria on nutrient acquisition process. A review. *Biol. Fertil. Soils* 51, 403–415. doi: 10.1007/s00374-015-0996-1

Ping, L., and Boland, W. (2004). Signals from the underground: bacterial volatiles promote growth in Arabidopsis. *Trends Plant Sci.* 9, 263–266. doi: 10.1016/j.tplants.2004.04.008

Poli, A., Lazzari, A., Prigione, V., Voyron, S., Spadaro, D., and Varese, G. C. (2016). Influence of plant genotype on the cultivable fungi associated to tomato rhizosphere and roots in different soils. *Fungal Biol.* 120, 862–872. doi: 10.1016/j.funbio.2016.03.008

Prashar, P., Kapoor, N., and Sachdeva, S. (2013). "Biocontrol of plant pathogens using plant growth promoting bacteria," in *Sustainable Agriculture Reviews*, ed. E. Lichtfouse (Berlin: Springer), 319–360.

Prudent, M., Salon, C., Souleimanov, A., Emery, R. J. N., and Smith, D. L. (2015). Soybean is less impacted by water stress using *Bradyrhizobium japonicum* and thuricin-17 from *Bacillus thuringiensis*. *Agron. Sustain. Dev.* 35, 749–757. doi: 10.1007/s13593-014-0256-z

Quiza, L., St-Arnaud, M., and Yergeau, E. (2015). Harnessing phytomicrobiome signaling for rhizosphere microbiome engineering. *Front. Plant Sci.* 6:507. doi: 10.3389/fpls.2015.00507

Ramesh, A., Sharma, S. K., Sharma, M. P., Yadav, N., and Joshi, O. P. (2014). Inoculation of zinc solubilizing *Bacillus aryabhattai* strains for improved growth, mobilization and biofortification of zinc in soybean and wheat cultivated in Vertisols of central India. *Appl. Soil Ecol.* 73, 87–96. doi: 10.1016/j.apsoil.2013.08.009

Ramey, B. E., Koutsoudis, M., Von Bodman, S. B., and Fuqua, C. (2004). Biofilm formation in plant-microbe associations. *Curr. Opin. Microbiol.* 7, 602–609. doi: 10.1016/j.mib.2004.10.014

Resch, A., Rosenstein, R., Nerz, C., and Gotz, F. (2005). Differential gene expression profiling of *Staphylococcus aureus* cultivated under biofilm and planktonic conditions. *Appl. Environ. Microbiol.* 71, 2663–2676. doi: 10.1128/AEM.71.5.2663-2676.2005

Ricci, E. C. (2015). *Investigating the Role of Pseudomonas sp. and Bacillus sp. Biofilms as Plant Growth Promoting Inoculants*. Master's thesis, McGill University, Montreal, QC.

Rijavec, T., and Lapanje, A. (2016). Hydrogen cyanide in the rhizosphere: not suppressing plant pathogens, but rather regulating availability of phosphate. *Front. Microbiol.* 7:1785. doi: 10.3389/fmicb.2016.01785

Rodriguez, R., and Redman, R. (2008). More than 400 million years of evolution and some plants still can't make it on their own: plant stress tolerance via fungal symbiosis. *J. Exp. Bot.* 59, 1109–1114. doi: 10.1093/jxb/erm342

Rokem, J. S., and Greenblatt, C. L. (2015). Making biofuels competitive: the limitations of biology for fuel production. *JSM Microbiol.* 3:1023.

Rondon, M. A., Lehmann, J., Ramirez, J., and Hurtado, M. (2007). Biological nitrogen fixation by common beans (*Phaseolus vulgaris* L.) increases with bio-char additions. *Biol. Fertil. Soils* 43, 699–708. doi: 10.1007/s00374-006-0152-z

Rubin, R. L., Van Groenigen, K. J., and Hungate, B. A. (2017). Plant growth promoting rhizobacteria are more effective under drought: a meta-analysis. *Plant Soil* 416, 309–323. doi: 10.1007/s11104-017-3199-8

Ruzzi, M., and Aroca, R. (2015). Plant growth-promoting rhizobacteria act as biostimulants in horticulture. *Sci. Hortic.* 196, 124–134. doi: 10.1016/j.scienta.2015.08.042

Saha, M., Sarkar, S., Sarkar, B., Sharma, B. K., Bhattacharjee, S., and Tribedi, P. (2016). Microbial siderophores and their potential applications: a review. *Environ. Sci. Pollut. Res.* 23, 3984–3999. doi: 10.1007/s11356-015-4294-0

Santoyo, G., Orozco-Mosqueda, M. D., and Govindappa, M. (2012). Mechanisms of biocontrol and plant growth-promoting activity in soil bacterial species of *Bacillus* and *Pseudomonas*: a review. *Biocontrol Sci. Technol.* 22, 855–872. doi: 10.1016/j.micres.2008.08.007

Schiltz, S., Gaillard, I., Pawlicki-Jullian, N., Thiombiano, B., Mesnard, F., and Gontier, E. (2015). A review: what is the spermosphere and how can it be studied? *J. Appl. Microbiol.* 119, 1467–1481. doi: 10.1111/jam.12946

Schuhegger, R., Ihring, A., Gantner, S., Bahnweg, G., Knappe, C., Vogg, G., et al. (2006). Induction of systemic resistance in tomato by N-acyl-L-homoserine lactone-producing rhizosphere bacteria. *Plant Cell Environ.* 29, 909–918. doi: 10.1111/j.1365-3040.2005.01471.x

Sessitsch, A., and Mitter, B. (2015). 21st century agriculture: integration of plant microbiomes for improved crop production and food security. *Microb. Biotechnol.* 8, 32–33. doi: 10.1111/1751-7915.12180

Shakeel, M., Rais, A., Hassan, M. N., and Hafeez, F. Y. (2015). Root associated *Bacillus* sp. improves growth, yield and zinc translocation for basmati rice (*Oryza sativa*) varieties. *Front. Microbiol.* 6:1286. doi: 10.3389/fmicb.2015.01286

Shakeri, E., Modarres-Sanavy, S. A. M., Amini Dehaghi, M., Tabatabaei, S. A., and Moradi-Ghahderijani, M. (2016). Improvement of yield, yield components and oil quality in sesame (*Sesamum indicum* L.) by N-fixing bacteria fertilizers and urea. *Arch. Agron. Soil Sci.* 62, 547–560. doi: 10.1080/03650340.2015.1064901

Shanta, N., Schwinghamer, T., Backer, R., Allaire, S. E., Teshler, I., Vanasse, A., et al. (2016). Biochar and plant growth promoting rhizobacteria effects on switchgrass (*Panicum virgatum* cv. Cave-in-rock) for biomass production in southern Québec depend on soil type and location. *Biomass Bioenergy* 95, 167–173. doi: 10.1016/j.biombioe.2016.10.005

Shivlata, L., and Satyanarayana, T. (2017). "Actinobacteria in agricultural and environmental sustainability," in *Agro-Environmental Sustainability*, eds J. S. Singh and G. Seneviratne (Berlin: Springer), 173–218.

Siddiqui, A. R., Nazeer, S., Piracha, M. A., Saleem, M. M., Siddiqi, I., Shahzad, S. M., et al. (2016). The production of biochar and its possible effects on soil properties and phosphate solubilizing bacteria. *J. Appl. Agric. Biotechnol.* 1, 27–40.

Singh, B. P., Hatton, B. J., Balwant, S., Cowie, A. L., and Kathuria, A. (2010). Influence of biochars on nitrous oxide emission and nitrogen leaching from two contrasting soils. *J. Environ. Qual.* 39, 1224–1235. doi: 10.2134/jeq2009.0138

Sivasakthi, S., Usharani, G., and Saranraj, P. (2014). Biocontrol potentiality of plant growth promoting bacteria (PGPR)-*Pseudomonas* fluorescens and *Bacillus subtilis*: a review. *Afr. J. Agric. Res.* 9, 1265–1277.

Smith, D. L., Gravel, V., and Yergeau, E. (2017). Editorial: signaling in the phytomicrobiome. *Front. Plant Sci.* 8:611. doi: 10.3389/fpls.2017.00611

Smith, D. L., Praslickova, D., and Ilangumaran, G. (2015a). Inter-organismal signaling and management of the phytomicrobiome. *Front. Plant Sci.* 6:722. doi: 10.3389/fpls.2015.00722

Smith, D. L., Subramanian, S., Lamont, J. R., and Bywater-Ekegard, M. (2015b). Signaling in the phytomicrobiome: breadth and potential. *Front. Plant Sci.* 6:709. doi: 10.3389/fpls.2015.00709

Spaepen, S., Bossuyt, S., Engelen, K., Marchal, K., and Vanderleyden, J. (2014). Phenotypical and molecular responses of *Arabidopsis thaliana* roots as a result of inoculation with the auxin-producing bacterium *Azospirillum brasilense*. *New Phytol.* 201, 850–861. doi: 10.1111/nph.12590

Spaepen, S., and Vanderleyden, J. (2011). Auxin and plant-microbe interactions. *Cold Spring Harb. Perspect. Biol.* 3:a001438. doi: 10.1101/cshperspect.a001438

Spaink, H. P. (2004). Specific recognition of bacteria by plant LysM domain receptor kinases. *Trends Microbiol.* 12, 201–204. doi: 10.1016/j.tim.2004.03.001

Srivastava, S., Bist, V., Srivastava, S., Singh, P. C., Trivedi, P. K., Asif, M. H., et al. (2016). Unraveling aspects of *Bacillus amyloliquefaciens* mediated enhanced production of rice under biotic stress of *Rhizoctonia solani*. *Front. Plant Sci.* 7:587. doi: 10.3389/fpls.2016.00587

Subramanian, P., Mageswari, A., Kim, K., Lee, Y., and Sa, T. (2015). Psychrotolerant endophytic *Pseudomonas* sp strains OB155 and OS261 induced chilling resistance in tomato plants (*Solanum lycopersicum* mill.) by activation of their antioxidant capacity. *Mol. Plant Microbe Interact.* 28, 1073–1081. doi: 10.1094/MPMI-01-15-0021-R

Subramanian, S., Ricci, E., Souleimanov, A., and Smith, D. L. (2016a). A proteomic approach to lipo-chitooligosaccharide and thuricin 17 effects on soybean germination unstressed and salt stress. *PLoS One* 11:e0160660. doi: 10.1371/journal.pone.0160660

Subramanian, S., and Smith, D. L. (2015). Bacteriocins from the rhizosphere microbiome - from an agriculture perspective. *Front. Plant Sci.* 6:909. doi: 10.3389/fpls.2015.00909

Subramanian, S., Souleimanov, A., and Smith, D. L. (2016b). Proteomic studies on the effects of lipo-chitooligosaccharide and thuricin 17 under unstressed and salt stressed conditions in *Arabidopsis thaliana*. *Front. Plant Sci.* 7:1314. doi: 10.3389/fpls.2016.01314

Sun, D. Q., Hale, L., and Crowley, D. (2016). Nutrient supplementation of pinewood biochar for use as a bacterial inoculum carrier. *Biol. Fertil. Soils* 52, 515–522. doi: 10.1007/s00374-016-1093-9

Tachibana, C. (2013). Opening industry-academic partnerships. *Science* 340, 225–227. doi: 10.1126/science.opms.r1300132

Takishita, Y., Charron, J., and Smith, D. L. (2018). (Pseudomonas Entomophila 23S, a PGPR with Potential for Control of Bacterial Canker Disease in Tomato (Solanum lycopersicum L.) Against Clavibacter Michiganensis Subsp. Michiganensis. Doctoral thesis, McGill University, Montreal, QC.

Tariq, M., Hameed, S., Malik, K. A., and Hafeez, F. Y. (2007). Plant root associated bacteria for zinc mobilization in rice. *Pak. J. Bot.* 39, 245–253.

Teplitski, M., Robinson, J. B., and Bauer, W. D. (2000). Plants secrete substances that mimic bacterial N-acyl homoserine lactone signal activities and affect population density-dependent behaviors in associated bacteria. *Mol. Plant Microbe Interact.* 13, 637–648. doi: 10.1094/MPMI.2000.13.6.637

Theis, K. R., Dheilly, N. M., Klassen, J. L., Brucker, R. M., Baines, J. F., Bosch, T. C. G., et al. (2016). Getting the hologenome concept right: an eco-evolutionary framework for hosts and their microbiomes. *msystems* 1:e00028-16. doi: 10.1128/mSystems.00028-16

Timmusk, S., Behers, L., Muthoni, J., Muraya, A., and Aronsson, A. C. (2017). Perspectives and challenges of microbial application for crop improvement. *Front. Plant Sci.* 8:49. doi: 10.3389/fpls.2017.00049

Tiwari, S., Lata, C., Chauhan, P. S., and Nautiyal, C. S. (2016). *Pseudomonas* putida attunes morphophysiological, biochemical and molecular responses in *Cicer arietinum* L. during drought stress and recovery. *Plant Physiol. Biochem.* 99, 108–117. doi: 10.1016/j.plaphy.2015.11.001

Toju, H., Peay, K. G., Yamamichi, M., Narisawa, K., Hiruma, K., Naito, K., et al. (2018). Core microbiomes for sustainable agroecosystems. *Nat. Plants* 4, 247–257. doi: 10.1038/s41477-018-0139-4

Trabelsi, D., and Mhamdi, R. (2013). Microbial inoculants and their impact on soil microbial communities: a review. *Biomed. Res. Int.* 2013:863240. doi: 10.1155/2013/863240

Traxler, V., Kim, H. S., Malyala, R., Thompson, T. A., Buege, B., and Jarand, M. L. (2016). Biochar coated seeds. U.S. Provisional Patent Application Series No. 62/180,525. Denver, CO: Cool Planet Energy Systems, Inc.

Trivedi, P., Schenk, P. M., Wallenstein, M. D., and Singh, B. K. (2017). Tiny microbes, big yields: enhancing food crop production with biological solutions. *Microb. Biotechnol.* 10, 999–1003. doi: 10.1111/1751-7915.12804

Triveni, S., Prasanna, R., and Saxena, A. K. (2012). Optimization of conditions for in vitro development of *Trichoderma viride*-based biofilms as potential inoculants. *Folia Microbiol.* 57, 431–437. doi: 10.1007/s12223-012-0154-1

Turner, T. R., James, E. K., and Poole, P. S. (2013). The plant microbiome. *Genome Biol.* 14:209. doi: 10.1186/gb-2013-14-6-209

Ude, S., Arnold, D. L., Moon, C. D., Timms-Wilson, T., and Spiers, A. J. (2006). Biofilm formation and cellulose expression among diverse environmental *Pseudomonas* isolates. *Environ. Microbiol.* 8, 1997–2011. doi: 10.1111/j.1462-2920.2006.01080.x

Vacheron, J., Desbrosses, G., Bouffaud, M. L., Touraine, B., Moenne-Loccoz, Y., Muller, D., et al. (2013). Plant growth-promoting rhizobacteria and root system functioning. *Front. Plant Sci.* 4:356. doi: 10.3389/fpls.2013.00356

van der Heijden, M. G., and Hartmann, M. (2016). Networking in the plant microbiome. *PLoS Biol.* 14:e1002378. doi: 10.1371/journal.pbio.1002378

Van Zwieten, L., Kimber, S., Downie, A., Morris, S., Petty, S., Rust, J., et al. (2010). A glasshouse study on the interaction of low mineral ash biochar with nitrogen in a sandy soil. *Soil Res.* 48, 569–576. doi: 10.1071/SR10003

Vecstaudza, D., Senkovs, M., Nikolajeva, V., Kasparinskis, R., and Muter, O. (2017). Wooden biochar as a carrier for endophytic isolates. *Rhizosphere* 3, 126–127. doi: 10.1016/j.rhisph.2017.04.002

Vejan, P., Abdullah, R., Khadiran, T., Ismail, S., and Nasrulhaq Boyce, A. (2016). Role of plant growth promoting rhizobacteria in agricultural sustainability—a review. *Molecules* 21:573. doi: 10.3390/molecules21050573

Velivelli, S. L., De Vos, P., Kromann, P., Declerck, S., and Prestwich, B. D. (2014). Biological control agents: from field to market, problems, and challenges. *Trends Biotechnol.* 32, 493–496. doi: 10.1016/j.tibtech.2014.07.002

Vessey, J. K. (2003). Plant growth promoting rhizobacteria as biofertilizers. *Plant Soil* 255, 571–586. doi: 10.1023/A:1026037216893

Wagner, M. R., Lundberg, D. S., Coleman-Derr, D., Tringe, S. G., Dangl, J. L., and Mitchell-Olds, T. (2014). Natural soil microbes alter flowering phenology and the intensity of selection on flowering time in a wild Arabidopsis relative. *Ecol. Lett.* 17, 717–726. doi: 10.1111/ele.12276

Walters, M. C. III, Roe, F., Bugnicourt, A., Franklin, M. J., and Stewart, P. S. (2003). Contributions of antibiotic penetration, oxygen limitation, and low metabolic activity to tolerance of *Pseudomonas aeruginosa* biofilms to ciprofloxacin and tobramycin. *Antimicrob. Agents Chemother.* 47, 317–323. doi: 10.1128/AAC.47.1.317-323.2003

Wang, Q. Y., Dodd, I. C., Belimov, A. A., and Jiang, F. (2016). Rhizosphere bacteria containing 1-aminocyclopropane-1-carboxylate deaminase increase growth and photosynthesis of pea plants under salt stress by limiting Na^+ accumulation. *Funct. Plant Biol.* 43, 161–172. doi: 10.1071/FP15200

Wang, Y. Y., Jing, X. R., Li, L. L., Liu, W. J., Tong, Z. H., and Jiang, H. (2017). Biotoxicity evaluations of three typical biochars using a simulated system of fast pyrolytic biochar extracts on organisms of three kingdoms. *ACS Sustain. Chem. Eng.* 5, 481–488. doi: 10.1021/acssuschemeng.6b01859

Wani, S. H., Kumar, V., Shriram, V., and Sah, S. K. (2016). Phytohormones and their metabolic engineering for abiotic stress tolerance in crop plants. *Crop J.* 4, 162–176. doi: 10.1016/j.cj.2016.01.010

Weyens, N., Van Der Lelie, D., Taghavi, S., Newman, L., and Vangronsveld, J. (2009a). Exploiting plant-microbe partnerships to improve biomass production and remediation. *Trends Biotechnol.* 27, 591–598. doi: 10.1016/j.tibtech.2009.07.006

Weyens, N., Van Der Lelie, D., Taghavi, S., and Vangronsveld, J. (2009b). Phytoremediation: plant–endophyte partnerships take the challenge. *Curr. Opin. Biotechnol.* 20, 248–254. doi: 10.1016/j.copbio.2009.02.012

Zhou, C., Ma, Z. Y., Zhu, L., Xiao, X., Xie, Y., Zhu, J., et al. (2016). Rhizobacterial strain *Bacillus megaterium* bofc15 induces cellular polyamine changes that improve plant growth and drought resistance. *Int. J. Mol. Sci.* 17:976. doi: 10.3390/ijms17060976

Whiteley, M., Bangera, M. G., Bumgarner, R. E., Parsek, M. R., Teitzel, G. M., Lory, S., et al. (2001). Gene expression in *Pseudomonas aeruginosa* biofilms. *Nature* 413, 860–864. doi: 10.1038/35101627

Winston, M. E., Hampton-Marcell, J., Zarraonaindia, I., Owens, S. M., Moreau, C. S., Gilbert, J. A., et al. (2014). Understanding cultivar-specificity and soil determinants of the cannabis microbiome. *PLoS One* 9:e99641. doi: 10.1371/journal.pone.0099641

Wintermans, P. C., Bakker, P. A., and Pieterse, C. M. (2016). Natural genetic variation in Arabidopsis for responsiveness to plant growth-promoting rhizobacteria. *Plant Mol. Biol.* 90, 623–634. doi: 10.1007/s11103-016-0442-2

Wu, C. H., Bernard, S. M., Andersen, G. L., and Chen, W. (2009). Developing microbe-plant interactions for applications in plant-growth promotion and disease control, production of useful compounds, remediation and carbon sequestration. *Microb. Biotechnol.* 2, 428–440. doi: 10.1111/j.1751-7915.2009.00109.x

Ye, J., Zhang, R., Nielsen, S., Joseph, S. D., Huang, D., and Thomas, T. (2016). A combination of biochar-mineral complexes and compost improves soil bacterial processes, soil quality, and plant properties. *Front. Microbiol.* 7:372. doi: 10.3389/fmicb.2016.00372

Zebelo, S., Song, Y., Kloepper, J. W., and Fadamiro, H. (2016). Rhizobacteria activates (+)-delta-cadinene synthase genes and induces systemic resistance in cotton against beet armyworm (*Spodoptera exigua*). *Plant Cell Environ.* 39, 935–943. doi: 10.1111/pce.12704

Zhang, L., and Mah, T. F. (2008). Involvement of a novel efflux system in biofilm-specific resistance to antibiotics. *J. Bacteriol.* 190, 4447–4452. doi: 10.1128/JB.01655-07

Zhang, R., Vivanco, J. M., and Shen, Q. (2017). The unseen rhizosphere root-soil-microbe interactions for crop production. *Curr. Opin. Microbiol.* 37, 8–14. doi: 10.1016/j.mib.2017.03.008

Zipfel, C., and Oldroyd, G. E. (2017). Plant signalling in symbiosis and immunity. *Nature* 543, 328–336. doi: 10.1038/nature22009

Conflict of Interest Statement: The authors declare that the research was conducted in the absence of any commercial or financial relationships that could be construed as a potential conflict of interest.

frontiers in Plant Science

REVIEW
published: 30 October 2018
doi: 10.3389/fpls.2018.01567

Developing Biostimulants From Agro-Food and Industrial By-Products

*Lin Xu and Danny Geelen**

Horticell, Department of Plant and Crops, Faculty of Bioscience Engineering, Ghent University, Ghent, Belgium

In modern agriculture, seeking eco-friendly ways to promote plant growth and enhance crop productivity is of priority. Biostimulants are a group of substances from natural origin that contribute to boosting plant yield and nutrient uptake, while reducing the dependency on chemical fertilizers. Developing biostimulants from by-products paves the path to waste recycling and reduction, generating benefits for growers, food industry, registration and distribution companies, as well as consumers. The criteria to select designated by-products for valorizing as biostimulant are: absence of pesticide residue, low cost of collection and storage, sufficient supply and synergy with other valorization paths. Over the years, projects on national and international levels such as NOSHAN, SUNNIVA, and Bio2Bio have been initiated (i) to explore valorization of by-products for food and agriculture industries; (ii) to investigate mode of action of biostimulants from organic waste streams. Several classes of waste-derived biostimulants or raw organic material with biostimulant components were shown to be effective in agriculture and horticulture, including vermicompost, composted urban waste, sewage sludge, protein hydrolysate, and chitin/chitosan derivatives. As the global market for biostimulants continues to rise, it is expected that more research and development will expand the list of biostimulants from by-products. Global nutrient imbalance also requires biostimulant to be developed for targeted market. Here, we review examples of biostimulants derived from agricultural by-products and discuss why agricultural biomass is a particularly valuable source for the development of new agrochemical products.

Keywords: biofertilizer, agrochemical, organic waste stream, bio-economy, sustainable farming, plant extract

OPEN ACCESS

Edited by:
Giuseppe Colla,
Università degli Studi della Tuscia, Italy

Reviewed by:
Roberta Bulgari,
Università degli Studi di Milano, Italy
Ebrahim Hadavi,
Islamic Azad University of Karaj, Iran

***Correspondence:**
Danny Geelen
danny.geelen@ugent.be

Specialty section:
This article was submitted to Crop and Product Physiology, a section of the journal Frontiers in Plant Science

Received: *27 July 2018*
Accepted: *08 October 2018*
Published: *30 October 2018*

Citation:
Xu L and Geelen D (2018) Developing Biostimulants From Agro-Food and Industrial By-Products. Front. Plant Sci. 9:1567. doi: 10.3389/fpls.2018.01567

INTRODUCTION

Due to increasing demand for better yield and quality of food and crops, seeking eco-friendly and sustainable ways to produce fertilization reagents of biological origins has become a major goal in agriculture. Biostimulants are products able to act on plants' metabolic and enzymatic processes improving productivity and crop quality. It also assists plants to cope with abiotic stress, especially in the early stage of plant development. European Biostimulants Industry Council (EBIC) defines biostimulants as "substance(s) and/or micro-organisms whose function when applied to plants or the rhizosphere is to stimulate natural processes to enhance/benefit nutrient uptake, nutrient efficiency, tolerance to abiotic stress, and crop quality[1]." The economic relevance of such products

[1]http://www.biostimulants.eu

is not insignificant as the global market for biostimulants has been projected to reach $ 2,241 million by 2018, having a compound annual growth rate of 12.5% from 2013 to 2018 (Calvo et al., 2014). In the European Union, the economic value of biostimulants is estimated to be between 200 and 400 million euros (with a yearly growth of 10%) (EBIC). Owing to the positive effects of biostimulant on plant growth, reduction of stress and disease prevention, the use of biostimulant contributes to boosting plant production, yield and quality.

Over the years, several types of biostimulants have been defined by different authors, based on source material, mode of action and other parameters [Reviewed in (Yakhin et al., 2016)]. For example, du Jardin (2015) categorized biostimulants into 7 classes: humic acid (HA) and fulvic acid (FA), protein hydrolysates (PHs), seaweed extracts, chitosan, inorganic compounds, beneficial fungi and bacteria (du Jardin, 2015). The primary sources of biostimulants also display various origins and physiological characteristics. It reflects on the categorization of biostimulants as well. For example, macroalgae and their extracts have long been used for biostimulant production (McHugh, 2013).

The active ingredients present in different types of biomass with potential biostimulant activity fall into a diverse range of molecules that includes phytohormones (cytokinin, auxins, gibberellins, brassinosteriods, ethylene, and abscisic acids) (Letham and Palni, 1983; Mussig et al., 2006; Pimenta Lange and Lange, 2006; Werner and Schmulling, 2009; Wolters and Jurgens, 2009; Zhao, 2010; Pacifici et al., 2015), amino acids (Hoque et al., 2007; Colla et al., 2015; Colla et al., 2017), polyamine (Fuell et al., 2010), etc. In seaweed extracts, phytohormones have been demonstrated to be present and are considered as putative bioactive ingredients of this category of biostimulants (Khan et al., 2009; Stirk et al., 2014; Stirk and Van Staden, 2014). In addition to hormones, algal extracts also contain a range of carbohydrates such as alginate, fucoidan, betaines as well as proteins and minerals which promote plant growth (Sharma et al., 2014).

It should be noted that waste streams from food and agricultural industries are equally important sources for biostimulant development. The biostimulants generated from waste streams are extracts from food waste, composts, manures, vermicompost, aquaculture waste streams and sewage treatments (Yakhin et al., 2016). PHs are commonly used biostimulants, which are a group of polypeptides, oligopeptides, and amino acids that are manufactured from hydrolyzed protein-rich waste (Schaafsma, 2009).

Recycling of organic waste as biofertilizers is historically a common practice. Manure has been and is applied to the field as soil fertilizers across the world. However, environmental problems occur when the local production exceeds storage and local application capacity, resulting in careless disposal and associated environmental spillage with consequent water pollution, over-fertilization, ammonia toxicity, and infestation of human pathogens, etc. (Dominguez and Edwards, 2010). To avoid environmental contamination, finding alternative ways to reuse such material in the agricultural production system is necessary. The concept of circular economy emphasizes the process of converting waste materials and products that have reached the end of their life cycle into new sources. A new EU regulation that entered into force on January 1, 2018 aims at boosting the use of bio-base wastes as new types of fertilizers (European Commission, 2016a). These waste-originated fertilizers usually contain biostimulatory substances, and we will discuss further in this review when placing composts as important sources of biostimulants. When a waste turns into raw material for industrial or agricultural application, then it is no longer waste. As the definition of "waste" describes the material to be discarded, it is sometimes more appropriate to label the material as by-product. A by-product is lawfully used, not deliberately produced, of certain use, ready for use without further processing and produced as an integral part of the production process (European Commission, 2007).

Through research and development of using industrial waste for re-manufacturing, reuse and recycling, one can make fundamental steps toward biomass optimization and resources use efficiency and sustainability (European Commission, 2017). Therefore, developing biostimulants from by-products provides the innovative methods to prevent inadvertent disposal and results in environmental-friendly solutions for waste re-use. From a legal point of view, the European Commission is proposing a Regulation which will ease the access of organic and waste-based fertilizers to the EU market (European Commission, 2016b). In this review, we give a current view of strategies employed to turn organic wastes into added-value products, especially in the field of biostimulant development. A number of categories of biostimulants is presented, providing a glimpse of biostimulant products currently on the market.

DEFINING BIOSTIMULANT DERIVED FROM WASTE STREAM

By-Products and Their Utilization in Food and Feed

With the demand for larger quantities of healthy and fresh food, the agricultural and food industry companies are faced with tremendous amount of organic biomass, coming from the production or processing procedures. Depending on the types of crop and its processing, the scale of biomass production may substantially vary. For example, fish by-product consists of over 60% of the biomass, including head, skin, fins, frames, etc. (Chalamaiah et al., 2012). The fishing industry produces vast amounts of the exoskeletons of crustaceans, coming from shrimp, crab and lobster, mounting to a global production of 5.9 Mt, with 35–45% of it being discarded as waste (head and thorax) (Sharp, 2013). A lot of these sources are enriched in secondary metabolites because they originate from cells and tissues exposed to the exterior of the organisms' body which is developed to keep off attackers and pathogens. For instance, potato peel is enriched in steroidal alkaloids which are associated with defense against

bacterial, fungal, and insect pathogens [Reviewed in (Fritsch et al., 2017)]. In tanning industry, which treats animal skins and hides to produce leather, it generates large quantities of by-products: one metric ton of wet salted hides produces 200 kg of leather and 450–600 kg solid waste, leaving more than 60% of biomass, if not converted, will be disposed to the environment as waste (Alexander et al., 1992; Verheijen et al., 1996). Thus, the sheer volume of by-product is vast and the presence of a complex mix of metabolites profile is favoring the exploitation of these products.

Coming to terming with the problem of disposing or re-using industrial by-products, techniques have been developed to process fish by-products, such as enzymatic hydrolysis, autolysis and thermal hydrolysis (Halim et al., 2016). The human health benefits associated with fish PHs (FPHs) are demonstrated by the commercial preparations used as healthy food and/or nutraceuticals in many countries (Chalamaiah et al., 2012). The antioxidant properties of FPHs are associated with protecting the human body from oxidative stress (Chalamaiah et al., 2012). Other positive effects on human physiology are attributed to fish by-product as well, including the prevention of high blood pressure (hypertension), as well as anti-cancer activity (Halim et al., 2016). Besides development of human consumption, by-products are also being used as animal feed. FPHs have been used in aquaculture feed to enhance the growth and survival of fish (Kotzamanis et al., 2007).

Another waste-turn-into-feed example comes from feather waste that is enriched in keratin, a nutrient source for animals and plants (Korniłłowicz-Kowalska and Bohacz, 2011). Chicken feathers are the most common keratin waste product with high amounts being produced in poultry slaughterhouses (Korniłłowicz-Kowalska and Bohacz, 2011). Owing to its high protein content, feather waste has been largely converted to feedstock. However, the application of animal by-product used as feedstock is declining over the years because of more stringent regulations. Besides, valorization strategies of animal feed come with limited financial return. On the other hand, PHs are now accepted as components of plant biostimulants in agriculture that increase productivity and quality of crops (Colla et al., 2017). It should be noted that animal by-products not intended for human consumption are potential sources of risks to the public and to animals. An example of the problems that may arise because of recycling animal waste is the use of certain animal by-products that gave rise to outbreaks of foot-and-mouth disease, bovine spongiform encephalopathy (BSE) (European Commission, 2009).

Suitability of Waste Streams Used for Biostimulant Development

As the first step toward developing biostimulants from organic waste, the choice of biomass resource is critical. Various active ingredients found in industrial waste streams and by-products of biological origins pose the perfect opportunity to extract molecules for better growth and pathogen resistance of valuable crop plants. However, some understanding of the intrinsic biochemical characteristics of raw materials is needed, such as preserving the specific bioactive ingredients (Povero et al., 2016). The environmental and economic evaluation must be carried out as well, to assess whether a new biostimulant product has good prospect to become successful. To assess if the raw material is suitable for the development of biostimulants, several factors must be taken into consideration.

Absence of Pesticides

It is expected that during conventional pest management, pesticides have been used to safeguard crop production. By-product derived from plant species that has been treated with pesticides could potentially cause problems for biostimulants production, as it no longer will be seen as a "natural" product and will be considered as an alternative preparation of the regulated agrochemical. In most EU member states, it is specified that biostimulant-like substances are different from plant protection products and they must not harm the wellbeing of humans, animals and the environment (La Torre et al., 2016). Hence, contamination by pesticides should be prevented with maximal measures when obtaining the source material. Even the smallest amount of contamination with pesticides may cause problems with the legislation. Since during the extraction procedure, solvents (e.g., n-hexane) may selectively solubilize and concentrate pesticides from water matrices (Zayats et al., 2013). To this end, the source of plant biostimulants should not contain pesticides that otherwise leads to problems with the registration and permission to use in sustainable agriculture.

Low Cost of Collection and Storage

The initial economic value of the waste material should be low and it preferably requires additional processing to dispose of at a financial cost. The reason for this is that the yield increase attributed to biostimulant application is typically in the range of 5–10% and this limits the profitability of biostimulant sales. The economic burden of a by-product resource can be broken down into three main components: collection, conservation and storage, and transport. Taking the example of fish by-products, the intrinsic value for the production of food and animal feed, coupled with the high cost to preserve fish biomass at low temperature, all lead to the recovery of by-products for further valorization unprofitable. To achieve the recovery of by-product, some fish species are often brought ashore for processing, in order to overcome limited preservation space of by-product while at sea [reviewed in (Olsen et al., 2014)]. Despite the complexity of organizing the logistic of storage for perishable biomass, agricultural production is typically linked with seasonal production activities and poor conservation methods may in addition result in deterioration of the material that could affect the stability of bioactive ingredients. Many natural products are unstable and undergo chemical modification when exposed to heat, light or oxygen, and result in loss of bioactivity (Turek and Stintzing, 2013). A sound business model that addresses the logistic problems of collection and storage of resources is more likely to become successful if it is derived from a stable source material.

Sufficient Availability

An effective agrochemical product is preferably available in sufficient quantities to accommodate the market demand. It is therefore critical that the biomass from which a biostimulant product is derived is available in large quantities and readily available for processing. A good example of an abundant waste product, yet highly perishable, is the heads and shells of crustaceans that are a source of chitin. It should also be avoided to develop biostimulants derived from by-products which are prone to perturbation, i.e., the material that strongly varies in composition or for which the supply is uncertain. An abundant product has, in addition, the advantage of attracting further valorization and development for other usage. The ample quantity and diversified applications can be demonstrated in the case of seaweed. As an essential source of biostimulants with already commercialized products, the seaweed industry provides an annual input of 7.5–8 million tons of wet seaweed, which is used for food, fertilizers, feed, biofuel, and cosmetics, etc. (McHugh, 2013). Among them, brown algae are the groups of seaweed having large tonnage whose extracts are exploited widely as a source of biostimulants. For sewage sludge, less exploited but with great potential for nutrient recovery, the annual flux is around 9.5 million tons in EU member states (Buckwell and Nadeu, 2016). Taken these issues into consideration, it is advised that a careful selection of materials is necessary for generating new types of biostimulant: from processing, extraction, formulation to marketing and distribution.

Positive and Negative Impact of Competing Use: A Double-Edged Sword

If different valorization strategies applied to the same waste stream are in competition with each other, which may turn out to have a negative impact on the development of a biostimulant product. This is because biostimulants are typically less valuable than animal feed and much less than valorization through the isolation of fine chemicals as pharmaceutical agents. Consequently, to be suitable as biostimulant source, the waste source is preferably not intensively used for feed or other higher value uses. An overview of the biomass value pyramid has been presented by Meyer (2017) in the context of developing bio-based economy (Meyer, 2017). Although there's no clear prioritization of different sectors of biomass use, it is most valuable when biomass is used as pharmaceuticals or fine chemicals to benefit health and lifestyle (Meyer, 2017). The waste-derived biostimulants currently on the market originate from only a few sources (e.g., plant- and animal-derived PHs). Further valorization through development of biostimulants from the same sources may be difficult due to intellectual property issues.

However, on the other hand, when a biomass is being used for other purposes, development of a new biostimulant from that source remains an option. To avoid the competition, it requires high compatibility of the two processing technologies, and when integration is feasible, it may even generate greater benefit. Such synergistic situations are currently still rare and further maturing of the biostimulant research is likely to generate opportunities for process integration. This is for example the case for various seaweeds that are now well accepted to harbor biostimulatory activity, as dozens of manufacturers around the world have launched formulated seaweed products specifically tailored for various crops (Sharma et al., 2014). In olive oil industry, 95% of olive by-product are currently consumed as low-value uses (energy generation, composting), with the remaining 5% as animal feed (Berbel and Posadillo, 2018). However, it doesn't hamper the processing development of olive biomass for high-value uses such as extraction of bioactive compounds (i.e., phenol) (Berbel and Posadillo, 2018). Consequently, there are both positive and negative aspects to consider when a given biomass is used in diversified sectors. Researchers and entrepreneurs are suggested to carry out a SWOT analysis, identifying the pros and cons when developing biostimulants from material that is being exploited for different purposes already.

VALORIZATION OF ORGANIC WASTES: EXAMPLES OF CROSS-REGIONAL FUNDED PROJECTS

Valorization is the process of converting by-products to high value substances. The development of novel tools to secure crop yield are objectives that are of high importance on the agenda of the research funding bodies across Europe and worldwide. On the other hand, increasing price of oil directly affects the cost of fertilizers, which urges farmers to look for novel ways to reduce the input cost. The concept of circular economy creates opportunities for the agricultural and food sector aiming at valorizing by-products. Indeed, recycling raw organic material from waste is a first step toward reducing energy and material input cost in the production process. Besides feedstocks, by-products can be converted to bio-based chemicals, biostimulants and soil amendments, as well as value-added products in biomaterial industries (e.g., bioplastic, lignin and alginate) (De Corato et al., 2018).

To promote the research and development toward valorizing organic wastes, a few funded projects are published within European Commission. The NOSHAN Project (Functional and Safe Feed from Food Waste, EU FP7 Grant No. 312140) investigated food waste processing and technologies to use for feed production at low cost. Food waste has good nutritional value provided it is treated and conserved correctly[2]. An example is pectin that has been characterized in 26 different food waste streams. The NOSHAN project revealed that the pectin structures and yield are highly diverse (Muller-Maatsch et al., 2016). Furthermore, the study provides insight into using pectin as food additive from a variety of waste source material (Muller-Maatsch et al., 2016). SUNNIVA focused on reducing waste by providing valorization strategies in vegetable processing[3]. It envisioned optimizing strategies to exploit by-product from food production and develop fertilizers from waste (field and storage) and processing side-flow. For instance, vegetable waste and side-flow were assessed for their use as organic fertilizers.

[2]http://noshan.eu/index.php/en/

[3]http://sunnivaproject.eu/

The raw material fractions were tested to identify their effects on plant growth and defense and their suitability to host beneficial microorganisms. Within Bio-Based Industries Joint Undertaking (BBI-JU), operating under H2020 Framework Projects, one of the key areas is to find solutions for waste reduction and strategies to valorize waste. In this regard, AgriMax[4], FUNGUSCHAIN[5], and NEWFERT[6] focus on recovering organic waste, filling the gaps of nutrient cycle for the food and fertilizer industries. It is postulated that waste and by-products from potato, tomato, cereals and olive have great potential to be valorized on the EU market as food additives, agricultural materials, packaging materials and biofertilizers (Fritsch et al., 2017). **Figure 1** illustrates the valorization chain starting from the suppliers of waste material to end users, illustrating the economic potential of biostimulants from waste streams.

[4]http://www.agrimax-project.eu
[5]http://www.funguschain.eu
[6]http://newfert.org

TACKLING MODES OF ACTION OF BIOSTIMULANTS FROM ORGANIC WASTE

"Mode of action" implies the detailed biochemical and physiological changes of the plants upon application of biostimulants. Despite the strong interest in developing new biostimulants, few well-characterized products with reliable performance are on the market. Generally there is limited insight into the mode of action, largely due to the diversity of source material and the complexity of the resulting product which is typically complex and chemically poorly characterized (Brown and Saa, 2015). Especially when material comes from living microbial cultures, macro and micro-algae, PHs, vermicompost and other types of industrial wastes, complexity is extremely high and multiple components are likely to be involved (Brown and Saa, 2015). Despite the complexity of the substance mixture, attempts to characterize bioactive ingredients are highly relevant to gain trust and reliability of the product

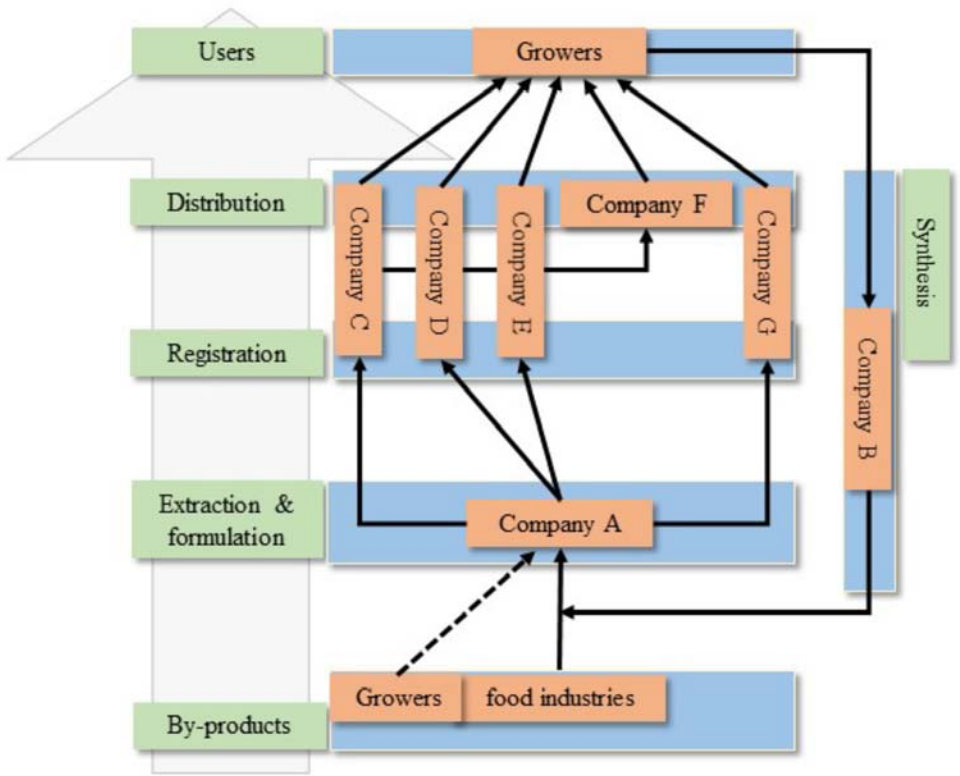


FIGURE 1 | Possible scenario in valorization chain of biostimulants from waste streams. Food industries and growers generate substantial amount of waste material and they are seeking methods to valorize as it costs to process the by-products. Food industries have the capacity to transport the by-product out of sites. Growers, however, usually lack the logistic means to process the by-products. Thus, a limiting factor for growers to valorize the crop residues is the cost of transport. As the first step toward valorization, Company a has the expertise in extraction and formulation from by-products. Company C–E, and G are heavily involved in the production and marketing of biostimulant products and they invest in registration and distribution. In spite of the ability to produce bioactive ingredients, these companies might still need assistance from Company a in extraction and formulation. Company C–E are also selling biostimulants to intermediate companies (Company F), who produce seeds, substrates or fertilizers. It is likely that one new biostimulant will be marketed as substrate, soil improvers or seed-coating. The ultimate target groups of biostimulants are crop growers. The proved efficacy of one biostimulant from growers will inspire the identification of chemical structure by Company B, carrying out synthesis of analogs. Consequently, it provides further knowledge into the valorization chain since the extraction and formulation can be designed to isolate the targeted bioactive ingredients.

marketed. Furthermore, subsequent stages of action in the plants after application also need to be taken into account: penetration into tissues, reaction with plant metabolites and chemical stability, binding to metabolic enzymes and impact of the compound on the plant's physiology and modulation of gene expression (Yakhin et al., 2016). It requires a systematic and multidisciplinary approach whereby various technologies in the fields of chemistry, biology and genomics need to be combined (Povero et al., 2016).

The investigation of the mode of action of a biostimulant requires two consecutive steps: determination of active ingredient(s) and its/their mode of action on plants. Omics approaches are routinely employed in modern life science and they involve high-throughput technologies that are capable of measuring global changes in the abundance of mRNA transcripts (transcriptome), proteome, and metabolome in complex biological systems as a result of biochemical stimulations or perturbation. They provide reliable and informative tools to unveil the mode of action of biostimulants and hence mode-of-action studies rely heavily on omics tools. A transcriptomic study revealed that HS extracted from vermicompost modified plant physiology and metabolism by regulating expression levels of genes involved in cell cycle and meristem and cytoskeleton organization (Trevisan et al., 2011). Similarly, proteomic data showed that chitosan modulates protein abundance belonging to primary metabolism pathways in grape (Ferri et al., 2014). We have recently embarked on a project with the objective to identify biostimulants and biopesticides from agricultural waste streams[7]. The project "Bio2Bio" investigates material from organic waste streams and by-products from food industries in Flanders, creating a library of formulated extracts. With the help of a bioassay screening platform, promising extracts and fractions are being characterized. The screening platform gathers the expertise from a variety of *in vitro*, greenhouse and field tests to score the materials for biostimulatory activity. Most importantly, Bio2Bio aims to determine bio-active ingredients and studies their mode of action. The impact of the project lies in the discovery of potentially new bioactive compounds of natural origin. The new ideas from the research part of the project will provide valuable leads for the agrochemical companies to develop new biostimulant products, benefiting the different target groups. The valorization potential can also be benefited from the mode of action studies.

CURRENT EXAMPLES OF BIOSTIMULANTS FROM ORGANIC WASTE AND BY-PRODUCTS

One of the important valorization strategies for by-products is to exploit bioactive compounds to improve plant growth and resistance to pathogens. Several classes of organic wastes or by-products that are currently valorized as plant biostimulants, which are claimed to render better plant growth and increased pest tolerance. In this section, we are introducing the biostimulant substances processed or extracted from different classes of source material that are considered to be wastes and by-products in the agriculture and food sectors, including substances from composting, PH, chitin and chitosan, and other by-products from biological origins. According to EBIC definition, compost doesn't fall into the category of the biostimulants and adheres better to biofertilizers. However, compost is a source for agrochemicals or microbes that potentially display biostimulant properties, such as HS, phytohormones, amino acids and other substances that can be extracted from it. These substances function by interacting with plant signaling processes and reducing negative response to stress, rather than by virtue of these substances (Brown and Saa, 2015). Typical biostimulant effects have been ascribed to composts, including enhancement of nutrient uptake, improved pathogen defense, etc. Moreover, the application methods, whether foliar spray or adding to soil, do not determine whether a product display biostimulatory activity or not. To this end, composted material exemplifies waste-derived sources of biostimulants, as various biostimulant-related studies have utilized active ingredients from compost to promote plant growth and protect against disease. In addition, composting is conforming to the concept of circular bio-economy.

Biostimulants From Vermicompost

Vermicompost is the organic matter processed by earthworms. The techniques of vermicomposting have been widely applied to reduce the volume of plant organic waste, manure, paper, food and sewage sludge (Dominguez and Edwards, 2010; Allardice et al., 2015). Vermicomposting helps to eliminate the occurrence of human pathogens present in manure, including fecal coliforms, *Salmonella* species, enteric viruses and helminthes (Eastman et al., 2001; Edwards and Subler, 2010). It can also be used as a substitute for peat, which is usually mined in unique wetland ecosystems, in potting media with biostimulatory effects on seedling performance, altering fruit quality (Zaller, 2007). As a result, the process of vermicomposting brings about more sustainable waste management strategies that may otherwise pose health risks, contaminating our living environment (Eastman et al., 2001; Edwards and Subler, 2010).

Biostimulants can be extracted from vermicompost, which are utilized in plant growth media and soil amendment, alleviating of nutrient deficiency and abiotic stress (Aremu et al., 2012; Chinsamy et al., 2013; Aremu et al., 2014). The biostimulatory effects of vermicompost are attributed to the presence of substances with phytohormonal activity. A mixture of plant growth regulators (PGRs), cytokinins, auxins, abscisic acid, gibberellins, and brassinosteroids were shown to occur in leachate of commercially produced vermicomposted garden waste (Aremu et al., 2015). Also HA and FA are present and are potentially responsible for the biostimulant activities of vermicompost. The composting process provides more stable and mature organic material, and in term results in enrichment of humic substances (HS) (Provenzano et al., 2001). The direct effect of HS on plant growth is suggested to come from its interaction with the plant membrane

[7]http://www.horticell.ugent.be/project/bio2bio-biological-waste-streams-biostimulants-and-biopesticides

transporters. This supposedly increases nutrient uptake and trigger membrane-associated signal transduction cascades which regulate growth and development (Canellas et al., 2015). Most reported beneficial effects of HS correlate with changes in root architecture. HS enhances the H^+ efflux activity in the elongation and differentiation root zone which has a stimulatory effect on nutrient uptake and lateral root emergence and root hair [Reviewed in (Canellas et al., 2015)]. In maize (*Zea mays* L.), a single application of HS, which is extracted from vermicomposted cattle manure, decreases the leaf total carbohydrate content, fructose and glucose contents, while increases starch content, indicating a role in N and C metabolism (Canellas et al., 2013). The application of HS isolated from vermicompost, in combination with diazotrophic endophytic bacterial inoculation was shown to strongly promote plant growth (Canellas et al., 2015).

There are several beneficial implications of vermicomposting in sustainable agriculture and nutrient recycling. Vermicomposting is a cost-effective and sustainable waste management tool that can be readily scaled up (Pathma and Sakthivel, 2012; Yadav and Garg, 2013). It circumvents many of the challenges associated with handling raw organic wastes (Dominguez and Edwards, 2010). Importantly, vermicomposting allows growers to recycle wastes from their own farming activities (e.g., plant materials and animal manure), as well as turning suitable wastes into effective organic fertilizers that improve crop production yield, while improving soil fertility at the same time (Brown et al., 2004; Laossi et al., 2010; Van Groenigen et al., 2014).

Biostimulants From Other Types of Compost

Composting of organic material is the aerobic degradation through a series of microbial reactions transforming the organic material to molecules of smaller size. Suitable as a source of plant biostimulant, composted municipal solid waste are useful for HA extraction (Jindo et al., 2012). When amended with bulking agents, it improves the growth of ornamental plants (Zhang et al., 2013; Zhang et al., 2014). Compost of municipal organic waste was also shown to improve Fe uptake of pear trees and augmented fruit quality (i.e., size, soluble solid concentration) (Sorrenti et al., 2012). In another report, the small molecule size of the composted garden waste was proposed to allow faster uptake by the tomato plants and improved the productivity (Sortino et al., 2014). Depending on the anaerobic or aerobic pre-treatment, urban organic waste is a source of different products (Massa et al., 2016). It is however recommended that such material be tailored to address the needs of specific plant species or cultivation conditions. The composition of bioactive ingredients varies in different composted organic waste, as HS extracted from composts of a number of organic waste sources (i.e., residues from artichoke, funnel, tomato, and cauliflower) were evaluated to harbor different biostimulant capacities (Monda et al., 2018). Most recently, composted olive mill waste water, a by-product from the olive oil industry, is used for HS extraction (Palumbo et al., 2018). Furthermore, although the mechanism remains elusive, the combinatory application of composted organic waste and *Trichoderma* stimulates the level of antioxidant enzyme *in planta* (Bernal-Vicente et al., 2015). This type of synergic biostimulatory capability between microorganisms and compost (or vermicompost) has also been reported previously, such as *Herbaspirillum seropedicae* and HS induced growth and pathogen resistance, possibly by auxin-like and membrane H^+-ATPase activities (Canellas et al., 2013).

Sewage sludge is the left-over residue generated at centralized wastewater treatment plants (Harrison et al., 2006). Although sewage sludge is used widely in agriculture to recycle mineral nutrients, the presence of heavy metals, organic contaminants and pathogenic bacteria may create risks (Zuloaga et al., 2012). Thus, an EU Directive prohibits the use of untreated sludge for agricultural land applications, and demands the removal of heavy metal and contaminants before using it as biofertilizer (European Commission, 1986). Composting is the main approach to stabilize the sewage sludge for use in sustainable agriculture. Composted sewage sludge (CSS) has been evaluated for the cultivation of vegetable species (Cai et al., 2010). CSS is tested as potting media for lettuce, to replace peat (Jayasinghe, 2012). Biostimulant substances can be found in CSS as well. HS extracted from CSS has been shown to promote root growth and proton pump activity in maize vesicles (Jindo et al., 2012).. In addition, two sanitized sewage sludge streams are used to improve growth and yield of pepper (*Capsicum annuum* L. cv. Piquillo), possibly by promoting rhizosphere microorganism activity and HS (Pascual et al., 2010). As these studies demonstrate the utility of sewage sludge as biofertilizer or source of plant biostimulants, the focus should be on finding solutions for the potential contamination with pathogenic microbes and toxic chemicals, in order to rule out all health risks.

Protein Hydrolysates

PHs are mainly produced from the chemical and/or enzymatic hydrolysis of proteins from by-products from the agriculture industries, both animal- and plant- origins [reviewed in (Colla et al., 2014)]. The production process and protein source are the major factors determining the chemical properties of PHs (Colla et al., 2015). PHs of animal origin are chemically hydrolyzed by acids and alkalis which increases their salinity (Colla et al., 2015). Considered to be more environmental-friendly, PHs of plant origin are produced by enzymatic hydrolysis, which results in mixture of amino acids and peptides of different lengths with low salinity (Colla et al., 2015). The sources of PHs are diverse, including animal epithelial or connective tissues, animal collagen and elastin, carob germ proteins, alfalfa residue, wheat-condensed distiller solubles, *Nicotiana* cell wall glycoproteins and algal proteins [Reviewed in (Calvo et al., 2014)].

PHs converted from organic waste have been widely promoted as plant biostimulants. Typically, insoluble and soluble fractions are obtained by alkaline hydrolysis. Different material sources are used, such as the remaining biomass from tomato plants at the end of cropping seasons (Baglieri et al., 2014), by-product extracts from apple seeds, rapeseeds, and rice husks (Donno et al., 2013). The enzymatic process to produce amino acids and proteins from carob germs, the by-products of carob fruit, and

its positive impact on tomato growth parameters, has also been described (Parrado et al., 2008). PHs from enzymatic extract of vegetable by-products, together with the phytohormone (auxins, gibberellins, cytokinins), improved the anthocyanin levels in grapes (Parrado et al., 2007). Moreover, to avoid heavy metal precipitations, the hydrolysis of sewage sludge has been reported to consist of peptides and free amino acids, and it has potentially biostimulatory effects (Tejada et al., 2013;Tejada et al., 2016). On the other hand, it contributes to alleviating the negative effects on soil enzymatic activities and microbial diversity imposed by herbicide application (Rodríguez-Morgado et al., 2014;Tejada et al., 2014).

When it comes to PHs of animal origin, waste management from the tanning industry turns PHs into a product that was shown to have biostimulants activity (Ertani et al., 2013a;Vaskova et al., 2013). PHs derived from chicken feather result in increased maize yield (Tejada et al., 2018). Siapton, a product from animal PH, acts as an alleviator of salt stress (Mladenova et al., 1998). Another PH with great potential as plant biostimulants is fish PHs. Fish PHs can be derived from fish skin, a rich source of collagen and gelatin (Chalamaiah et al., 2012). Other by-products from the fish processing industries include fish head, muscle, viscera, liver, bone, frame, and roe/egg [Reviewed in (Chalamaiah et al., 2012)]. Fish by-products are enriched in proteins, fat, amino acids (after hydrolysis), antioxidant, which are already valorized as food or feed (Halim et al., 2016). It is expected that they can equally provide nutrient sources, increase immunity for crops as well. A recent study suggested that FPHs act to promote lettuce growth and stomatal conductance (Xu and Mou, 2017).

Currently, most PHs are from chemically hydrolyzed leather waste (Colantoni et al., 2017). In terms of energy use and environmental impact, animal-derived PHs (e.g., leather by-product) showed higher ecological footprint than plant-derived PHs (Colantoni et al., 2017). As a result, for building sustainable farming, it is preferred to generate biostimulants by enzymatically hydrolyzing plant proteins over animal by-products. By switching to plant-based PHs, it also avoids risks of possible contamination with pathogens.

Several PH biostimulant products have demonstrated activity in enhancing N and C metabolism in plants. For example, a plant-derived PH "Trainer®" was used to evaluate the plant growth promoting effects (Colla et al., 2014). The application of Trainer® on maize coleoptiles and dwarf pea induced auxin- and gibberellin-like activities, respectively (Colla et al., 2014). It promotes elongation of maize coleoptiles and shoot length of gibberellin-deficient dwarf pea (Colla et al., 2014). Moreover, it also improves dry weight of shoots and roots, total biomass, cholorphyll content and leaf N content in tomato plants (Colla et al., 2014). The treatment of maize seedlings with one PH derived from hydrolyzed tanning residues resulted in decrease NO^{3-}, $PO_4{}^{3-}$, and $SO_4{}^{2-}$ concentrations (Ertani et al., 2013a). The decreased level of these ions is due to changes of transcripts of genes involved in N metabolism (nitrate reductase, glutamine synthetase, glutamate synthase and asparate aminotransferase) and TCA cycle (malate dehydrogenase, isocitrate dehydrogenase and citrate synthase) (Ertani et al., 2013a).

Chitin and Chitosan

Chitin, a biopolymer from crustaceans shells, and chitosan, the deacetylated form of chitin, have potential applications in food, cosmetics and industrial processes [Reviewed in (Olsen et al., 2014)]. Chitin and chitosan are co-polymers of N-acetyl-d-glucosamine and d-glucosamine, where the ratio of each monomer in the polymer chain defines its physical, chemical and biological properties (Pichyangkura and Chadchawan, 2015). The binding of chitin and chitosan to cell receptors induces physiological changes, triggering an oxidative burst reaction with H_2O_2 accumulation and Ca^{2+} leakage into the cell which are similar to signaling of stress response and in the developmental regulation (du Jardin, 2015). Phenylalanine ammonia-lyase (PAL) is a key plant defense enzyme induced upon contact with chitin molecules, leading to the accumulation of phenolic compounds. Most plant species respond in a similar manner including papaya (*Carica papaya* L.), sweet basil (*Ocimum basilicum* L.), sunflower (*Helianthus annuus* L.), litchi (*Litchi chinensis* Sonn.), grape (*Vitis vinifera* L.), etc. [Reviewed in (Pichyangkura and Chadchawan, 2015)]. The cellular response to chitosan involves also NO, and the phytohormone regulators jasmonic acid (JA), abscisic acid (ABA), and phosphatidic acid (PA), which all relate to abiotic stress gene regulation (Pichyangkura and Chadchawan, 2015). By proteomic approach, a recent study uncovered that enzymes involved in phenylpropanoids biosynthesis in grapes were accumulated in response to chitosan (Lucini et al., 2018).

Due to its potency to induce defense mechanisms and stress response pathways, chitin and chitosan are used to improve crop resilience to pathogen attack and abiotic stress conditions. Chitosan, for example, has been found to be effective against biotrophic and necrotrophic pathogens (Sharp, 2013). Moreover, fungal infection may be affected directly by oligochitosan as the endomembrane system of *Phytophthora capsici* is disrupted, especially the integrity of vacuoles (Xu et al., 2007). The activity can also be indirect, as it involves chitinolytic microbes which form mutualistic relationship with plants by producing chitinase enzymes that degrade chitin-rich tissues from other organism (Sharp, 2013). In terms of yield, preharvest application of chitosan increases fruit yield at harvest (Bautista-Baños et al., 2006). The yield of tomato increases by applying chitosan to soil with *F. oxysporum* f. sp. *radicis-lycopersici* fungal inoculation (Lafontaine and Benhamou, 1996; Bautista-Baños et al., 2006). Chitosan could reduce post-harvesting disease as well, as indicated by several studies showing that chitosan effectively prevents postharvest decay during storage and delays microbial infection [Reviewed in (Bautista-Baños et al., 2006)].

Other Types of By-Products

Several other types of by-products enhancing plant production and food quality have been reported in recent years. Sugarcane vinasse, a by-product mainly of the sugar-ethanol industry, has been evaluated as a nutrient source for microorganisms to detoxify or remove xenobiotics from the environment, promoting bioremediation of the soil (Christofoletti et al., 2013). Vine shoots generated during the pruning process, have been shown to contain phenolic, volatile and mineral compounds

that were applied as grapevine biostimulant and foliar fertilizer (Sánchez-Gómez et al., 2014; Sanchez-Gomez et al., 2017). Aqueous extracts of by-products from fennel, lemon and barley grains were shown to enhance tomato yield and fruit quality (Abou Chehade et al., 2018). Furthermore, it's not surprising that HS extracted from the by-products of rape, castor oil and flax showed bioactivity in maize plantlet growth, attributed to the phytohormone-like activity (Ertani et al., 2013b).

Aquaponics is an integrated system combining hydroponic crop cultivation with fish cultivation, using the same water for both systems [Reviewed in (Tyson et al., 2011)]. The nutrient by-products generated by the aquaculture could have potential biostimulatory properties, although relative reports are scarce and there's no solid evidence to substantiate the biostimulatory effects. It is speculated that boosted nutrient uptake of crops was connected with biostimulant elements in the aquaponic water supplemented with macro-nutrients (Nicoletto et al., 2018). It might be due to microorganisms and organic matter dissolved in water that are responsible for the biostimulatory effects (Delaide et al., 2016).

THE USE OF BIOSTIMULANTS COMPLEMENTS CONVENTIONAL FERTILIZERS

The increasing use of organic fertilizers is driven by the awareness to quest for organic food industry, replacing conventional farming methods relying heavily on chemical fertilizers. In terms of yield, organic farming performs differently with conventional farming. A 21-year study of agronomic and ecological performance of organic and conventional farming systems revealed that legume-based crop rotations to fertilize the soil organically reduces the input of fertilizer at an expense of 20% lower crop yield compared to conventional fertilization systems (Mader et al., 2002). A comprehensive synthesis of scientific literature on organic farming found that the average organic yield is 25% lower than conventional farming (Seufert et al., 2012). However, the performance of organic farming systems varies substantially depending on the contexts and crops cultivated. Organic fruits and oilseed crops, for instance, are most often as profitable as conventional cultivation (Seufert et al., 2012). The organic systems show efficient resource utilization, increased soil fertility and enhanced floral and faunal diversity (Mader et al., 2002; Diacono and Montemurro, 2011). Another study also indicates that the population of *Trichoderma* species, thermophilic microorganism and enteric bacteria were in greater numbers in organic soil amendment (i.e., composted organic waste) (Bulluck et al., 2002). Meanwhile, in other cases, the yield drop was compensated by the application of vermicompost when applied under specific growing conditions (Arancon et al., 2004; Arancon et al., 2005; Gutiérrez-Miceli et al., 2007). It indicates that by using composts as sources of biostimulants, it can increase yield and decrease loss due to infestation. These findings demonstrate that biostimulants or biofertilizers are relevant components of sustainable agriculture and have the potential to tip the balance in favor of organic cultivation methods, in addition to the fact that comparing yield between different systems is highly subject to contexts. As variations in waste being used to generate vermicompost and biostimulants are a complex mixture of substances showing batch differences that may influence performance, studies to optimize the robustness are needed to avoid variability in the effectiveness of biostimulants products.

The fertilization of soil containing microbial biomass is pH dependent, meaning that urea and ammonium input can lower soil pH over time, posing negative effects on beneficial soil microorganism and crop yield (Geisseler and Scow, 2014). Thus, chemical fertilizers should be applied considering the impact on the environment. Biostimulants could play an important role in improved nutrient uptake by the crop, hereby increase yield, which in turn reduces the dependence on traditional fertilizers. In a study of hydroponically grown rocket (*Eruca sativa* Mill.), biostimulant Actiwave® was added to the nutrient solution of plants grown in floating system (Vernieri et al., 2006). The result showed that Actiwave® increased nutrient uptake and nutrient use efficiency, reduced leaf nitrate content and increased chlorophyll and carotenoids contents (Vernieri et al., 2006). GroZyme®, a microbial fermentation product, enriched Zn concentration in the phloem and xylem/collenchyma region of the petiole vascular bundle in sunflower (*H. annuus* L.) (Tian et al., 2014). Field trials of GroZyme® have shown the positive growth effects and elevated K translocation and other nutrient elements, possibly by the microbial extracts forming meal complexes that enhance mineral uptake or mobility (Tian et al., 2014). HS extract, HA7 has been found to stimulate plant growth and leaf chlorophyll content by enhancing N, C and S assimilation in rapeseed (*Brassica napus*) (Jannin et al., 2012). Taken together, sustainable food production and security with minimal impact on the environment will require a combination of organic and conventional cropping systems, which take benefit from the fact that biostimulant application improves mineral absorption by modifying plant innate metabolic pathways and compensates for the reduced use of chemical fertilizers. An important notice is however, that yield increase in organic farming usually takes years to be detected (Bulluck et al., 2002; Seufert et al., 2012).

CONCLUSION AND PERSPECTIVES

Under the framework of circular economy, the development of biostimulants from organic waste has important valorization potential. One of the drivers of market development is the companies in research and development contributing to the expanding list of biostimulants, as well as production and formulation processes (du Jardin, 2015). Currently, there have been some studies on biostimulants originated from organic waste streams, including vermicompost, composted urban waste, sewage sludge. PHs, chitin and chitosan represent the group of biostimulants generated from organic waste. However, a broader profile encompassing molecular and physiological impact of biostimulant on targeted plants is needed. By mode of action

analysis, novel groups of compounds with biostimulant activities will be discovered from a variety sources of feedstocks.

The European Commission has proposed to revise EU legislation on waste to set clear targets to establish a long-term goal for waste management (European Commission, 2017). It is expected that the prospect of a circular economy require more research and technology development, focusing on bringing up sustainable products onto the market. Biostimulants extracted from waste streams provides a path for research and industrial partners, from lab research, prototyping, to commercialization and benefit the crop growers and consumers. Eventually, it creates incentives for the society to involve in the sustainable development scheme. Higher relevance to growth parameters (e.g., biomass, SPAD index, plant height, and growth index) can lead to better selection of biostimulatory components from source material, as these components (e.g., soluble or insoluble PH) offer better value and development potentials (Massa et al., 2016).

Agricultural nutrient imbalance is substantial with economic development, from inadequate input to maintain soil fertility in sub-Saharan Africa, to excessive surpluses in many areas in the more developed world (Vitousek et al., 2009). As a result, the use of biostimulants, as an element to increase nutrient use efficiency, should also consider the geo-economic differences across different areas around the world. Monitoring tools for the efficacy of biostimulants are needed to adapt to local and temporal use of biostimulants in agriculture and horticulture (du Jardin, 2015). It is expected that, with the trend of replacing chemical fertilizers, biostimulant derived from by-products will be more commonly used if valorization chain is well established and mode of action is further investigated.

AUTHOR CONTRIBUTIONS

LX collected the literature mentioned in the manuscript. LX and DG conceptualized and wrote the manuscript.

FUNDING

This work was funded by the Flemish Research Foundation (FWO) – Strategic Basic Research (SBO), under the file number S006017N.

ACKNOWLEDGMENTS

We wish to thank Mr. Christophe El-Nakhel (University of Naples Federico II and Ghent University) for assistance in gathering information on biostimulants from by-products. We also wish to thank Dr. Maaike Perneel (CropFit, Ghent University), Mr. El-Nakhel, and Dr. Stefano D'Alessandro for critical review of the manuscript.

REFERENCES

Abou Chehade, L., Al Chami, Z., De Pascali, S. A., Cavoski, I., and Fanizzi, F. P. (2018). Biostimulants from food processing by-products: agronomic, quality and metabolic impacts on organic tomato (*Solanum lycopersicum* L.). *J. Sci. Food Agric.* 98, 1426–1436. doi: 10.1002/jsfa.8610

Alexander, K. T. W., Corning, D. R., Cory, N. J., Donohue, V. J., and Sykes, R. L. (1992). Environmental and safety issues – Clean technology and environmental auditing. *J. Soc. Leather Technol. Chem.* 76, 17–23.

Allardice, R. P., Kapp, C., Botha, A., and Valentine, A. (2015). Optimizing vermicompost concentrations for the N nutrition and production of the legume lupinus angustifolius. *Compos. Sci. Util.* 23, 217–236. doi: 10.1080/1065657X.2015.1038399

Arancon, N. Q., Edwards, C. A., Atiyeh, R., and Metzger, J. D. (2004). Effects of vermicomposts produced from food waste on the growth and yields of greenhouse peppers. *Bioresour. Technol.* 93, 139–144. doi: 10.1016/j.biortech.2003.10.015

Arancon, N. Q., Galvis, P. A., and Edwards, C. A. (2005). Suppression of insect pest populations and damage to plants by vermicomposts. *Bioresour. Technol.* 96, 1137–1142. doi: 10.1016/j.biortech.2004.10.004

Aremu, A. O., Kulkarni, M. G., Bairu, M. W., Finnie, J. F., and Van Staden, J. (2012). Growth stimulation effects of smoke-water and vermicompost leachate on greenhouse grown-tissue-cultured 'Williams' bananas. *Plant Growth Regul.* 66, 111–118. doi: 10.1007/s10725-011-9634-6

Aremu, A. O., Masondo, N. A., and Van Staden, J. (2014). Physiological and phytochemical responses of three nutrient-stressed bulbous plants subjected to vermicompost leachate treatment. *Acta Physiol. Plant.* 36, 721–731. doi: 10.1007/s11738-013-1450-3

Aremu, A. O., Stirk, W. A., Kulkarni, M. G., Tarkowska, D., Tureckova, V., Gruz, J., et al. (2015). Evidence of phytohormones and phenolic acids variability in garden-waste-derived vermicompost leachate, a well-known plant growth stimulant. *Plant Growth Regul.* 75, 483–492. doi: 10.1007/s10725-014-0011-0

Baglieri, A., Cadili, V., Mozzetti Monterumici, C., Gennari, M., Tabasso, S., Montoneri, E., et al. (2014). Fertilization of bean plants with tomato plants hydrolysates. Effect on biomass production, chlorophyll content and N assimilation. *Sci. Hortic.* 176, 194–199. doi: 10.1016/j.scienta.2014.07.002

Bautista-Baños, S., Hernández-Lauzardo, A. N., Velázquez-Del Valle, M. G., Hernández-López, M., Ait Barka, E., Bosquez-Molina, E., et al. (2006). Chitosan as a potential natural compound to control pre and postharvest diseases of horticultural commodities. *Crop Protect.* 25, 108–118. doi: 10.1016/j.cropro.2005.03.010

Berbel, J., and Posadillo, A. (2018). Review and analysis of alternatives for the valorisation of agro-industrial olive oil by-products. *Sustainability* 10:237. doi: 10.3390/su10010237

Bernal-Vicente, A., Pascual, J. A., Tittarelli, F., Hernández, J. A., and Diaz-Vivancos, P. (2015). Trichoderma harzianum T-78 supplementation of compost stimulates the antioxidant defence system in melon plants. *J. Sci. Food Agric.* 95, 2208–2214. doi: 10.1002/jsfa.6936

Brown, G. G., Edwards, C. A., and Brussaard, L. (2004). "How earthworms affect plant growth: burrowing into the mechanisms," in *Earthworm Ecology*, ed. C. A. Edwards (Boca Raton, FL: CRC Press), 13–49.

Brown, P., and Saa, S. (2015). Biostimulants in agriculture. *Front. Plant Sci.* 6:671. doi: 10.3389/fpls.2015.00671

Buckwell, A., and Nadeu, E. (2016). *Nutrient Recovery and Reuse (NRR) in European Agriculture. A Review of the Issues, Opportunities, and Actions*. Brussels: RISE Foundation.

Bulluck, L. R., Brosius, M., Evanylo, G. K., and Ristaino, J. B. (2002). Organic and synthetic fertility amendments influence soil microbial, physical and chemical properties on organic and conventional farms. *Appl. Soil Ecol.* 19, 147–160. doi: 10.1016/S0929-1393(01)00187-1

Cai, H., Chen, T., Liu, H., Gao, D., Zheng, G., and Zhang, J. (2010). The effect of salinity and porosity of sewage sludge compost on the growth of vegetable seedlings. *Sci. Hortic.* 124, 381–386. doi: 10.1016/j.scienta.2010.01.009

Calvo, P., Nelson, L., and Kloepper, J. W. (2014). Agricultural uses of plant biostimulants. *Plant Soil* 383, 3–41. doi: 10.1007/s11104-014-2131-8

Canellas, L. P., Balmori, D. M., Médici, L. O., Aguiar, N. O., Campostrini, E., Rosa, P. C. C., et al. (2013). A combination of humic substances and *Herbaspirillum seropedicae* inoculation enhances the growth of maize (*Zea mays* L.). *Plant Soil* 366, 119–132. doi: 10.1007/s11104-012-1382-5

Canellas, L. P., Olivares, F. L., Aguiar, N. O., Jones, D. L., Nebbioso, A., Mazzei, P., et al. (2015). Humic and fulvic acids as biostimulants in horticulture. *Sci. Hortic.* 196, 15–27. doi: 10.1016/j.scienta.2015.09.013

Chalamaiah, M., Dinesh Kumar, B., Hemalatha, R., and Jyothirmayi, T. (2012). Fish protein hydrolysates: proximate composition, amino acid composition, antioxidant activities and applications: a review. *Food Chem.* 135, 3020–3038. doi: 10.1016/j.foodchem.2012.06.100

Chinsamy, M., Kulkarni, M. G., and Van Staden, J. (2013). Garden-waste-vermicompost leachate alleviates salinity stress in tomato seedlings by mobilizing salt tolerance mechanisms. *Plant Growth Regul.* 71, 41–47. doi: 10.1007/s10725-013-9807-6

Christofoletti, C. A., Escher, J. P., Correia, J. E., Marinho, J. F. U., and Fontanetti, C. S. (2013). Sugarcane vinasse: environmental implications of its use. *Waste Manage.* 33, 2752–2761. doi: 10.1016/j.wasman.2013.09.005

Colantoni, A., Recchia, L., Bernabei, G., Cardarelli, M., Rouphael, Y., and Colla, G. (2017). Analyzing the environmental impact of chemically-produced protein hydrolysate from leather waste vs. enzymatically-produced protein hydrolysate from legume grains. *Agriculture* 7:62. doi: 10.3390/agriculture7080062

Colla, G., Hoagland, L., Ruzzi, M., Cardarelli, M., Bonini, P., Canaguier, R., et al. (2017). Biostimulant action of protein hydrolysates: unraveling their effects on plant physiology and microbiome. *Front. Plant Sci.* 8:2202. doi: 10.3389/fpls.2017.02202

Colla, G., Nardi, S., Cardarelli, M., Ertani, A., Lucini, L., Canaguier, R., et al. (2015). Protein hydrolysates as biostimulants in horticulture. *Sci. Hortic.* 196, 28–38. doi: 10.1016/j.scienta.2015.08.037

Colla, G., Rouphael, Y., Canaguier, R., Svecova, E., and Cardarelli, M. (2014). Biostimulant action of a plant-derived protein hydrolysate produced through enzymatic hydrolysis. *Front. Plant Sci.* 5:448. doi: 10.3389/fpls.2014.00448

European Commission (ed.). (2007). *Communication from the Commission To The Council and the European Parliament on the Interpretative Communication On Waste And By-Products. COM (2007) 59 Final*. Strasbourg: European Parliment.

European Commission (ed.). (1986). *Coucil Directive on the Protection of the Environment, and in Particular of the Soil, When Sewage Sludge is Used in Agriculture*. Luxembourg: European Commission.

European Commission (2009). *Regulation (EC) No 1069/2009 of the European Parliament and of the Council: Laying Down Health Rules as Regards Animal by-Products and Derived Products Not Intended for Human Consumption and Repealing Regulation,* ed. E. Parliament. Brussels: European Commission.

European Commission (ed.). (2016a). *Circular Economy Package – Proposal for a Regulation of the European Parliament and of the Council: In: 2016/0084*. Brussels: European Commission.

European Commission (2016b). *Circular Economy: New Regulation to Boost the Use of Organic and Waste-Based Fertilisers*. Brussels: European Commission.

European Commission (2017). *Circular Economy Research and Innovation – Connecting Economic & Environmental Gains*. Brussels: European Commission.

De Corato, U., De Bari, I., Viola, E., and Pugliese, M. (2018). Assessing the main opportunities of integrated biorefining from agro-bioenergy co/by-products and agroindustrial residues into high-value added products associated to some emerging markets: a review. *Renew. Sustain. Energy Rev.* 88, 326–346. doi: 10.1016/j.rser.2018.02.041

Delaide, B., Goddek, S., Gott, J., Soyeurt, H., and Jijakli, M. (2016). Lettuce (*Lactuca sativa* L. var. Sucrine) growth performance in complemented aquaponic solution outperforms hydroponics. *Water* 8:467. doi: 10.3390/w8100467

Diacono, M., and Montemurro, F. (2011). "Long-term effects of organic amendments on soil fertility," in *Sustainable Agriculture*, Vol. 2, eds E. Lichtfouse, M. Hamelin, M. Navarrete, and P. Debaeke (Dordrecht: Springer Netherlands), 761–786.

Dominguez, J., and Edwards, C. A. (2010). "Relationships between composting and vermicomposting," in *Vermiculture Technology Earthworms, Organic Wastes, and Environmental Management*, eds C. A. Edwards, N. Q. Arancon, and R. Sherman (Boca Raton, FL: CRC Press), 11–26. doi: 10.1201/b10453-3

Donno, D., Beccaro, G. L., Mellano, M. G., Canterino, S., Cerutti, A. K., and Bounous, G. (2013). Improving the nutritional value of kiwifruit with the application of agroindustry waste extracts. *J. Appl. Bot. Food Qual.* 86, 11–15.

du Jardin, P. (2015). Plant biostimulants: definition, concept, main categories and regulation. *Sci. Hortic.* 196, 3–14. doi: 10.1016/j.scienta.2015.09.021

Eastman, B. R., Kane, P. N., Edwards, C. A., Trytek, L., Gunadi, B., Stermer, A. L., et al. (2001). The effectiveness of vermiculture in human pathogen reduction for USEPA biosolids stabilization. *Compos. Sci. Util.* 9, 38–49. doi: 10.1080/1065657X.2001.10702015

Edwards, C. A., and Subler, S. (2010). "Human pathogen reduction during vermicomposting," in *Vermiculture Technology Earthworms, Organic Wastes, and Environmental Management*, eds C. A. Edwards, N. Q. Arancon, and R. Sherman (Boca Raton, FL: CRC Press), 249–261. doi: 10.1201/b10453-17

Ertani, A., Pizzeghello, D., Altissimo, A., and Nardi, S. (2013a). Use of meat hydrolyzate derived from tanning residues as plant biostimulant for hydroponically grown maize. *J. Plant Nutr. Soil Sci.* 176, 287–295. doi: 10.1002/jpln.201200020

Ertani, A., Pizzeghello, D., Baglieri, A., Cadili, V., Tambone, F., Gennari, M., et al. (2013b). Humic-like substances from agro-industrial residues affect growth and nitrogen assimilation in maize (*Zea mays* L.) plantlets. *J. Geochem. Explor.* 129, 103–111. doi: 10.1016/j.gexplo.2012.10.001

Ferri, M., Franceschetti, M., Naldrett, M. J., Saalbach, G., and Tassoni, A. (2014). Effects of chitosan on the protein profile of grape cell culture subcellular fractions. *Electrophoresis* 35, 1685–1692. doi: 10.1002/elps.201300624

Fritsch, C., Staebler, A., Happel, A., Marquez, M. A. C., Aguilo-Aguayo, I., Abadias, M., et al. (2017). Processing, valorization and application of bio-waste derived compounds from Potato, Tomato, olive and cereals: a review. *Sustainability* 9:1492. doi: 10.3390/su9081492

Fuell, C., Elliott, K. A., Hanfrey, C. C., Franceschetti, M., and Michael, A. J. (2010). Polyamine biosynthetic diversity in plants and algae. *Plant Physiol. Biochem.* 48, 513–520. doi: 10.1016/j.plaphy.2010.02.008

Geisseler, D., and Scow, K. M. (2014). Long-term effects of mineral fertilizers on soil microorganisms – A review. *Soil Biol. Biochem.* 75, 54–63. doi: 10.1016/j.soilbio.2014.03.023

Gutiérrez-Miceli, F. A., Santiago-Borraz, J., Montes Molina, J. A., Nafate, C. C., Abud-Archila, M., Oliva Llaven, M. A., et al. (2007). Vermicompost as a soil supplement to improve growth, yield and fruit quality of tomato (*Lycopersicum esculentum*). *Bioresour. Technol.* 98, 2781–2786. doi: 10.1016/j.biortech.2006.02.032

Halim, N. R. A., Yusof, H. M., and Sarbon, N. M. (2016). Functional and bioactive properties of fish protein hydolysates and peptides: a comprehensive review. *Trends Food Sci. Technol.* 51, 24–33. doi: 10.1016/j.tifs.2016.02.007

Harrison, E. Z., Oakes, S. R., Hysell, M., and Hay, A. (2006). Organic chemicals in sewage sludges. *Sci. Total Environ.* 367, 481–497. doi: 10.1016/j.scitotenv.2006.04.002

Hoque, M. A., Okuma, E., Banu, M. N. A., Nakamura, Y., Shimoishi, Y., and Murata, Y. (2007). Exogenous proline mitigates the detrimental effects of salt stress more than exogenous betaine by increasing antioxidant enzyme activities. *J. Plant Physiol.* 164, 553–561. doi: 10.1016/j.jplph.2006.03.010

Jannin, L., Arkoun, M., Ourry, A., Laîné, P., Goux, D., Garnica, M., et al. (2012). Microarray analysis of humic acid effects on *Brassica napus* growth: involvement of N, C and S metabolisms. *Plant Soil* 359, 297–319. doi: 10.1007/s11104-012-1191-x

Jayasinghe, G. Y. (2012). Composted sewage sludge as an alternative potting media for lettuce cultivation. *Commun. Soil Sci. Plant Anal.* 43, 2878–2887. doi: 10.1080/00103624.2012.728264

Jindo, K., Martim, S. A., Navarro, E. C., Pérez-Alfocea, F., Hernandez, T., Garcia, C., et al. (2012). Root growth promotion by humic acids from composted and non-composted urban organic wastes. *Plant Soil* 353, 209–220. doi: 10.1007/s11104-011-1024-3

Khan, W., Rayirath, U. P., Subramanian, S., Jithesh, M. N., Rayorath, P., Hodges, D. M., et al. (2009). Seaweed extracts as biostimulants of plant growth and development. *J. Plant Growth Regul.* 28, 386–399. doi: 10.1007/s00344-009-9103-x

Korniłowicz-Kowalska, T., and Bohacz, J. (2011). Biodegradation of keratin waste: theory and practical aspects. *Waste Manage.* 31, 1689–1701. doi: 10.1016/j.wasman.2011.03.024

Kotzamanis, Y. P., Gisbert, E., Gatesoupe, F. J., Infante, J. Z., and Cahu, C. (2007). Effects of different dietary levels of fish protein hydrolysates on growth, digestive enzymes, gut microbiota, and resistance to *Vibrio anguillarum* in

European sea bass (*Dicentrarchus labrax*) larvae. *Compar. Biochem. Physiol. -Mol. Integr. Physiol.* 147, 205–214. doi: 10.1016/j.cbpa.2006.12.037

La Torre, A., Battaglia, V., and Caradonia, F. (2016). An overview of the current plant biostimulant legislations in different European Member States. *J. Sci. Food Agric.* 96, 727–734. doi: 10.1002/jsfa.7358

Lafontaine, P. J., and Benhamou, N. (1996). Chitosan treatment: an emerging strategy for enhancing resistance of greenhouse tomato plants to infection by *Fusarium oxysporum* f sp radicis-lycopersici. *Biocontrol Sci. Technol.* 6, 111–124. doi: 10.1080/09583159650039575

Laossi, K. R., Ginot, A., Noguera, D. C., Blouin, M., and Barot, S. (2010). Earthworm effects on plant growth do not necessarily decrease with soil fertility. *Plant Soil* 328, 109–118. doi: 10.1007/s11104-009-0086-y

Letham, D. S., and Palni, L. M. S. (1983). The biosynthesis and metabolism of cytokinins. *Ann. Rev. Plant Physiol. Plant Mol. Biol.* 34, 163–197. doi: 10.1146/annurev.pp.34.060183.001115

Lucini, L., Baccolo, G., Rouphael, Y., Colla, G., Bavaresco, L., and Trevisan, M. (2018). Chitosan treatment elicited defence mechanisms, pentacyclic triterpenoids and stilbene accumulation in grape (*Vitis vinifera* L.) bunches. *Phytochemistry* 156, 1–8. doi: 10.1016/j.phytochem.2018.08.011

Mader, P., Fliessbach, A., Dubois, D., Gunst, L., Fried, P., and Niggli, U. (2002). Soil fertility and biodiversity in organic farming. *Science* 296, 1694–1697. doi: 10.1126/science.1071148

Massa, D., Prisa, D., Montoneri, E., Battaglini, D., Ginepro, M., Negre, M., et al. (2016). Application of municipal biowaste derived products in *Hibiscus* cultivation: effect on leaf gaseous exchange activity, and plant biomass accumulation and quality. *Sci. Hortic.* 205, 59–69. doi: 10.1016/j.scienta.2016.03.033

McHugh, D. J. (2013). *A Guide to the Seaweed Industry*. Rome: Food and Agriculture Organization of the United Nations.

Meyer, R. (2017). Bioeconomy strategies: contexts, visions, guiding implementation principles and resulting debates. *Sustainability* 9:1031. doi: 10.3390/su9061031

Mladenova, Y. I., Maini, P., Mallegni, C., Goltsev, V., Vladova, R., Vinarova, K., et al. (1998). Siapton – An amino-acid-based biostimulant reducing osmostress metabolic changes in maize. *Agro Food Indus. Hi-Tech* 9, 18–22.

Monda, H., Cozzolino, V., Vinci, G., Drosos, M., Savy, D., and Piccolo, A. (2018). Molecular composition of the Humeome extracted from different green composts and their biostimulation on early growth of maize. *Plant Soil* 429, 407–424. doi: 10.1007/s11104-018-3642-5

Muller-Maatsch, J., Bencivenni, M., Caligiani, A., Tedeschi, T., Bruggeman, G., Bosch, M., et al. (2016). Pectin content and composition from different food waste streams. *Food Chem.* 201, 37–45. doi: 10.1016/j.foodchem.2016.01.012

Mussig, C., Lisso, J., Coll-Garcia, D., and Altmann, T. (2006). Molecular analysis of brassinosteroid action. *Plant Biol. (Stuttg.)* 8, 291–296. doi: 10.1055/s-2005-873043

Nicoletto, C., Maucieri, C., Mathis, A., Schmautz, Z., Komives, T., Sambo, P., et al. (2018). Extension of aquaponic water use for NFT baby-leaf production: mizuna and rocket salad. *Agronomy* 8:75. doi: 10.3390/agronomy8050075

Olsen, R. L., Toppe, J., and Karunasagar, I. (2014). Challenges and realistic opportunities in the use of by-products from processing of fish and shellfish. *Trends Food Sci. Technol.* 36, 144–151. doi: 10.1016/j.tifs.2014.01.007

Pacifici, E., Polverari, L., and Sabatini, S. (2015). Plant hormone cross-talk: the pivot of root growth. *J. Exp. Bot.* 66, 1113–1121. doi: 10.1093/jxb/eru534

Palumbo, G., Schiavon, M., Nardi, S., Ertani, A., Celano, G., and Colombo, C. M. (2018). Biostimulant potential of humic acids extracted from an amendment obtained via combination of olive mill wastewaters (OMW) and a pre-treated organic material derived from municipal solid waste (MSW). *Fron. Plant Sci.* 9:1028. doi: 10.3389/fpls.2018.01028

Parrado, J., Bautista, J., Romero, E. J., García-Martínez, A. M., Friaza, V., and Tejada, M. (2008). Production of a carob enzymatic extract: potential use as a biofertilizer. *Bioresour. Technol.* 99, 2312–2318. doi: 10.1016/j.biortech.2007.05.029

Parrado, J., Escudero-Gilete, M. L., Friaza, V., García-Martínez, A., González-Miret, M. L., Bautista, J. D., et al. (2007). Enzymatic vegetable extract with bioactive components: influence of fertiliser on the colour and anthocyanins of red grapes. *J. Sci. Food Agric.* 87, 2310–2318. doi: 10.1002/jsfa.2989

Pascual, I., Azcona, I., Aguirreolea, J., Morales, F., Corpas, F. J., Palma, J. M., et al. (2010). Growth, yield, and fruit quality of pepper plants amended with two sanitized sewage sludges. *J. Agric. Food Chem.* 58, 6951–6959. doi: 10.1021/jf100282f

Pathma, J., and Sakthivel, N. (2012). Microbial diversity of vermicompost bacteria that exhibit useful agricultural traits and waste management potential. *Springerplus* 1:26. doi: 10.1186/2193-1801-1-26

Pichyangkura, R., and Chadchawan, S. (2015). Biostimulant activity of chitosan in horticulture. *Sci. Hortic.* 196, 49–65. doi: 10.1016/j.scienta.2015.09.031

Pimenta Lange, M. J., and Lange, T. (2006). Gibberellin biosynthesis and the regulation of plant development. *Plant Biol. (Stuttg)* 8, 281–290. doi: 10.1055/s-2006-923882

Povero, G., Mejia, J. F., Di Tommaso, D., Piaggesi, A., and Warrior, P. (2016). A systematic approach to discover and characterize natural plant biostimulants. *Front. Plant Sci.* 7:435. doi: 10.3389/fpls.2016.00435

Provenzano, M. R., De Oliveira, S. C., Santiago Silva, M. R., and Senesi, N. (2001). Assessment of maturity degree of composts from domestic solid wastes by fluorescence and fourier transform infrared spectroscopies. *J. Agric. Food Chem.* 49, 5874–5879. doi: 10.1021/jf0106796

Rodríguez-Morgado, B., Gómez, I., Parrado, J., and Tejada, M. (2014). Behaviour of oxyfluorfen in soils amended with edaphic biostimulants/biofertilizers obtained from sewage sludge and chicken feathers, *effects on soil biological properties. Environ. Sci. Pollut. Res.* 21, 11027–11035. doi: 10.1007/s11356-014-3040-3

Sánchez-Gómez, R., Zalacain, A., Alonso, G. L., and Salinas, M. R. (2014). Vine-shoot waste aqueous extracts for re-use in agriculture obtained by different extraction techniques: phenolic, volatile, and mineral compounds. *J. Agric. Food Chem.* 62, 10861–10872. doi: 10.1021/jf503929v

Sanchez-Gomez, R., Zalacain, A., Pardo, F., Alonso, G. L., and Salinas, M. R. (2017). Moscatel vine-shoot extracts as a grapevine biostimulant to enhance wine quality. *Food Res. Int.* 98, 40–49. doi: 10.1016/j.foodres.2017.01.004

Schaafsma, G. (2009). Safety of protein hydrolysates, fractions thereof and bioactive peptides in human nutrition. *Eur. J. Clin. Nutr.* 63, 1161–1168. doi: 10.1038/ejcn.2009.56

Seufert, V., Ramankutty, N., and Foley, J. A. (2012). Comparing the yields of organic and conventional agriculture. *Nature* 485:229. doi: 10.1038/nature11069

Sharma, H. S. S., Fleming, C., Selby, C., Rao, J. R., and Martin, T. (2014). Plant biostimulants: a review on the processing of macroalgae and use of extracts for crop management to reduce abiotic and biotic stresses. *J. Appl. Phycol.* 26, 465–490. doi: 10.1007/s10811-013-0101-9

Sharp, R. (2013). A review of the applications of chitin and its derivatives in agriculture to modify plant-microbial interactions and improve crop yields. *Agronomy* 3:757. doi: 10.3390/agronomy3040757

Sorrenti, G., Toselli, M., and Marangoni, B. (2012). Use of compost to manage Fe nutrition of pear trees grown in calcareous soil. *Sci. Horticul.* 136, 87–94. doi: 10.1016/j.scienta.2011.12.033

Sortino, O., Montoneri, E., Patanè, C., Rosato, R., Tabasso, S., and Ginepro, M. (2014). Benefits for agriculture and the environment from urban waste. *Sci. Total Environ.* 487, 443–451. doi: 10.1016/j.scitotenv.2014.04.027

Stirk, W. A., Tarkowska, D., Turecova, V., Strnad, M., and Van Staden, J. (2014). Abscisic acid, gibberellins and brassinosteroids in Kelpak (R), a commercial seaweed extract made from *Ecklonia maxima*. *J. Appl. Phycol.* 26, 561–567. doi: 10.1007/s10811-013-0062-z

Stirk, W. A., and Van Staden, J. (2014). Plant growth regulators in seaweeds: occurrence, regulation and functions. *Sea Plants* 71, 125–159. doi: 10.1016/B978-0-12-408062-1.00005-6

Tejada, M., García-Martínez, A. M., Rodríguez-Morgado, B., Carballo, M., García-Antras, D., Aragón, C., et al. (2013). Obtaining biostimulant products for land application from the sewage sludge of small populations. *Ecol. Eng.* 50, 31–36. doi: 10.1016/j.ecoleng.2012.07.006

Tejada, M., Rodríguez-Morgado, B., Gómez, I., Franco-Andreu, L., Benítez, C., and Parrado, J. (2016). Use of biofertilizers obtained from sewage sludges on maize yield. *Eur. J. Agron.* 78, 13–19. doi: 10.1016/j.eja.2016.04.014

Tejada, M., Rodríguez-Morgado, B., Gómez, I., and Parrado, J. (2014). Degradation of chlorpyrifos using different biostimulants/biofertilizers: effects on soil biochemical properties and microbial community. *Appl. Soil Ecol.* 84, 158–165. doi: 10.1016/j.apsoil.2014.07.007

Tejada, M., Rodríguez-Morgado, B., Paneque, P., and Parrado, J. (2018). Effects of foliar fertilization of a biostimulant obtained from chicken feathers on maize yield. *Eur. J. Agron.* 96, 54–59. doi: 10.1016/j.eja.2018.03.003

Tian, S., Lu, L., Xie, R., Zhang, M., Jernstedt, J. A., Hou, D., et al. (2014). Supplemental macronutrients and microbial fermentation products improve the uptake and transport of foliar applied zinc in sunflower (*Helianthus annuus* L.) *plants*, studies utilizing micro x-ray florescence. *Front. Plant Sci.* 5:808. doi: 10.3389/fpls.2014.00808

Trevisan, S., Botton, A., Vaccaro, S., Vezzaro, A., Quaggiotti, S., and Nardi, S. (2011). Humic substances affect *Arabidopsis* physiology by altering the expression of genes involved in primary metabolism, growth and development. *Environ. Exp. Bot.* 74, 45–55. doi: 10.1016/j.envexpbot.2011.04.017

Turek, C., and Stintzing, F. C. (2013). Stability of essential oils: a review. *Comprehen. Rev. Food Sci. Food Saf.* 12, 40–53. doi: 10.1111/1541-4337.12006

Tyson, R. V., Treadwell, D. D., and Simonne, E. H. (2011). Opportunities and challenges to sustainability in aquaponic systems. *HortTechnology* 21, 6–13.

Van Groenigen, J. W., Lubbers, I. M., Vos, H. M. J., Brown, G. G., De Deyn, G. B., and Van Groenigen, K. J. (2014). Earthworms increase plant production: a meta-analysis. *Sci. Rep.* 4:6365. doi: 10.1038/srep06365

Vaskova, H., Kolomaznik, K., and Vasek, V. (2013). Hydrolysis process of collagen protein from tannery waste materials for production of biostimulator and its mathematical model. *Int. J. Mathemat. Models Methods Appl. Sci.* 7, 568–575.

Verheijen, L. A. H. M., Wiersema, D., and Hulshoff Pol, L. W. (1996). *Management of Waste from Animal Product Processing*. Canberra, ACT: International Agriculture Centre.

Vernieri, P., Borghesi, E., Tognoni, F., Serra, G., Ferrante, A., and Piagessi, A. (2006). "Use of Biostimulants for the reducing nutrient solution concentration in floating system," in *Proceedings of the III International Symposium on Models for Plant Growth, Environmental Control and Farm Management in Protected Cultivation: International Society for Horticultural Science (ISHS)*, Leuven, 477–484. doi: 10.17660/ActaHortic.2006.718.55

Vitousek, P. M., Naylor, R., Crews, T., David, M. B., Drinkwater, L. E., Holland, E., et al. (2009). Nutrient imbalances in agricultural development. *Science* 324, 1519–1520. doi: 10.1126/science.1170261

Werner, T., and Schmulling, T. (2009). Cytokinin action in plant development. *Curr. Opin. Plant Biol.* 12, 527–538. doi: 10.1016/j.pbi.2009.07.002

Wolters, H., and Jurgens, G. (2009). Survival of the flexible: hormonal growth control and adaptation in plant development. *Nat. Rev. Genet.* 10, 305–317. doi: 10.1038/nrg2558

Xu, C. P., and Mou, B. Q. (2017). Drench application of fish-derived protein hydrolysates affects lettuce growth, chlorophyll content, and gas exchange. *Horttechnology* 27, 539–543. doi: 10.21273/HORTTECH03723-17

Xu, J. G., Zhao, X. M., Han, X. W., and Du, Y. G. (2007). Antifungal activity of oligochitosan against *Phytophthora capsici* and other plant pathogenic fungi in vitro. *Pesticide Biochem. Physiol.* 87, 220–228. doi: 10.1016/j.pestbp.2006.07.013

Yadav, A., and Garg, V. K. (2013). Nutrient recycling from industrial solid wastes and weeds by vermiprocessing using earthworms. *Pedosphere* 23, 668–677. doi: 10.1016/S1002-0160(13)60059-4

Yakhin, O. I., Lubyanov, A. A., Yakhin, I. A., and Brown, P. H. (2016). Biostimulants in plant science: a global perspective. *Front. Plant Sci.* 7:2049. 10.3389/fpls.2016.02049 doi: 10.3389/fpls.2016.02049

Zaller, J. G. (2007). Vermicompost as a substitute for peat in potting media: effects on germination, biomass allocation, yields and fruit quality of three tomato varieties. *Sci. Hortic.* 112, 191–199. doi: 10.1016/j.scienta.2006.12.023

Zayats, M. F., Leschev, S. M., Petrashkevich, N. V., Zayats, M. A., Kadenczki, L., Szitas, R., et al. (2013). Distribution of pesticides in n-hexane/water and n-hexane/acetonitrile systems and estimation of possibilities of their extraction isolation and preconcentration from various matrices. *Anal. Chim. Acta* 774, 33–43. doi: 10.1016/j.aca.2013.03.003

Zhang, L., Sun, X. Y., Tian, Y., and Gong, X. Q. (2013). Composted green waste as a substitute for peat in growth media: effects on growth and nutrition of calathea insignis. *PLoS One* 8:e78121. doi: 10.1371/journal.pone.0078121

Zhang, L., Sun, X.-Y., Tian, Y., and Gong, X.-Q. (2014). Biochar and humic acid amendments improve the quality of composted green waste as a growth medium for the ornamental plant *Calathea insignis*. *Sci. Horticul.* 176, 70–78. doi: 10.1016/j.scienta.2014.06.021

Zhao, Y. (2010). Auxin biosynthesis and its role in plant development. *Annu. Rev. Plant Biol.* 61, 49–64. doi: 10.1146/annurev-arplant-042809-112308

Zuloaga, O., Navarro, P., Bizkarguenaga, E., Iparraguirre, A., Vallejo, A., Olivares, M., et al. (2012). Overview of extraction, clean-up and detection techniques for the determination of organic pollutants in sewage sludge: a review. *Anal. Chim. Acta* 736, 7–29. doi: 10.1016/j.aca.2012.05.016

Conflict of Interest Statement: The authors declare that the research was conducted in the absence of any commercial or financial relationships that could be construed as a potential conflict of interest.

frontiers in Plant Science

ORIGINAL RESEARCH
published: 02 November 2018
doi: 10.3389/fpls.2018.01609

Jasmonic Acid Seed Treatment Stimulates Insecticide Detoxification in *Brassica juncea* L.

Anket Sharma[1,2], Vinod Kumar[3], Huwei Yuan[1], Mukesh Kumar Kanwar[4], Renu Bhardwaj[2], Ashwani Kumar Thukral[2] and Bingsong Zheng[1]**

[1] State Key Laboratory of Subtropical Silviculture, Zhejiang A&F University, Hangzhou, China, [2] Plant Stress Physiology Lab, Department of Botanical and Environmental Sciences, Guru Nanak Dev University, Amritsar, India, [3] Department of Botany & Environment Studies, DAV University, Jalandhar, India, [4] Department of Horticulture, Zhejiang University, Hangzhou, China

The present study focused on assessing the effects of jasmonic acid (JA) seed treatment on the physiology of *Brassica juncea* seedlings grown under imidacloprid (IMI) toxicity. It has been observed that IMI application declined the chlorophyll content and growth of seedlings. However, JA seed treatment resulted in the significant recovery of chlorophyll content and seedling growth. Contents of oxidative stress markers like superoxide anion, hydrogen peroxide, and malondialdehyde were enhanced with IMI application, but JA seed treatment significantly reduced their contents. Antioxidative defense system was activated with IMI application which was further triggered after JA seed treatment. Activities of antioxidative enzymes and contents of non-enzymatic antioxidants were enhanced with the application of IMI as well as JA seed treatment. JA seed treatment also regulated the gene expression of various enzymes under IMI stress. These enzymes included respiratory burst oxidase (*RBO*), Ribulose-1,5-bisphosphate carboxylase/oxygenase (*RUBISCO*), NADH-ubiquinone oxidoreductase (*NADH*), carboxylesterase (*CXE*), chlorophyllase (*CHLASE*), cytochrome P450 monooxygenase (*P450*). JA seed treatment up-regulated the expressions of *RUBISCO*, *NADH*, *CXE,* and *P450* under IMI toxicity. However, expressions of *RBO* and *CHLASE* were down-regulated in seedlings germinated from JA seed treatment and grown in presence of IMI. Seed soaking with JA also resulted in a significant reduction of IMI residues in *B. juncea* seedlings. The present study concluded that seed soaking with JA could efficiently reduce the IMI toxicity by triggering the IMI detoxification system in intact plants.

Keywords: insecticide, jasmonic acid, oxidative stress, *Brassica juncea*, imidacloprid

OPEN ACCESS

Edited by:
Giuseppe Colla, Università degli Studi della Tuscia, Italy

Reviewed by:
Hsu-Liang Hsieh, National Taiwan University, Taiwan
Renu Deswal, University of Delhi, India

****Correspondence:***
Anket Sharma
anketsharma@gmail.com
Bingsong Zheng
bszheng@zafu.edu.cn

Specialty section:
This article was submitted to Crop and Product Physiology, a section of the journal Frontiers in Plant Science

Received: *24 July 2018*
Accepted: *17 October 2018*
Published: *02 November 2018*

Citation:
Sharma A, Kumar V, Yuan H, Kanwar MK, Bhardwaj R, Thukral AK and Zheng B (2018) Jasmonic Acid Seed Treatment Stimulates Insecticide Detoxification in Brassica juncea L. Front. Plant Sci. 9:1609. doi: 10.3389/fpls.2018.01609

INTRODUCTION

Food demands are continuously increasing due to the rapid population growth throughout the globe. To meet the food requirements, there is a need to check the loss of crop yield caused by the attack of pests (Kular and Kumar, 2011; Razaq et al., 2011). Pesticides are widely used to control these pests. However, overuse of pesticides has a negative impact on the environment as well as on agricultural products because pesticides act as source of environmental pollution and their residues contaminate food stuffs (Shahzad et al., 2018).

Brassica juncea is an important vegetable as well as oil yielding crop and is generally attacked by insects like aphids. This insect attack drastically reduces the yield of *Brassica* plants (Kular and Kumar, 2011; Razaq et al., 2011). Imidacloprid (IMI) is a neonicotinoid insecticide and was the first member of its family which came into the market in 1991 (Cox, 2001; Stamm et al., 2016). IMI is systemic in nature which is more effective against sucking insects like aphids as compared to other insecticides (El-Naggar and Zidan, 2013). Moreover, it is also much effective against those insects which have got resistance for other insecticides like carbamates, organophosphates, and pyrethroids (Cox, 2001). IMI is usually applied into soil, which results in protection of the whole plant as it is distributed into plant tissues via xylem (Bonmatin et al., 2005; Alsayeda et al., 2008; Sharma et al., 2016a,b, 2017a). Another reason which favors the soil application of IMI is that, persistence of IMI in form of residues in plant parts is less in case of soil applied IMI as compared to foliar application (Juraske et al., 2009). Similar to other xenobiotic compounds, translocation of IMI from soil to the aerial parts (mainly leaves) of plants takes place through xylem. Further, it moves into the flowers, fruits and other plant parts via phloem sap (Laurent and Rathahao, 2003; Alsayeda et al., 2008). Residues of this neonicotinoid insecticide are also translocated to the pollens as well as to the nectar (Stoner and Eitzer, 2012). This results in toxicity causing colony collapse disorder to the not-target insects which are mainly pollinators like honey bees (Tapparo et al., 2012; Whitehorn et al., 2012). IMI is considered as one of the most toxic insecticide which badly affects the bee population (Seifrtova et al., 2017). Residues of IMI may enter into the soil and water ecosystem indirectly through leaf fall or directly via off-site leaching, and their persistence is more in those soils which are deficit in organic matter or containing high percentage of clay (Felsot et al., 1998; Wilkins, 2000; Smelt et al., 2003). Persistence of IMI is so long that in some cases, even no IMI is used in a particular season (but applied in previous seasons), its residues still persist in soil and get translocated into plant parts (Benton et al., 2015).

Pesticide application also causes negative effects on plants by generating harmful reactive oxygen species (ROS) which leads to oxidative stress in plant cells (Zhou et al., 2015; Sharma et al., 2018). This ultimately results in the reduction of plant growth accompanied by decline in photosynthetic efficiency of plants (Xia et al., 2006; Sharma et al., 2016a). In order to minimize the oxidative damages caused by pesticides, plants have their internal defense system (enzymatic and non-enzymatic antioxidative defense system) which gets activated under stress conditions (Sharma et al., 2018). Plants can also detoxify xenobiotics via enzymatic mediated detoxification system which includes enzymes like peroxidases, monooxygenases, carboxylesterase and glutathione-*S*-transferase (Coleman et al., 1997; Cherian and Oliveira, 2005; Sharma et al., 2017c).

Phytohormones including jasmonic acid (JA) are known to ameliorate the negative effects of various abiotic stresses on plants (Vardhini and Anjum, 2015; Kaya and Doganlar, 2016; Rajewska et al., 2016; Bali et al., 2018a; Per et al., 2018). These hormones modulate the antioxidative defense system of plants, resulting in reduction of the oxidative stress under abiotic stress conditions. Earlier studies have also reported the protective role of JA in *Brassica* plants under different abiotic stress conditions like salt (Kaur et al., 2017), cadmium (Ali et al., 2018), arsenic (Farooq et al., 2016), and lead (Bali et al., 2018a). JA also regulates the plant defense system under herbicide toxicity by modulating the biochemical and physiological responses of tobacco plants (Kaya and Doganlar, 2016). As exogenous application of JA helps in ameliorating toxicity in plants caused by abiotic stresses, there is a limited work done on the functions of JA in plants under pesticide stress. Keeping this fact in mind, the current experiment attempts to study the roles of exogenously applied JA on the physiological responses of *B. juncea* seedlings subjected to IMI treatment.

MATERIALS AND METHODS

Plant Material

Brassica juncea L. seeds (Var. RLC-1) were given pre-sowing treatment with 0 and 100 nM JA for 8 h. The working concentration of JA (aqueous solution) was made from the stock solution of JA (prepared in ethanol). Petri-plates containing filter papers (Whatman#1) were given IMI treatments (with the concentrations of 0, 200, and 250 $mg{\cdot}L^{-1}$, where 200 $mg{\cdot}L^{-1}$ is the IC_{50} concentration and one above this concentration, i.e., 250 $mg{\cdot}L^{-1}$ was also selected for better comparison). The JA concentration (100 nM) was selected on the basis of its effectiveness as compared to other trial concentrations (**Supplementary Figure S1**). IMI used in the current investigation was purchased from K.P.R. Fertilizers limited, Tata Nagar, India (17.8% S.L.). After this, JA treated seeds were sown in IMI supplemented Petri-plates and were placed in seed germinator (light intensity, 175 $\mu mol\ m^{-2}\ s^{-1}$; photoperiod, 16 h; temperature, 25 $\pm$ 0.5°C). Harvesting of seedlings was done after 7 days of seed sowing for further analysis. All the analysis was done using three replicates.

Growth Parameters

Seed germination, hypocotyl length, radicle length and fresh weight of hypocotyl were measured after 7 days of seed sowing.

Gene Expression Analysis

In order to study the mechanism behind the role of JA in *B. juncea*, we studied the expression of some important genes encoding enzymes which are responsive to oxidative stress (respiratory burst oxidase, chlorophyllase, ribulose-1,5-bisphosphate carboxylase/oxygenase) and pesticide detoxification (NADH-ubiquinone oxidoreductase, carboxylesterase and cytochrome P450 monooxygenase).

Trizol method (Invitrogen) was used to isolate total RNA from the fresh seedlings. Isolated RNA was reverse transcribed into cDNA using cDNA kit (Invitrogen). Primers (gene specific) used were taken from earlier studies (Sharma et al., 2017c) and *actin* was used as reference gene. Criteria for primer designing included product size which was mainly ranged between 100 and 170 bp, primer length 20 bp, and GC content 40–60%. Details about primers used are given in **Table 1**. Quantitative real time PCR was done using StepOne^{TM} system (Applied Biosystems) and

TABLE 1 | Primers used in relative gene expression analysis.

Name	Sequence	Annealing temperature
actin	Forward primer 5′ CTTGCACCTAGCAGCATGAA 3′ Reverse primer 5′ GGACAATGGATGGACCTGAC 3′	52
CHLASE	Forward primer 5′ GAATATCCGGTGGTGATGCT 3′ Reverse primer 5′ TCCGCCGTTGATTTTATCTC 3′	49
RBO	Forward primer 5′ACGGGGTGTGATAGAGATGC 3′ Reverse primer 5′TTTTTCCAGTTGGGTCTTGC 3′	50
RUBISCO	Forward primer 5′TTAGCTGCATGAAGGTGTGG 3′ Reverse primer 5′TCCATGCTCACGGTAAACAA 3′	53
NADH	Forward primer 5′CTCGGCCTTTCTCAACAGAC 3′ Reverse primer 5′CATTTCCCAAGTTTCCCAGA 3′	49
CXE	Forward primer 5′ GGCGCTAACATGACTCATCA 3′ Reverse primer 5′ CTCCCAGAGTTGAGCGATTC 3′	53
P450	Forward primer 5′ CATTTGTTCTCACCCACACG 3′ Reverse primer 5′ CACAACCGAGTTCGTGAATG 3′	53

CHLASE, chlorophyllase; RBO, respiratory burst oxidase; RUBISCO, ribulose-1,5-bisphosphate carboxylase/oxygenase; NADH, NADH-ubiquinone oxidoreductase; CXE, carboxylesterase; P450, cytochrome P450 monooxygenase (Primers are taken from Sharma et al., 2016c, 2017b, except for RUBISCO).

following the procedure described by Sharma et al. (2017c). The relative gene expression (fold change) was calculated according to Livak and Schmittgen (2001).

Estimation of Pigment Contents

Chlorophyll and Carotenoid Content

Chlorophyll content was estimated according to the method given by Arnon (1949) whereas carotenoid content was estimated by following the method of Maclachlan and Zalik (1963). One g of fresh seedlings were homogenized in 4 mL of 80% acetone and centrifuged at 1,500 *g* for 20 min at 4°C. The supernatant was collected and absorbance was taken at 645 nm and 663 nm (for chlorophyll) and 480 and 510 nm (for carotenoid).

Anthocyanin Content

Method given by Mancinelli (1984) was followed to determine anthocyanin content. Fresh seedlings (1 g) were crushed in 3 mL solution (0.03 mL of HCl, 0.6 mL of H_2O, 2.37 mL of CH_3OH). The homogenized sample was centrifuged for 20 min at 4°C (1,500 *g*). The supernatant was used to take absorbance at 530 and 651 nm.

Estimation of the Activities of Antioxidative Enzymes

The catalase (CAT) activity was determined by following the method given by Aebi (1984). Three mL of 100 mM potassium phosphate buffer (PPB, pH = 7.0) was used for the crushing of 1 g seedlings. The homogenate was centrifuged at 12,000 *g* for 20 min (4°C), and the supernatant was used for the estimation of enzyme activity. The reaction mixture consisted of 1,500 μL of potassium phosphate buffer (50 mM), 930 μL of hydrogen peroxide (15 mM) and 70 μL of sample. The absorbance was observed at 240 nm.

Peroxidase (POD) activity was determined according to the method of Putter (1974). Three mL of PPB (100 mM, pH = 7.0) was used for the crushing of 1 g seedlings, followed by centrifugation at 12,000 *g* for 20 min (4°C). The supernatant was used for the estimation of POD activity. The reaction mixture was prepared by adding 3 mL of phosphate buffer, 50 μL guaiacol solution, 100 μL enzyme extract and 30 μL H_2O_2. The absorbance was taken at 436 nm.

Dehydroascorbate reductase (DHAR) activity was determined according to the method of Dalton et al. (1986). Fresh seedlings (1 g) were crushed in 3 mL of 100 mM PPB (pH = 7.0). The mixture was centrifuged at 12,000 *g* for 20 min (4°C). The supernatant was collected and utilized for determining the activity of DHAR. The reaction mixture contained 1.5 mL of phosphate buffer, 300 μL dehydroascorbate and 400 μL plant extract. The absorbance was measured at 265 nm.

Glutathione-*S*-transferase (GST) activity was determined according to the method of Habig and Jakoby (1981). One g of fresh seedlings were crushed in 3 mL of PPB (100 mM, pH = 7.5), followed by centrifugation at 12,000 *g* for 20 min at 4°C. The reaction mixture was prepared by adding 70 μL of sample, 1,930 μL potassium phosphate buffer (10 mM), and 250 μL each of reduced glutathione (10 mM) and 1-chloro-2, 4-dinitrobenzene (10 mM). The absorbance was taken at 340 nm.

Superoxide dismutase (SOD) activity was determined by following the method of Kono (1978). One g seedlings was crushed in 3 mL of sodium carbonate buffer and centrifuged at 12,000 rpm for 20 min at 4°C. Supernatant was used to check the SOD activity. The reaction mixture was prepared by adding 1,630 μL of sodium carbonate buffer (pH = 10.2), 500 μL of nitroblue tetrazolium (24 μM), 100 μL of EDTA (0.1 mM), 100 μL of hydroxylamine hydrochloride (1 mM), 100 μL of Triton-X-100 (0.03%) and 70 μL of sample. The absorbance was taken at 560 nm.

Oxidative Stress Markers

Superoxide Anions ($O_2^{\cdot -}$)

$O_2^{\cdot -}$ content was determined by following the method of Wu et al. (2010). One g of seedlings was crushed in 6 mL of phosphate buffer (pH = 7.8, 65 mM) containing 1% of polyvinylpyrrolidone. The crushed sample was centrifuged at 5,000 *g* for 15 min at 4°C. To 0.5 mL of supernatant, 0.5 mL of phosphate buffer and 0.1 mL of hydroxylamine hydrochloride (10 mM) were added.

After that, the mixture was incubated for 30 min at 25°C. The absorbance was taken at 530 nm. The content of superoxide was calculated from the standard curve of sodium nitrite and content was expressed as $\mu mol \cdot g^{-1}$ FW of seedlings.

Hydrogen Peroxide (H_2O_2)

Hydrogen peroxide content was determined by following the method of Patterson et al. (1984). Half g of seedlings were homogenized in 1 mL of acetone and centrifuged at 5,000 *g* for 15 min at 4°C. To the supernatant, 20 μL of 20% titanium chloride in concentrated HCl were added. After that, 200 μL of ammonia solution (17 M) was added, followed by repeated washing of the mixture with acetone. Washed precipitates were dissolved in 1.5 mL of sulphuric acid (2 N) and absorbance was measured at 410 nm. H_2O_2 content was determined from the standard curve of H_2O_2 and represented as $\mu mol \cdot g^{-1}$ FW of seedlings.

Malondialdehyde (MDA)

Procedure mentioned in Heath and Packer (1968) was referred to determine MDA content. Trichloroacetate (TCA, 0.1%) was used to crush 1 g of fresh seedlings. Sample was then centrifuged for 15 min at 10000 *g* (4°C) followed by mixing supernatant (1 mL) with 4 mL of TCA mixture (20% TCA + 0.5% thiobarbituric acid). Sample was then incubated for 30 min (95°C) followed by cooling down and another centrifugation for 15 min at 10000 *g* (4°C). The supernatant was used to record absorbance at 532 and 600 nm.

Non-enzymatic Antioxidants

Glutathione (GSH)

Glutathione (GSH) content was estimated according to the method given by Sedlak and Lindsay (1968). One g of fresh seedlings were crushed in 3 mL of Tris buffer (50 mM, pH = 10) containing 1 mM EDTA. After that, homogenate was centrifuged at 12,000 *g* for 15 min, and the supernatant was used to determine the GSH content. The reaction mixture was prepared by adding 1 mL of Tris buffer, 50 μL Ellman's reagent, 4 mL absolute methanol and 100 μL of plant extract, followed by centrifugation at 3,000 *g* for 15 min. The absorbance was taken at 412 nm.

Ascorbate

Ascorbate content was estimated according to procedure mentioned by Roe and Kuether (1943). One g of fresh plant material was crushed in 3 mL of 50 mM tris-buffer (pH 10.0) having 1 mM EDTA. The mixture was then centrifuged at 12,000 *g* for 15 min (4°C). Activated charcoal (0.1 g) was added to reaction mixture containing 0.5 mL plant sample (supernatant), 0.5 mL TCA (50%) and 4 mL H_2O, followed by filtration using Whatman#1 filter paper. To the filtrate (1 mL), 2,4-dinitrophenylhydrazine was added, followed by incubation for 3 h (37°C). The mixture was cooled down and 65% H_2SO_4 (1.6 mL) was added followed by another incubation at room temperature (30 min). Absorbance of the mixture was recorded at 520 nm.

Tocopherol

Estimation of tocopherol content was done according to Martinek (1964). Three mL of 50 mM tris-buffer (pH 10.0) with 1 mM EDTA were used to homogenize plant tissue followed by centrifugation at 12,000 *g* for 15 min (4°C). Half mL each of supernatant, H_2O, xylene and ethanol were mixed well. The mixture was then centrifuged at 12,000 *g* for 10 min (4°C). To the 0.5 mL of supernatant, 2,4,6-tripyridyl-s-triazine was added. The absorbance of reaction mixture was recorded at 600 nm.

Total Phenols

Procedure given by Singleton and Rossi (1965) was referred to estimate total phenolic content. Five mL of ethanol (60%) was used to crush seedlings, followed by incubation for 30 min (60°C). The mixture was centrifuged at 1,500 *g* for 10 min. 0.25 mL of supernatant was mixed with 1 mL of Na_2CO_3 (7.5%) and 1.25 mL of Folin–Ciocalteu reagent. The reaction mixture was further incubated at room temperature for 2 h and then absorbance was taken as 765 nm.

Organic Acid Quantification Using GC-MS (QP 2010 Plus, Shimadzu, Kyoto, Japan)

Organic acids extraction was done following the method described by Sharma et al. (2016d). Fifty mg dried seedlings powder was taken and to this, 0.5 mL of 0.5 N HCl and 0.5 mL of methanol were added. After that, samples were shaken for 3 h followed by centrifugation at 10,000 *g* for 10 min. To the supernatant, 300 μL of methanol and 100 μL of 50% sulfuric acid were added followed by overnight incubation in water bath at 60°C. The mixture was cooled down to 25°C and 800 μL of chloroform and 400 μL of distilled water were added to it followed by vortexing for 1 min. The lower chloroform layer was used to measure the organic acids. Instrument conditions mentioned by Sharma et al. (2016d) were used for analysis. The contents of organic acids (methylester derivatives of organic acids) were calculated using standard curve.

Analysis of IMI Residues Using GC-MS (QP 2010 Plus, Shimadzu, Kyoto, Japan)

Samples for GC-MS analysis were prepared after following Lehotay (2007). One ml of acetonitrile (containing 1% of glacial acetic acid) was used to homogenate 1 g of fresh seedlings, followed by addition of anhydrous $MgSO_4$ (200 mg) and sodium acetate (50 mg). After shaking the mixture (2 min), centrifugation of sample was carried out for 5 min (1,500 *g*). After centrifugation, to the upper layer (0.5 mL), anhydrous $MgSO_4$ (75 mg) and primary secondary amine sorbent (25 mg) were added. This was followed by vortexing and another centrifugation (1,500 *g* for 2 min) and the upper phase was collected for GC-MS analysis (8 μL injection volume). Instrument conditions were set according to Sharma et al. (2017c). Carrier gas used was helium. The initial temperature of the column oven was 50°C, first increased to 125°C (rate = 25°C min^{-1}) and finally enhanced to 300°C (rate = 10°C min^{-1}, hold time = 15 min). Temperature

of sample injector was 250°C, column gas flow rate was 1.7 Ml min^{-1}, DB-5 ms column was used, and the temperature of ion source and interface was 200 and 280°C, respectively. The IMI quantification was done using standard curve.

Statistical Analysis

Data was statistically analyzed using Two-way analysis of variance (ANOVA) followed by Tukey's multiple comparison test (honestly significant difference i.e., HSD) and multiple linear regression analysis (MLR) using self-coded softwares (MS-Excel 2010).

RESULTS

Growth Parameters

Seed germination (**Figure 1A**), hypocotyl length (**Figure 1B**), hypocotyl fresh weight (**Figure 1C**), radicle length (**Figure 1D**) and overall seedling growth (**Figure 1E**) were noticed to decrease drastically when IMI treated seedlings were compared with controls. However, JA seed application before sowing resulted in significant recovery of these growth parameters. A significant recovery by 50, 87.9, 56.6, and 48.52% was noticed in seed germination, hypocotyl length, hypocotyl fresh weight and radicle length, respectively, when seedlings raised from JA treated seeds and germinated in presence of IMI (200 $mg{\cdot}L^{-1}$) were compared to only IMI treated (200 $mg{\cdot}L^{-1}$) seedlings. Statistical analysis using Two-way ANOVA also showed a significant increase in these parameters after seed soaking with JA. MLR analysis showed that IMI application reduced Seed germination, hypocotyl length, hypocotyl fresh weight and radicle length (indicated by negative β-regression coefficient values), whereas JA seed soaking enhanced these parameters under IMI toxicity as indicated by positive β-regression coefficient values (**Figures 1A–D**).

Relative Gene Expression

Application of JA via seed soaking resulted in modulation of the gene expression of 7-day-old *B. juncea* seedlings under IMI stress. It was observed that relative expression of *CHLASE* and *RBO* was 3.11- and 6.24-fold after IMI application when compared with controls. However, JA seed soaking reduced their expression to 1.64-fold for *CHLASE* (**Figure 2A**) and 2.03-fold for *RBO* (**Figure 2C**) under IMI toxicity. Relative expression of *RUBISCO* (**Figure 2B**) was down-regulated (0.53-fold) with the application of IMI, but JA treatment up-regulated its expression to 1.51-fold in presence of IMI. It has been noticed that IMI up-regulates the expression of *CXE, NADH* and *P450* by 2.62-, 1.57-, and 1.99-fold, respectively. Moreover, JA seed treatment before sowing in IMI supplemented Petri-plates, further enhanced the relative expression of these genes, i.e., *CXE* (**Figure 2D**) by 5.94-fold, *NADH* (**Figure 2E**) by 11.33-fold and *P450* (**Figure 2F**) by 5.30-fold. Two-way ANOVA analysis also showed a significant difference in the relative expression of various genes among different treatments. MLR analysis of data also revealed the role of JA in regulating expression of these genes. Positive β-regression coefficient values indicate up-regulation of gene expression, whereas negative β-regression coefficient values indicate down-regulation of gene expression (**Figures 2A–F**).

Pigment Contents

Chlorophyll contents (chl-a, chl-b, and total chl) were decreased with increase in IMI toxicity (**Figures 3A–C**). As compared to control seedlings (324.57 $\mu g{\cdot}g^{-1}$ FW), total chlorophyll content was reduced to 179.06 $\mu g{\cdot}g^{-1}$ FW under IMI toxicity (200 $mg{\cdot}L^{-1}$). However, 61.66% recovery in total chlorophyll content (**Figure 3C**) was noticed when JA treated seeds were grown in presence of IMI (200 $mg{\cdot}L^{-1}$). Contents of carotenoid and anthocyanin were observed to increase with the application of IMI as well as JA. As compared to control seedlings, carotenoid and anthocyanin contents were maximum increased by 129.57 and 98.01%, respectively, in seedlings germinated from JA soaked-seeds and grown in IMI supplemented Petri-plates (**Figures 3D,E**). A significant difference in the contents of all the pigments was observed after analyzing data using Two-way ANOVA followed by Tukey's HSD. Moreover, MLR analysis revealed that IMI application resulted in reduction of chlorophyll contents (negative β-regression coefficient values), whereas JA seed soaking enhanced the chlorophyll contents (positive β-regression coefficient values). Additionally, both IMI and JA application resulted in the enhancement of carotenoid as well as anthocyanin contents as indicated by positive β-regression coefficient values (**Figures 3A–E**).

Oxidative Stress Markers

Contents of oxidative stress markers like superoxide anion, hydrogen peroxide and malondialdehyde were increased with the increasing concentrations of insecticide. However, a drastic decline in their concentration (55.76% in $O_2^{\cdot -}$, **Figure 4A**; 36.15% in H_2O_2, **Figure 4B**; and 45.76% for MDA, **Figure 4C**) was seen in seedlings raised from JA soaked-seeds and grown in presence of IMI (200 $mg{\cdot}L^{-1}$). Statistical analysis of data using Two-way ANOVA showed a significant change in the contents of all these stress markers among different treatments. MLR analysis revealed the increment in the contents of oxidative stress markers with IMI application (positive β-regression coefficients), whereas reduction in their contents (negative β-regression coefficients) after JA seed soaking and growing in presence of IMI (**Figures 4A–C**).

Antioxidative Enzymes

Activities of antioxidative enzymes (SOD, CAT, POD, DHAR, and GST) were observed to increase in *B. juncea* seedlings with the application of JA as well as IMI. Maximum enhancement in the activities of these enzymes (107.71% in SOD, **Figure 5A**; 92.32% in CAT, **Figure 5B**; 100.80% in POD, **Figure 5C**; 92.73% in DHAR, **Figure 5D** and 91.90% in GST, **Figure 5E**) was noticed when JA soaked-seeds were germinated in IMI containing Petri-plates (200 $mg{\cdot}L^{-1}$). Two-way ANOVA analysis showed a significant difference among the activities of enzymes under different experimental treatments. Positive β-regression coefficients were obtained from MLR analysis (for both JA and IMI), suggesting that JA and IMI application enhanced the activities of antioxidative enzymes (**Figures 5A–E**).

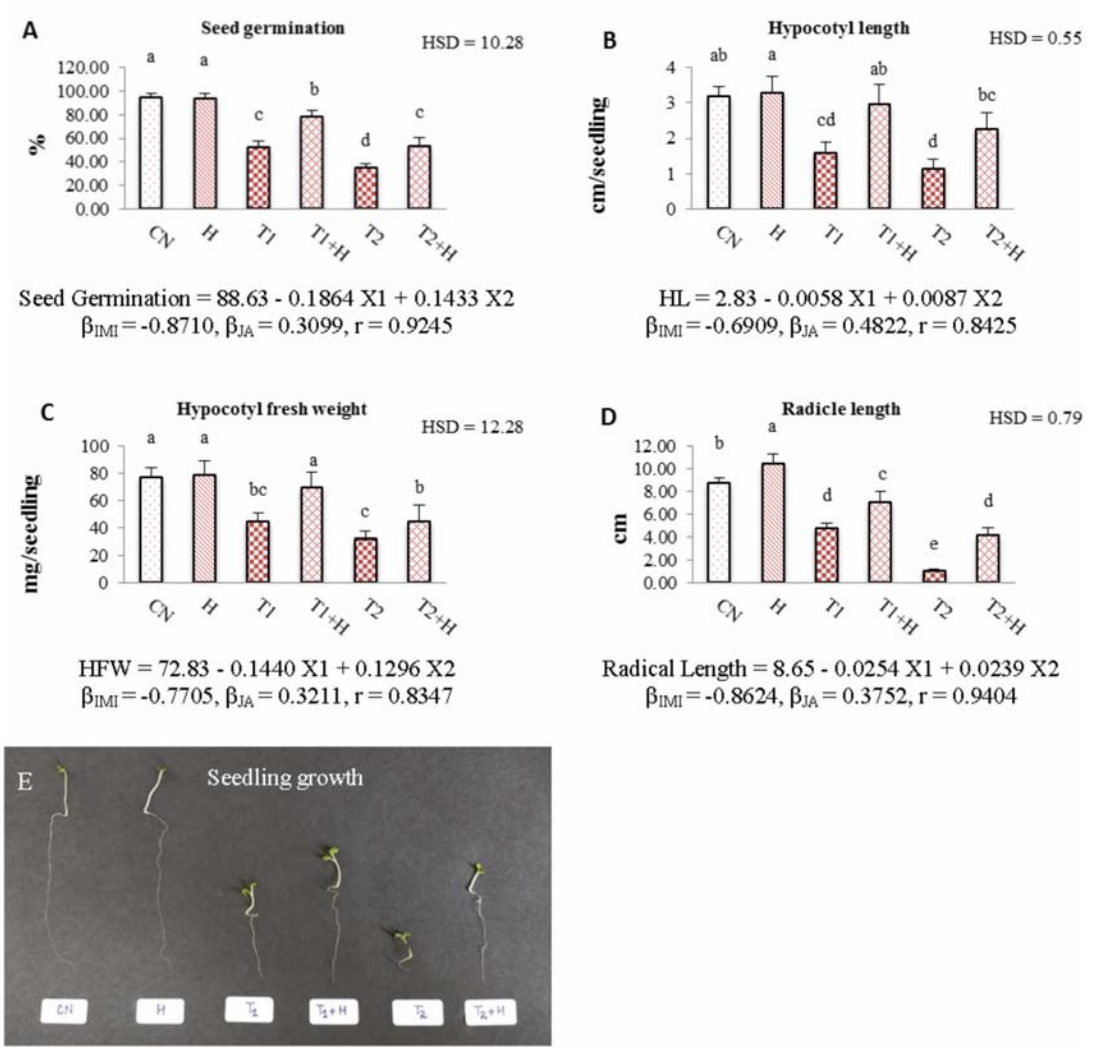


FIGURE 1 | Effect of JA seed soaking on growth parameters of *Brassica juncea* seedlings under IMI stress. Data shown here are mean ± SD (n = 10). Means with same letters are not significantly different from each other, IMI, imidacloprid (X1); JA, jasmonic acid (X2); T1, 200 mg·L^{-1} (IMI); T2, 250 mg·L^{-1} (IMI); *H*, 100 nM (JA); r, multiple correlation coefficient. β = β-regression coefficient. **A** = seed germination; **B** = hypocotyl length; **C** = hypocotyl fresh weight; **D** = radicle length; **E** = seedling growth.

Non-enzymatic Antioxidants

Contents of all the non-enzymatic antioxidants (ascorbic acid, tocopherol, glutathione and total phenolics) were enhanced with the application of both JA and IMI. The maximum enhancement (71.21% in ascorbic acid, **Figure 6A**; 125.51% in tocopherol, **Figure 6B**; 82.66% in glutathione, **Figure 6C** and 71.22% in total phenolic content, **Figure 6D**) in the contents of all these antioxidants was noticed in seedlings germinated from JA soaked-seeds and grown in Petri-plates supplemented with IMI (200 mg·L^{-1}). A significant difference in the contents of all these antioxidants was observed after analyzing data using Two-way ANOVA. Positive β-regression coefficients for both JA and IMI revealed that JA as well as IMI application triggered the biosynthesis of all these antioxidants in *B. juncea* seedlings (**Figures 6A–D**).

Organic Acid Contents

In comparison to control and only IMI treatments, contents of organic acids (citrate, succinate, fumarate, and malate) were observed to increase in seedlings raised from JA soaked-seeds and grown in presence of IMI. The maximum increase in the organic acid contents (55.23% in citrate, **Figure 7A**; 56.37% in succinate, **Figure 7B**; 50.22% in fumarate, **Figure 7C** and 55.25% in malate, **Figure 7D**) was seen in JA (100 nM) + IMI (200 mg·L^{-1}) combination. Two-way ANOVA analysis also showed a significant change in the contents of these organic acids on comparison of control/IMI with JA + IMI treatments. Positive β-regression coefficients for JA also revealed that JA is helpful in triggering the organic acid biosynthesis in *B. juncea* seedlings under IMI toxicity (**Figures 7A–D**).

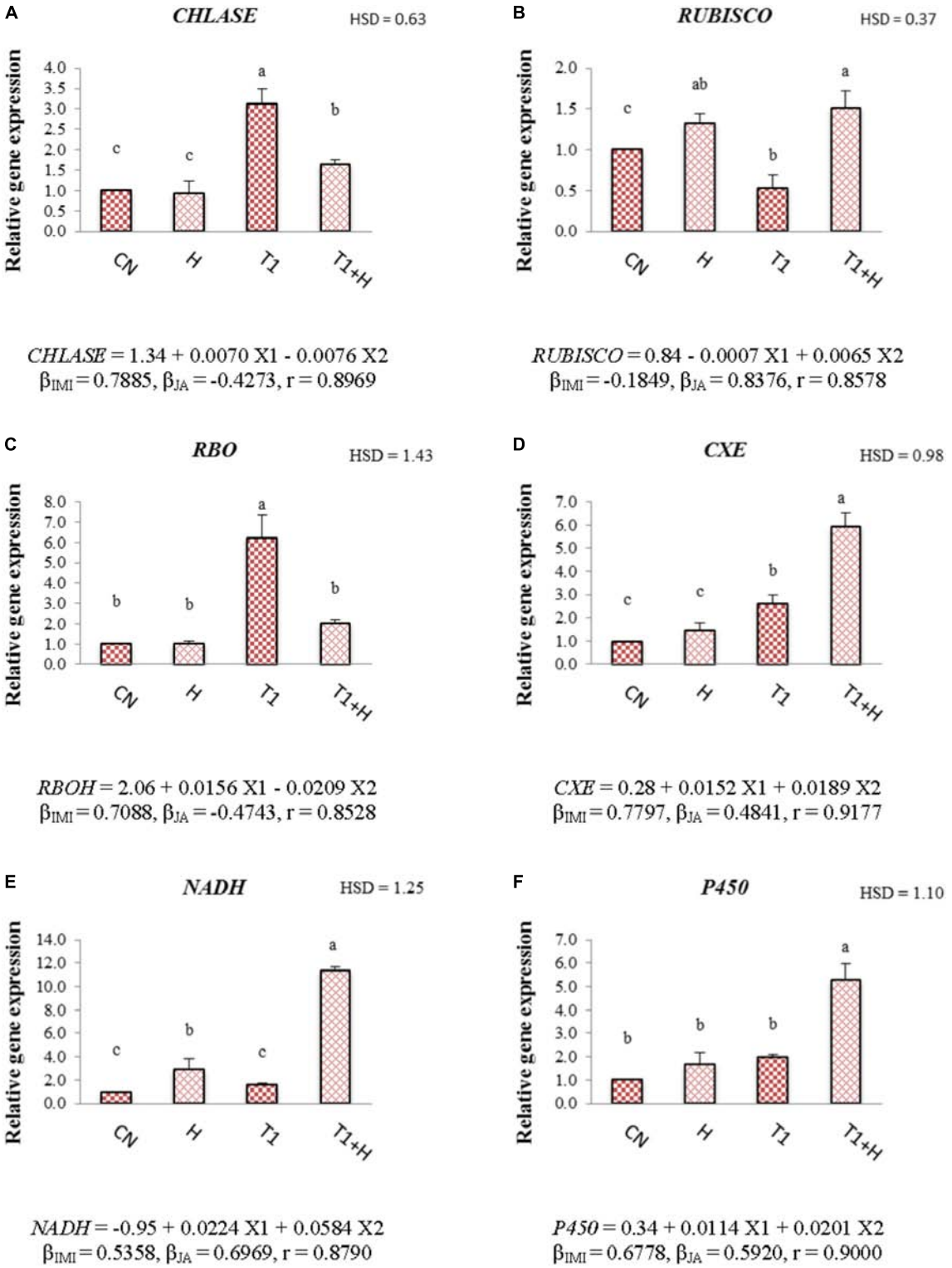


FIGURE 2 | Effect of JA seed soaking on the relative expression of various genes in *B. juncea* seedlings under IMI stress. Data shown here are mean ± SD (n = 3). Means with same letters are not significantly different from each other, IMI, imidacloprid (X1); JA, jasmonic acid (X2); T1, 200 mg·L^{-1} (IMI); *H*, 100 nM (JA), r, multiple correlation coefficient. *RBO*, respiratory burst oxidase; *RUBISCO*, ribulose-1,5-bisphosphate carboxylase/oxygenase; *NADH,* NADH-ubiquinone oxidoreductase*; CXE,* carboxylesterase; *CHLASE*, chlorophyllase; *P450*, cytochrome P450 monooxygenase. β = β-regression coefficient. **A** = *CHLASE*; **B** = *RUBISCO*; **C** = *RBO*; **D** = *CXE*; **E** = *NADH*; **F** = *P450*.

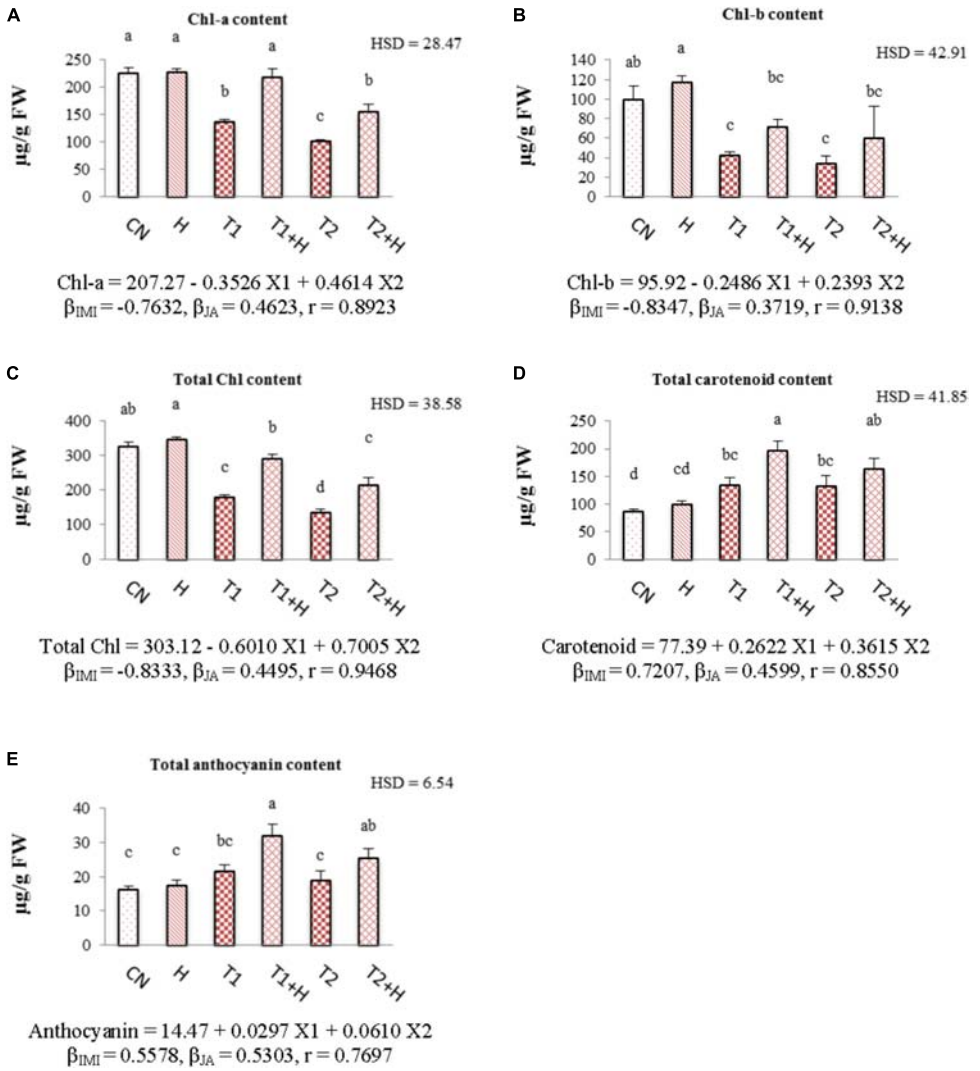


FIGURE 3 | Effect of JA seed soaking on the contents of pigment system in *B. juncea* seedlings under IMI stress. Data shown here are mean ± SD (n = 3). Means with same letters are not significantly different from each other, IMI, imidacloprid (X1); JA, jasmonic acid (X2); T1, 200 mg·L^{-1} (IMI); T2, 250 mg·L^{-1} (IMI); H, 100 nM (JA); r, multiple correlation coefficient. β = β-regression coefficient. **A** = chl-a; **B** = chl-b; **C** = total chl; **D** = carotenoid; **E** = anthocyanin.

IMI Residues

Exogenous application of JA via seed soaking has resulted in reduction of IMI residues in *B. juncea* seedlings. Maximum reduction (30.19%) was observed in seedlings raised from JA-soaked seeds and grown in IMI (200 mg·L^{-1}) containing Petri-plates (**Figure 7E**). Analysis of data using Two-way ANOVA also showed significant change in IMI residues for this treatment. MLR analysis revealed a significant reduction (indicated by negative β-regression coefficients) of IMI residues in *B. juncea* seedlings raised from JA soaked-seeds (**Figure 7E**).

DISCUSSION

The recovery in the seedling growth parameters after JA application might be due to the role of JA in enhancing the cell expansion, cell elongation and differentiation of vascular tissues (Kovač and Ravnikar, 1994; Cenzano et al., 2003). JA also plays an important role in regulating the primary root growth of plants (Huang et al., 2017). Additionally, Rubisco also involved in regulating the physiological processes of plants under stressful conditions (Perdomo et al., 2017). In the present study, JA application resulted in up-regulating the expression of *RUBISCO* (**Figure 2B**), suggesting a possible role of JA in regulating the growth of plants under environmental stress conditions. However, in the current study, JA treatment alone, showed no significant difference on the growth of seedlings. But, JA treatment under IMI stress conditions resulted in the significant recovery of seedling growth (**Figure 1E**). This could be due to the fact that in absence of any stimulant (e.g., absence of any environmental stress), JA responsive genes are not expressed, but in presence of any stimulant, JA responsive genes undergo

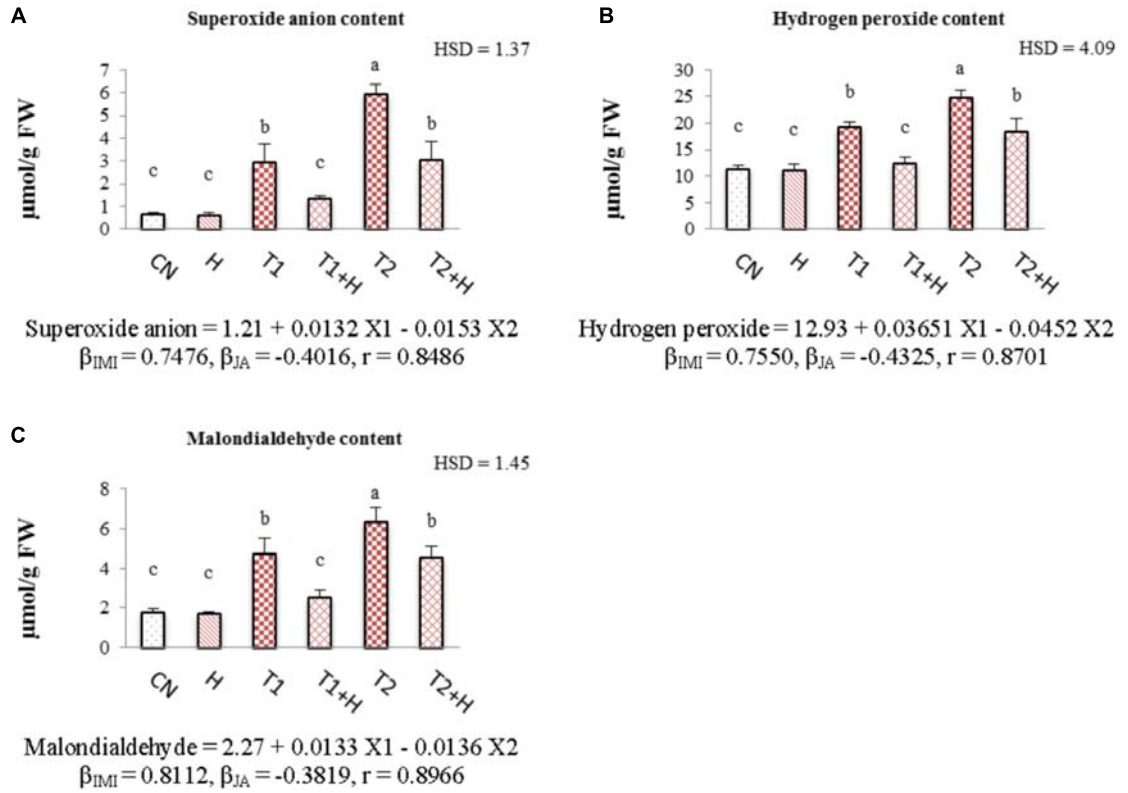


FIGURE 4 | Effect of JA seed soaking on the contents of oxidative stress markers in *B. juncea* seedlings under IMI stress. Data shown here are mean ± SD (n = 3). Means with same letters are not significantly different from each other, IMI, imidacloprid (X1); JA, jasmonic acid (X2); T1, 200 mg·L^{-1} (IMI); T2, 250 mg·L^{-1} (IMI); H, 100 nM (JA), r, multiple correlation coefficient. β = β-regression coefficient. **A** = superoxide anion; **B** = hydrogen peroxide; **C** = malondialdehyde.

transcription and hence regulate the physiological process under stress conditions (Wasternack and Hause, 2013).

Reduction in the content of chlorophyll might be due to the oxidative stress and enhanced activity of chlorophyllase enzyme under stress conditions (Santos, 2004; Li et al., 2010; Sakuraba et al., 2014; Sen et al., 2014). In the current study, expression of *CHLASE* (encoding chlorophyllase enzyme) was increased with the application of IMI (**Figure 2A**). Additionally, we also noticed that only JA treatment did not significantly affected the expression of *CHLASE* (0.93-fold) and this could be due to the fact that the concentration of JA used in current experiment is non-toxic to seedlings. However, JA application to plants in higher concentrations is toxic and can result in degradation of chlorophyll molecules due to the enhanced expression of *NYE1* gene involved in chlorophyll degradation (Zhu et al., 2015). JA application down-regulated the expression of *CHLASE* (**Figure 2A**) in *B. juncea* seedlings under IMI toxicity. This JA mediated regulation of gene expression (*CHLASE*) might be one of the reasons behind recovery of chlorophyll content under IMI toxicity. Additionally, some secondary metabolites like phenolics and anthocyanins are also involved in the protection of main photosynthetic pigments (Memelink et al., 2001). In the present study, contents of anthocyanins and total phenols were also observed to increase after JA treatment under IMI toxicity, indicating the role of these metabolites in protection of chlorophyll pigment from insecticide toxicity.

Carotenoids protect chlorophyll pigment from damage caused by photooxidation as a consequence of oxidative stress (Li et al., 2010). JA also triggers the accumulation of carotenoids in plant under pesticide stress (Kaya and Doganlar, 2016) and in the current investigation; we noticed a significant enhancement in carotenoid accumulation after JA application under IMI toxicity (**Figure 3D**). This JA-induced accumulation of carotenoids could be due to the JA mediated up-regulation of transcription patterns of key genes (*DXS, GGPS, PSY1,* and *PDS*) involved in carotenoid biosynthesis (Liu et al., 2012). In the current study, it has been noticed that carotenoid content was also enhanced in groups treated with IMI, as compared to control. Since carotenoids also acts as an antioxidant, their enhanced biosynthesis may help in ameliorating the pesticide toxicity (Kaya and Doganlar, 2016; Sharma et al., 2016b,d). Moreover, IMI application also up-regulates the expression of *PSY* (gene encoding enzyme phytoene synthase) which is involved in carotenoid biosynthesis (Sharma et al., 2016d), suggesting another possible reason for the enhanced carotenoid content under IMI treatment in the current investigation.

Anthocyanins also play a crucial role in protecting plant cells from oxidative stress caused by ROS generation (Nakabayashi et al., 2014). Their accumulation also increases in plant grown

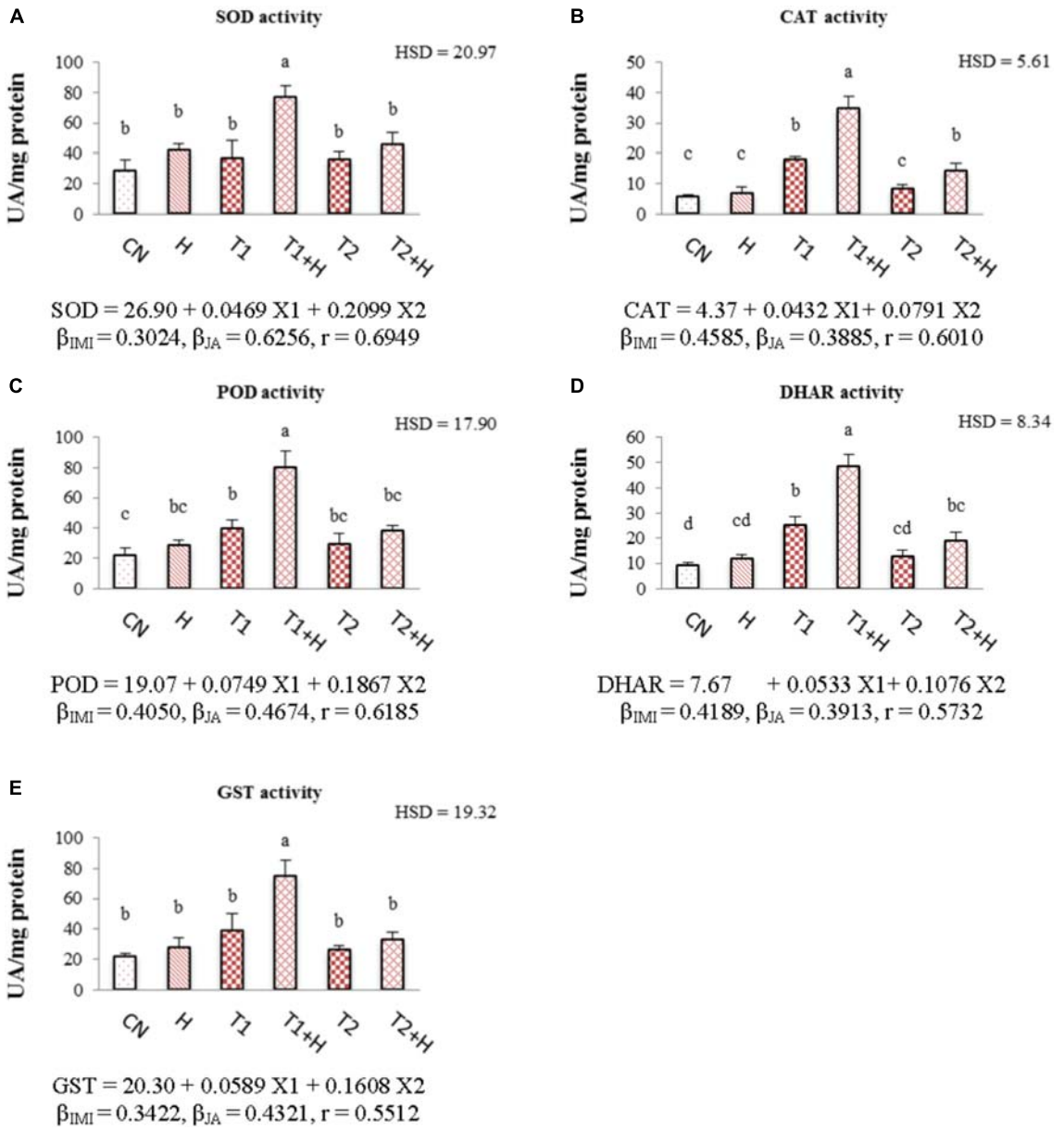


FIGURE 5 | Effect of JA seed soaking on the activities of antioxidative enzymes in *B. juncea* seedlings under IMI stress. Data shown here are mean ± SD (n = 3). Means with same letters are not significantly different from each other, IMI, imidacloprid (X1); JA, jasmonic acid (X2); T1, 200 mg·L^{-1} (IMI); T2, 250 mg·L^{-1} (IMI); H, 100 nM (JA); r, multiple correlation coefficient. β = β-regression coefficient. **A** = SOD; **B** = CAT; **C** = POD; **D** = DHAR; **E** = GST.

in presence of insecticide (Sharma et al., 2016b). Anthocyanins act as potential antioxidant and are helpful in scavenging the ROS like H2O2 (Gould et al., 2002; Agati et al., 2012). Their enhanced production in plants also triggers the biosynthesis of quercetin-3-*O*-glycoside, which also acts as an antioxidant (Tohge et al., 2005). Moreover, anthocyanins in combination with the tonoplast intrinsic proteins help in scavenging of harmful ROS (Schüssler et al., 2008; Maurel et al., 2009). This anthocyanin mediated ROS scavenging ultimately aid plants in amelioration of toxicity caused by abiotic stresses (Gould, 2004). JA modulates the biosynthesis of anthocyanins in plants by regulating various transcriptional factors which regulate the expression of key genes involved in anthocyanin biosynthesis (Shan et al., 2009). In the current study, JA application further enhanced the anthocyanin accumulation under IMI toxicity (**Figure 3E**), and this could be due to the JA mediated biosynthesis of anthocyanins in plants (Zhang et al., 2002).

In the current study, the generation of ROS was increased under IMI toxicity, leading to enhanced lipid peroxidation. This enhanced ROS production and lipid peroxidation might be due to the disruption of plant's internal defense mechanism as a result of oxidative stress (Mittler, 2002). Additionally, enzyme RBO

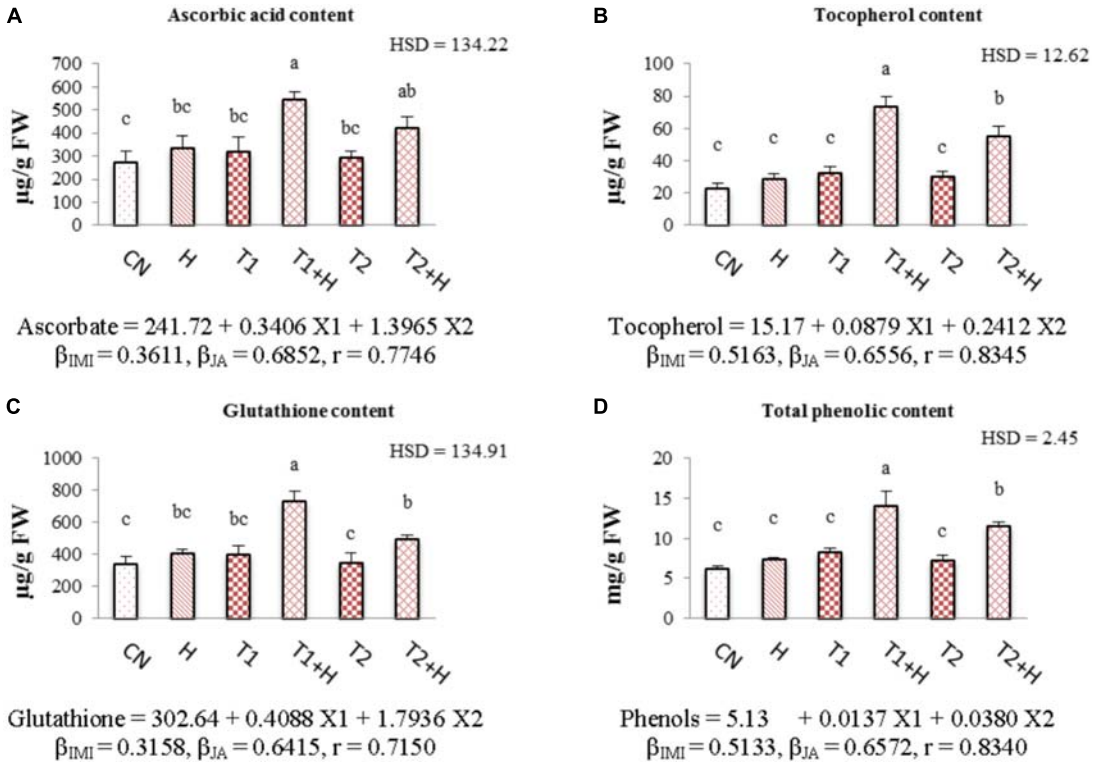


FIGURE 6 | Effect of JA seed soaking on the contents of non-enzymatic antioxidants in *B. juncea* seedlings under IMI stress. Data shown here are mean ± SD (n = 3). Means with same letters are not significantly different from each other, IMI, imidacloprid (X1); JA, jasmonic acid (X2); T1, 200 mg·L^{-1} (IMI); T2, 250 mg·L^{-1} (IMI); H, 100 nM (JA); r, multiple correlation coefficient. β = β-regression coefficient. **A** = ascorbic acid; **B** = tocopherol; **C** = glutathione; **D** = total phenolics.

(respiratory burst oxidase) accelerates the generation of hydrogen peroxide under abiotic stress and expression of gene encoding this enzyme (*RBO*) also gets up-regulated under pesticide stress (Zhou et al., 2015; Sharma et al., 2017c). Moreover, in the present study, *RBO* expression was significantly up-regulated under IMI toxicity (**Figure 2C**). The increased pesticide toxicity also leads to the accumulation of harmful ROS like superoxide anion radical (Sharma et al., 2017c) which could ultimately trigger the process of lipid peroxidation in plants (Kaya and Doganlar, 2016). Since IMI caused oxidative stress in the seedlings and in-order to scavenge harmful ROS, enzymatic antioxidative defense system of *B. juncea* got activated. This resulted in enhanced activities of antioxidative enzymes. Internal defense system of plants (including enzymatic antioxidants) helps to protect them from oxidative stress caused by pesticide toxicity (Zhou et al., 2015; Kaya and Doganlar, 2016; Sharma et al., 2018). Antioxidative enzymes like SOD, CAT, POD, and DHAR regulates the oxidative stress by scavenging harmful ROS in plants (Vardhini and Anjum, 2015; Mir et al., 2018). SOD reduces the cellular damage via scavenging harmful superoxide anion radicals by converting them into hydrogen peroxide, which is further converted into H_2O by CAT (Gill and Tuteja, 2010). DHAR is involved in the Ascorbic acid-glutathione cycle, which also helps plants to scavenge ROS (Gill and Tuteja, 2010). But, alone enhanced activities of enzymes were unable to reduce the oxidative stress which might be due to the high IMI toxicity (Sharma et al., 2017b,c). Exogenous application of JA to plants under abiotic stress conditions also enhances the activities of antioxidative enzymes and helps in enhancing the scavenging of ROS (Kaya and Doganlar, 2016; Mir et al., 2018). This ultimately results in reduction of oxidative stress in plants generated by pesticide toxicity (Kaya and Doganlar, 2016). After JA treatment, we also noticed a significant decline in the expression of *RBO* in IMI stressed seedlings. This could be another reason for the reduction of ROS accumulation in insecticide stressed seedlings, after JA treatment. In the current study, JA application also triggered the activities of antioxidative enzymes including SOD, CAT, POD, and DHAR under IMI toxicity (**Figures 5A–E**), suggesting a positive role of JA in regulation of antioxidative defense mechanism in plants under pesticide stress. JA also up-regulates the expression of genes like *SOD, POD, APX,* and *CAT* under abiotic stress (Sirhindi et al., 2016). This could be another reason for the JA mediated regulation of the activities of antioxidative enzymes in the current investigation. So it is anticipated that, JA application further enhances the activities of antioxidative enzymes, which aid in enhancing their efficiency to scavenge harmful ROS, leading to reduction of IMI-induced oxidative stress. However, JA application (alone) did not significantly regulate the activities of antioxidative enzymes. This can be explain by the fact that generally JA signaling gets activated only after any injury to plant cell caused by biotic or abiotic factors (Wasternack and Hause, 2013; Huang et al., 2017).

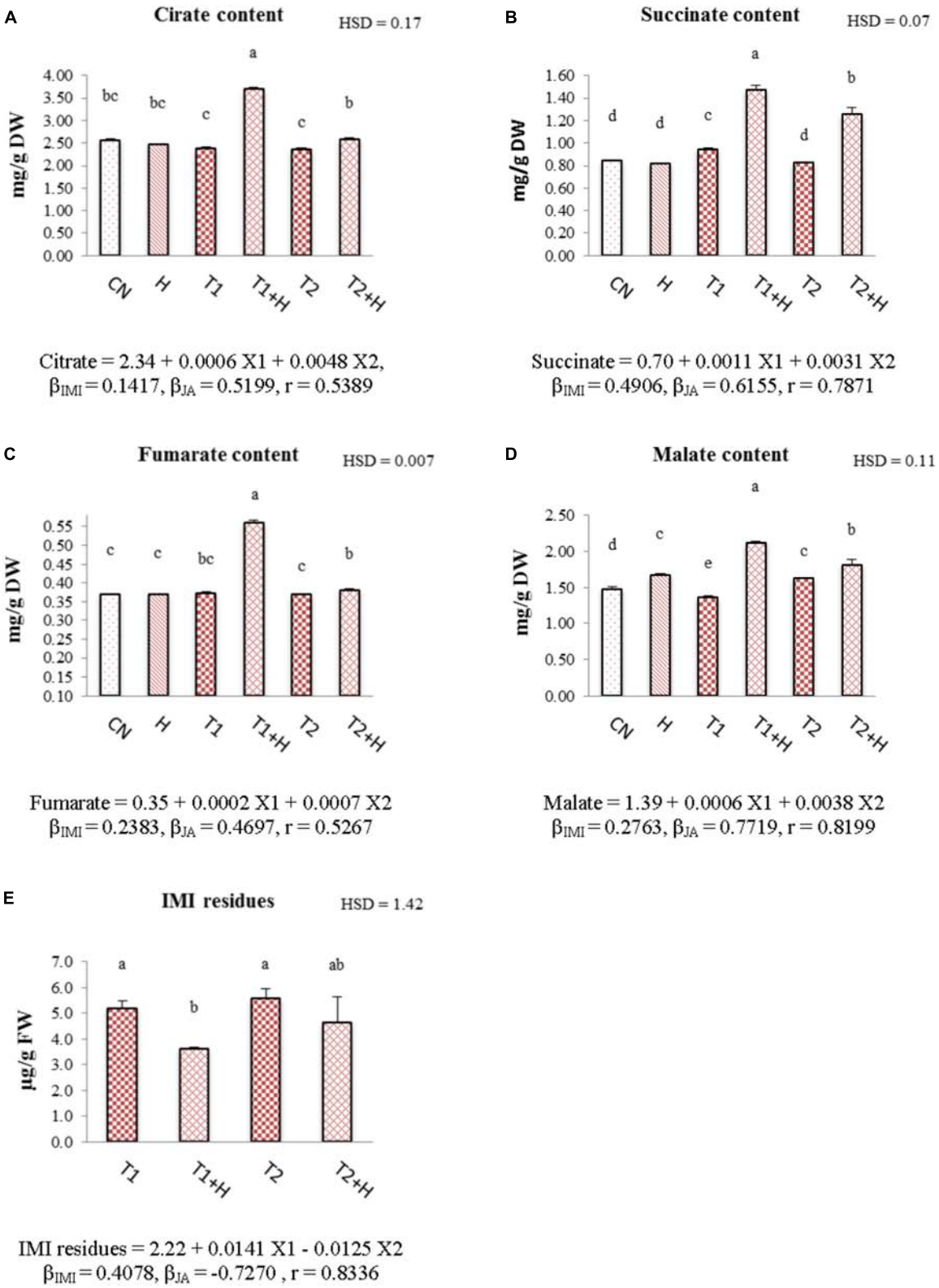


FIGURE 7 | Effect of JA seed soaking on the contents of organic acids and IMI residues in *B. juncea* seedlings under IMI stress. Data shown here are mean ± SD (n = 3). Means with same letters are not significantly different from each other, IMI, imidacloprid (X1); JA, jasmonic acid (X2); T1, 200 mg·L^{-1} (IMI); T2, 250 mg·L^{-1} (IMI); H, 100 nM (JA); r, multiple correlation coefficient. β = β-regression coefficient. **A** = citrate; **B** = succinate; **C** = fumarate; **D** = malate; **E** = IMI residues.

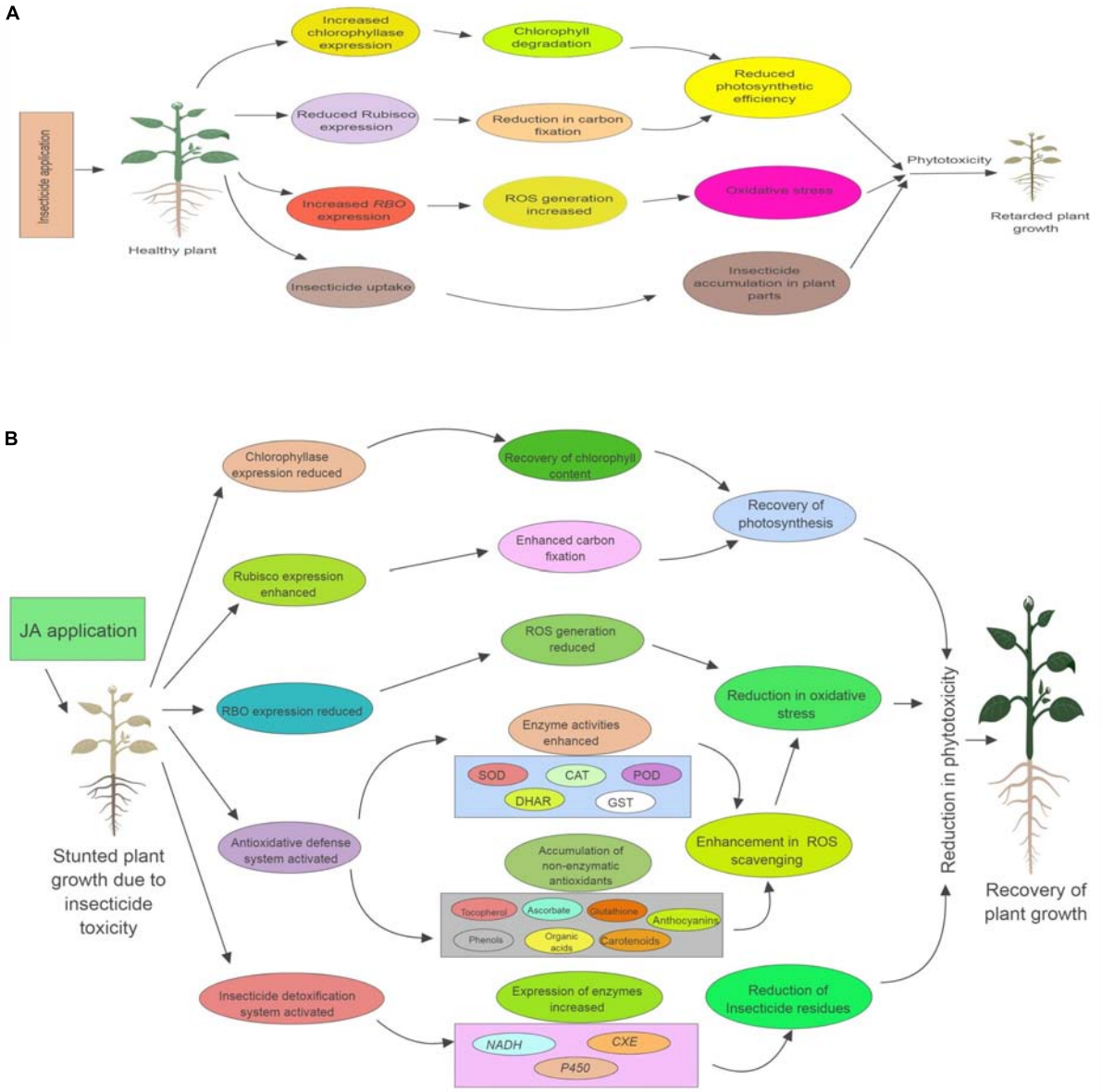


FIGURE 8 | Role of exogenous applied jasmonic acid (JA) in plants under insecticide toxicity. **(A)** Describes how insecticide causes toxicity to plants. **(B)** Describes the various physiological process regulated by JA application, which help in reducing the insecticide induced phytotoxicity. *RBO*, respiratory burst oxidase; Rubisco, Ribulose-1,5-bisphosphate carboxylase/oxygenase; *NADH,* NADH-ubiquinone oxidoreductase*; CXE,* carboxylesterase; *P450*, cytochrome P450 monooxygenase; ROS, reactive oxygen species; SOD, superoxide dismutase; CAT, catalase; POD, peroxidase; DHAR, dehydroascorbate reductase; GST, glutathione-*S*-transferase.

Additionally, in the present study, JA concentration used is non-toxic to seedlings and this might be the possible reason behind this non-regulation of enzyme activities by JA application (alone). This fact is also supported by the studies carried out on *Nicotiana tabacum*, in which JA treatment (alone) did not show any significant change in the activities of enzymes like CAT, GST, and GR after 9 days of treatment (Kaya and Doganlar, 2016). Moreover, JA is also known for its regulatory actions in antioxidative defense system under stressful conditions (Qiu et al., 2014). We noticed that lower IMI concentration (200 mg L^{-1}) resulted in significant enhancement in the activities of enzymes like CAT, POD, and DHAR but higher concentration of IMI (250 mg L^{-1}) did not result in any significant change in the activities of antioxidative enzymes as compared to control. It has also been observed that in the group treated with 250 mg L^{-1} IMI, activities of antioxidative enzymes were found to be lower as compared to that of group treated with 200 mg L^{-1} (**Figures 5A–E**). This difference in the enzymatic activities might be due to the fact that at higher pesticide toxicity, oxidative stress caused an imbalance in the antioxidative defense system of plants, resulting in reduction of enzyme efficiency (San Miguel et al., 2012; Wang et al., 2015; Sharma et al., 2017c). Zhang et al. (2012)

also reported that exposure of pesticide (Diclofop-methyl) to *Arabidopsis thaliana* for short time did not significantly affect the activities of antioxidative enzymes. So, the antioxidative defense system is not able to scavenge the ROS efficiently under high pesticide toxicity, leading to increase in the oxidative stress with the increasing application of pesticide (Kaya and Doganlar, 2016). This fact is also supported by the results obtained from current investigation, in which ROS content was gradually increased in seedlings with the increase in IMI application.

In addition to enzymatic antioxidants, we also noticed a considerable enhancement in the contents of non-enzymatic antioxidants like ascorbate, glutathione, tocopherol and total phenols, with the application of IMI as well as JA (**Figures 6A–D**). Due to its antioxidative properties, ascorbate is involved in reducing the oxidative stress in plants caused by the generation of ROS under abiotic stress conditions (Khan et al., 2011). Additionally, there exists a possible crosstalk between ascorbate and phytohormones including JA in plants under stress conditions, resulting in enhancement of plant resistance to these conditions (Fujita et al., 2006; Khan et al., 2011; Akram et al., 2017). Ascorbate-glutathione cycle also plays a key role in the regulation of ROS in plants (Foyer and Noctor, 2011). Moreover, it has also been established that a triad of antioxidants, comprised of ascorbate-glutathione-α tocopherol is also involved in the ROS scavenging in plants grown under stressful environments (Szarka et al., 2012). The concentration of these non-enzymatic antioxidants in plant parts increases by many folds under stress conditions, which aid in reducing the ROS, generated oxidative stress (Kanwischer et al., 2005). Pesticide application also increases the accumulation of these antioxidants in plant parts to increase the resistance of plants against pesticide toxicity (Kaya and Doganlar, 2016; Sharma et al., 2016c,d). Additionally the exogenous application of JA further triggers their accumulation in plants growing under adverse environmental conditions, which results in increasing plant's resistance against those conditions (Wolucka et al., 2005; Bali et al., 2018b; Mir et al., 2018). JA is also involved in the biosynthesis of phenolic compounds (Malekpoor et al., 2016; Mendoza et al., 2018). The fact behind JA mediated biosynthesis of phenolic compounds is that, JA enhances the activity of phenylalanine-ammonialyase enzyme, which ultimately activates the phenolic biosynthetic pathway (phenylpropanoid pathway), resulting in more accumulation of phenolics in presence of exogenous JA (Kim et al., 2007). So, it is anticipated that, exogenous JA application under pesticide exposure has triggered the biosynthesis of ascorbate, glutathione, tocopherol and phenolics, which plays an important role in the scavenging of ROS and leads to the enhancement of *B. juncea* resistance against insecticide stress.

In our study, we found that JA seed treatment enhanced the contents of organic acids including citrate, succinate, fumarate and malate in seedlings grown under IMI stress (**Figures 7A–D**). The concentration of these organic acids increases when plants encounter any abiotic or biotic stress (Li et al., 2000; Ma, 2000; Bali et al., 2018b). These organic acids may act as part of plant's defense system (Sharma et al., 2017b) and may also regulate the ionic balance and osmotic pressure under abiotic stress (Zeng et al., 2008). Exogenous application of JA triggers the biosynthesis of these organic acids (Bali et al., 2018b). JA enhances the resistance in plants against biotic and abiotic stress factors (Bali et al., 2018b; Mir et al., 2018), suggesting that accumulation of these organic acids may aid in boosting up the defense system of plants under pesticide toxicity.

Pesticide detoxification in the plants is catalyzed by various key enzymes including P450-monooxygenase (P450), glutathione-*S*-transferase (GST), peroxidases (POD), carboxylesterase (CXE), and oxidoreductase (Coleman et al., 1997; Cherian and Oliveira, 2005; Zhou et al., 2015). All these enzymes trigger the detoxification of pesticides resulting in reduction of harmful pesticide residues in intact plants (Sharma et al., 2017c). In the present study, a significant reduction in the IMI residues was observed in seedlings raised from JA treated seeds (**Figure 7E**). Also, the activities of POD and GST were noticed to increase in seedlings germinated from JA treated seeds. Moreover, JA application also up-regulated the expression of *P450*, *CXE,* and *NADH* (NADH-ubiquinone oxidoreductase) in *B. juncea* seedlings grown in presence of IMI (**Figures 2D–F**). This JA mediated regulation of the activities and gene expression of these pesticide detoxification enzymes might be the possible reason behind reduction of IMI residues.

In the current investigation, we observed that 100 nM JA application alone did not affect the most of parameters, but in combination with IMI, JA regulated most of physiological processes. The possible reason behind this can be the JA-responsive cell signaling (Wasternack and Hause, 2013). During low levels of JA-Ile (*iso*-jasmonoyl-L-isoleucine, a ligand of JA receptor), which is possibly in absence of any stress/stimulation, the JAZs (jasmonate ZIM domain proteins) gets bind to MYC2 (a basic-helix-loop-helix zip transcription factor). This results in the repression of the transcription of JA-responsive genes. But, upon getting a signal, possibly any stress/stimulation, COI1 (CORONATINE INSENSITIVE 1) recruits JAZs, resulting in ubiquitinylation of JAZs followed by their degradation. So, in this case MYC2 is able to stimulate the transcription of JA-responsive genes, which are responsible for the JA mediated regulation of physiological process under stress conditions.

CONCLUSION

From the present study, it has been concluded that exogenous application of JA can aid plants in recovering from the negative impacts of oxidative stress cause by pesticide toxicity. JA also enhances the pesticide detoxification potential of plants, resulting in reduction of pesticide residues. This JA assisted insecticide detoxification in *B. juncea* plants could be due to the JA mediated regulation of various physiological processes (**Figure 8**). However, more detailed studies on the JA mediated signaling mechanisms of various metabolic pathways are still needed to understand the exact mechanisms of enhanced

pesticide detoxification. Using mutants like *jar1* and *coi1* can help in confirmation of the fact that JA signaling is involved in the amelioration of insecticide induced toxicity in plants.

AUTHOR CONTRIBUTIONS

AS, VK, HY, and MK performed the experiments and were involved in writing of manuscript. AS, RB, AT, and BZ designed and critically checked the manuscript.

FUNDING

This study was supported by National Natural Science Foundation of China (31070604, 31270716, and 31470683), National High Technology Research and Development Program of China (863 Program) (2013AA102605), Key Project of Zhejiang Provincial Natural Science Foundation (LZ18C160001), The First-class General Financial Grant from the China Postdoctoral Science Foundation (2017M610377), Fruit Innovation Team Project of Zhejiang Province (2016C02052-12), Zhejiang Provincial Natural Science Foundation for Distinguished Young Scholar (LR13C160001), Open Foundation of First-class Discipline of Forestry, Zhejiang Province (201703), Preferential Funding of Postdoctoral Scientific Research Project in Zhejiang Province (2016-73-114-168961), National Undergraduate Innovation and Entrepreneurship Training Project (201610341010), Undergraduate Science and Technology Innovation Plan of Zhejiang Province (2017R412006), and Undergraduate Research Training Program in Zhejiang A & F University (102-2013200005, 102-2013200041, and 102-2013200042).

ACKNOWLEDGMENTS

We acknowledge UGC, New Delhi, India for providing necessary instrumentation to the Plant Stress Physiology Lab, Department of Botanical and Environmental Sciences, Guru Nanak Dev University, Punjab, India.

SUPPLEMENTARY MATERIAL

The Supplementary Material for this article can be found online at: https://www.frontiersin.org/articles/10.3389/fpls.2018.01609/full#supplementary-material

FIGURE S1 | Effect of JA seed soaking on growth parameters of *B. juncea* seedlings under IMI stress. Data shown here are mean ± S.D. Means with same letters are not significantly different from each other. CN = control, H1 = 1 nM JA, H2 = 100 nM JA, H3 = 200 nM JA, H4 = 300 nM JA, T1 = 200 mg·L^{-1} (IMI), T2 = 250 mg·L^{-1} (IMI), JA = jasmonic acid, IMI = imidacloprid.

REFERENCES

Aebi, H. (1984). Catalase in vitro. *Methods Enzymol.* 105, 121–126. doi: 10.1016/S0076-6879(84)05016-3

Agati, G., Azzarello, E., Pollastri, S., and Tattini, M. (2012). Flavonoids as antioxidants in plants: location and functional significance. *Plant Sci.* 196, 67–76. doi: 10.1016/j.plantsci.2012.07.014

Akram, N. A., Shafiq, F., and Ashraf, M. (2017). Ascorbic acid-a potential oxidant scavenger and its role in plant development and abiotic stress tolerance. *Front. Plant Sci.* 8:613. doi: 10.3389/fpls.2017.00613

Ali, E., Hussain, N., Shamsi, I. H., Jabeen, Z., Siddiqui, M. H., and Jiang, L. X. (2018). Role of jasmonic acid in improving tolerance of rapeseed (*Brassica napus* L.) to Cd toxicity. *J. Zhejiang Univ. Sci. B* 19, 130–146. doi: 10.1631/jzus.B1700191

Alsayeda, H., Pascal-Lorber, S., Nallanthigal, C., Debrauwer, L., and Laurent, F. (2008). Transfer of the insecticide [14 C] imidacloprid from soil to tomato plants. *Environ. Chem. Lett.* 6, 229–234. doi: 10.1007/s10311-007-0121-2

Arnon, D. I. (1949). Copper enzymes in isolated chloroplasts. Polyphenoloxidase in *Beta vulgaris*. *Plant Physiol.* 24, 1–15. doi: 10.1104/pp.24.1.1

Bali, S., Kaur, P., Kohli, S. K., Ohri, P., Thukral, A. K., Bhardwaj, R., et al. (2018a). Jasmonic acid induced changes in physio-biochemical attributes and ascorbate-glutathione pathway in *Lycopersicon esculentum* under lead stress at different growth stages. *Sci. Total Environ.* 645, 1344–1360. doi: 10.1016/j.scitotenv.2018.07.164

Bali, S., Kaur, P., Sharma, A., Ohri, P., Bhardwaj, R., Alyemeni, M. N., et al. (2018b). Jasmonic acid-induced tolerance to root-knot nematodes in tomato plants through altered photosynthetic and antioxidative defense mechanisms. *Protoplasma* 255, 471–484. doi: 10.1007/s00709-017-1160-6

Benton, E. P., Grant, J. F., Webster, R. J., Nichols, R. J., Cowles, R. S., Lagalante, A. F., et al. (2015). Assessment of imidacloprid and its metabolites in foliage of eastern hemlock multiple years following treatment for hemlock woolly Adelgid, *Adelges tsugae* (Hemiptera: Adelgidae), in forested conditions. *J. Econ. Entomol.* 108, 2672–2682. doi: 10.1093/jee/tov241

Bonmatin, J. M., Marchand, P. A., Charvet, R., Moineau, I., Bengsch, E. R., and Colin, M. E. (2005). Quantification of imidacloprid uptake in maize crops. *J. Agric. Food Chem.* 53, 5336–5341. doi: 10.1021/jf0479362

Cenzano, A., Vigliocco, A., Kraus, T., and Abdala, G. (2003). Exogenously applied jasmonic acid induces changes in apical meristem morphology of potato stolons. *Ann. Bot.* 91, 915–919. doi: 10.1093/aob/mcg098

Cherian, S., and Oliveira, M. M. (2005). Transgenic plants in phytoremediation: recent advances and new possibilities. *Environ. Sci. Technol.* 39, 9377–9390. doi: 10.1021/es051134l

Coleman, J., Blake-Kalff, M., and Davies, E. (1997). Detoxification of xenobiotics by plants: chemical modification and vacuolar compartmentation. *Trends Plant Sci.* 2, 144–151. doi: 10.1016/S1360-1385(97)01019-4

Cox, C. (2001). Insecticide factsheet: imidacloprid. *J. Pestic. Reform.* 21, 15–21.

Dalton, D. A., Russell, S. A., Hanus, F. J., Pascoe, G. A., and Evans, H. J. (1986). Enzymatic reactions of ascorbate and glutathione that prevent peroxide damage in soybean root nodules. *Proc. Natl. Acad. Sci. U.S.A.* 83, 3811–3815. doi: 10.1073/pnas.83.11.3811

El-Naggar, J. B., and Zidan, N. E. H. A. (2013). Field evaluation of imidacloprid and thiamethoxam against sucking insects and their side effects on soil fauna. *J. Plant Prot. Res.* 53, 375–387. doi: 10.2478/jppr-2013-0056

Farooq, M. A., Gill, R. A., Islam, F., Ali, B., Liu, H., Xu, J., et al. (2016). Methyl jasmonate regulates antioxidant defense and suppresses arsenic uptake in *Brassica napus* L. *Front. Plant Sci.* 7:468. doi: 10.3389/fpls.2016.00468

Felsot, A. S., Cone, W., Yu, J., and Ruppert, J. R. (1998). Distribution of imidacloprid in soil following subsurface drip chemigation. *Bull. Environ. Contam. Toxicol.* 60, 363–370. doi: 10.1007/s001289900635

Foyer, C. H., and Noctor, G. (2011). Ascorbate and glutathione: the heart of the redox hub. *Plant Physiol.* 155, 2–18. doi: 10.1104/pp.110.167569

Fujita, M., Fujita, Y., Noutoshi, Y., Takahashi, F., Narusaka, Y., Yamaguchi-Shinozaki, K., et al. (2006). Crosstalk between abiotic and biotic stress responses: a current view from the points of convergence in the stress signaling networks. *Curr. Opin. Plant Biol.* 9, 436–442. doi: 10.1016/j.pbi.2006.05.014

Gill, S. S., and Tuteja, N. (2010). Reactive oxygen species and antioxidant machinery in abiotic stress tolerance in crop plants. *Plant Physiol. Biochem.* 48, 909–930. doi: 10.1016/j.plaphy.2010.08.016

Gould, K. S. (2004). Nature's Swiss army knife: the diverse protective roles of anthocyanins in leaves. *Biomed Res. Int.* 2004, 314–320. doi: 10.1155/S1110724304406147

Gould, K. S., McKelvie, J., and Markham, K. R. (2002). Do anthocyanins function as antioxidants in leaves? Imaging of H_2O_2 in red and green leaves after mechanical injury. *Plant Cell Environ.* 25, 1261–1269. doi: 10.1046/j.1365-3040.2002.00905.x

Habig, W. H., and Jakoby, W. B. (1981). Glutathione S-transferases (rat and human). *Methods Enzymol.* 77, 218–231. doi: 10.1016/S0076-6879(81)77029-0

Heath, R. L., and Packer, L. (1968). Photoperoxidation in isolated chloroplasts. I. Kinetics and stoichiometry of fatty acid peroxidation. *Arch. Biochem. Biophy.* 125, 189–198. doi: 10.1016/0003-9861(68)90654-1

Huang, H., Liu, B., Liu, L., and Song, S. (2017). Jasmonate action in plant growth and development. *J. Exp. Bot.* 68, 1349–1359. doi: 10.1093/jxb/erw495

Juraske, R., Castells, F., Vijay, A., Muñoz, P., and Antón, A. (2009). Uptake and persistence of pesticides in plants: measurements and model estimates for imidacloprid after foliar and soil application. *J. Hazard. Mater.* 165, 683–689. doi: 10.1016/j.jhazmat.2008.10.043

Kanwischer, M., Porfirova, S., Bergmüller, E., and Dörmann, P. (2005). Alterations in tocopherol cyclase activity in transgenic and mutant plants of *Arabidopsis* affect tocopherol content, tocopherol composition, and oxidative stress. *Plant Physiol.* 137, 713–723. doi: 10.1104/pp.104.054908

Kaur, H., Sirhindi, G., and Sharma, P. (2017). Effect of jasmonic acid on some biochemical and physiological parameters in salt-stressed *Brassica napus* seedlings. *Int. J. Plant Physiol. Biochem.* 9, 36–42. doi: 10.5897/IJPPB2016.0245

Kaya, A., and Doganlar, Z. B. (2016). Exogenous jasmonic acid induces stress tolerance in tobacco (*Nicotiana tabacum*) exposed to imazapic. *Ecotoxicol. Environ. Saf.* 124, 470–479. doi: 10.1016/j.ecoenv.2015.11.026

Khan, T., Mazid, M., and Mohammad, F. (2011). A review of ascorbic acid potentialities against oxidative stress induced in plants. *J. Agrobiol.* 28, 97–111. doi: 10.2478/v10146-011-0011-x

Kim, H. J., Fonseca, J. M., Choi, J. H., and Kubota, C. (2007). Effect of methyl jasmonate on phenolic compounds and carotenoids of romaine lettuce (*Lactuca sativa* L.). *J. Agric. Food Chem.* 55, 10366–10372. doi: 10.1021/jf071927m

Kono, Y. (1978). Generation of superoxide radical during autooxidation of hydroxylamine and an assay for superoxide dismutase. *Arch. Biochem. Biophy.* 186, 189–195. doi: 10.1016/0003-9861(78)90479-4

Kovač, M., and Ravnikar, M. (1994). The effect of jasmonic acid on the photosynthetic pigments of potato plants grown in vitro. *Plant Sci.* 103, 11–17. doi: 10.1016/0168-9452(94)03974-7

Kular, J., and Kumar, S. (2011). Quantification of avoidable yield losses in oilseed *Brassica* caused by insect pests. *J. Plant Prot. Res.* 51, 38–43. doi: 10.2478/v10045-011-0007-y

Laurent, F. M., and Rathahao, E. (2003). Distribution of [14C] imidacloprid in sunflower (*Helianthus annuus* L.) following seed treatment. *J. Agric. Food Chem.* 51, 8005–8010. doi: 10.1021/jf034310n

Lehotay, S. J. (2007). *Pesticide Residues in Foods by Acetonitrile Extraction and Partitioning with Magnesium Sulfate*. Rockville, MD: AOAC International.

Li, G., Wan, S., Zhou, J., Yang, Z., and Qin, P. (2010). Leaf chlorophyll fluorescence, hyperspectral reflectance, pigments content, malondialdehyde and proline accumulation responses of castor bean (*Ricinus communis* L.) seedlings to salt stress levels. *Ind. Crops Prod.* 31, 13–19. doi: 10.1016/j.indcrop.2009.07.015

Li, X. F., Ma, J. F., and Matsumoto, H. (2000). Pattern of aluminum-induced secretion of organic acids differs between rye and wheat. *Plant Physiol.* 123, 1537–1544. doi: 10.1104/pp.123.4.1537

Liu, L., Wei, J., Zhang, M., Zhang, L., Li, C., and Wang, Q. (2012). Ethylene independent induction of lycopene biosynthesis in tomato fruits by jasmonates. *J. Exp. Bot.* 63, 5751–5761. doi: 10.1093/jxb/ers224

Livak, K. J., and Schmittgen, T. D. (2001). Analysis of relative gene expression data using real-time quantitative PCR and the 2-ΔΔCT method. *Methods* 25, 402–408. doi: 10.1006/meth.2001.1262

Ma, J. F. (2000). Role of organic acids in detoxification of aluminum in higher plants. *Plant Cell Physiol.* 41, 383–390. doi: 10.1093/pcp/41.xyb4.383

Maclachlan, C., and Zalik, S. (1963). Plastid structure chlorophyll concentration and free amino acid composition of a chlorophyll mutant of barley. *Can. J. Bot.* 41, 1053–1062.doi: 10.1139/b63-088

Malekpoor, F., Salimi, A., and Pirbalouti, A. G. (2016). Effect of jasmonic acid on total phenolic content and antioxidant activity of extract from the green and purple landraces of sweet basil. *Acta Pol. Pharm.* 73, 1229–1234.

Mancinelli, A. L. (1984). Photoregulation of anthocyanin synthesis VIII. Effect of light pretreatments. *Plant Physiol.* 75, 447–453.doi: 10.1104/pp.75.2.447

Martinek, R. G. (1964). Method for determination of vitamin E (total tocopherols) in serum. *Clin. Chem.* 10, 1078–1086.

Maurel, C., Santoni, V., Luu, D. T., Wudick, M. M., and Verdoucq, L. (2009). The cellular dynamics of plant aquaporin expression and functions. *Curr. Opin. Plant Biol.* 12, 690–698. doi: 10.1016/j.pbi.2009.09.002

Memelink, J., Verpoorte, R., and Kijne, J. W. (2001). ORCAnization of jasmonate-responsive gene expression in alkaloid metabolism. *Trends Plant Sci.* 6, 212–219. doi: 10.1016/S1360-1385(01)01924-0

Mendoza, D., Cuaspud, O., Arias, J. P., Ruiz, O., and Arias, M. (2018). Effect of salicylic acid and methyl jasmonate in the production of phenolic compounds in plant cell suspension cultures of *Thevetia peruviana*. *Biotechnol. Rep.* 19:e00273. doi: 10.1016/j.btre.2018.e00273

Mir, M. A., John, R., Alyemeni, M. N., Alam, P., and Ahmad, P. (2018). Jasmonic acid ameliorates alkaline stress by improving growth performance, ascorbate glutathione cycle and glyoxalase system in maize seedlings. *Sci. Rep.* 8:2831. doi: 10.1038/s41598-018-21097-3

Mittler, R. (2002). Oxidative stress, antioxidants and stress tolerance. *Trends Plant Sci.* 7, 405–410. doi: 10.1016/S1360-1385(02)02312-9

Nakabayashi, R., Yonekura-Sakakibara, K., Urano, K., Suzuki, M., Yamada, Y., Nishizawa, T., et al. (2014). Enhancement of oxidative and drought tolerance in Arabidopsis by overaccumulation of antioxidant flavonoids. *Plant J.* 77, 367–379. doi: 10.1111/tpj.12388

Patterson, B. D., Mackae, E. A., and Mackae, I. (1984). Estimation of hydrogen peroxide in plants extracts using titanium (IV). *Anal. Biochem.* 139, 487–492.doi: 10.1016/0003-2697(84)90039-3

Per, T. S., Khan, M. I. R., Anjum, N. A., Masood, A., Hussain, S. J., and Khan, N. A. (2018). Jasmonates in plants under abiotic stresses: crosstalk with other phytohormones matters. *Environ. Exp. Bot.* 145, 104–120. doi: 10.1016/j.envexpbot.2017.11.004

Perdomo, J. A., Capó-Bauçà, S., Carmo-Silva, E., and Galmés, J. (2017). Rubisco and rubisco activase play an important role in the biochemical limitations of photosynthesis in rice, wheat, and maize under high temperature and water deficit. *Front. Plant Sci.* 8:490. doi: 10.3389/fpls.2017.00490

Putter, J. (1974). "Peroxidase," in *Methods of Enzymatic Analysis*, ed. H. U. Bergmeyer (Weinheim: Verlag Chemie), 685–690.doi: 10.1016/B978-0-12-091302-2.50033-5

Qiu, Z., Guo, J., Zhu, A., Zhang, L., and Zhang, M. (2014). Exogenous jasmonic acid can enhance tolerance of wheat seedlings to salt stress. *Ecotoxicol. Environ. Saf.* 104, 202–208. doi: 10.1016/j.ecoenv.2014.03.014

Rajewska, I., Talarek, M., and Bajguz, A. (2016). Brassinosteroids and response of plants to heavy metals action. *Front. Plant Sci.* 7:629. doi: 10.3389/fpls.2016.00629

Razaq, M., Mehmood, A., Aslam, M., Ismail, M., Afzal, M., and Shad, S. A. (2011). Losses in yield and yield components caused by aphids to late sown *Brassica napus* L., *Brassica juncea* L. and Brassica carinata A. Braun at Multan, Punjab (Pakistan). *Pak. J. Bot.* 43, 319–324.

Roe, J., and Kuether, C. A. (1943). The determination of ascorbic acid in whole blood and urine through the 2, 4 dinitropheny 1 hydrazine derivative of dehydroascorbic acid. *J. Biol. Chem.* 147, 399–407.

Sakuraba, Y., Kim, D., Kim, Y. S., Hörtensteiner, S., and Paek, N. C. (2014). *Arabidopsis* STAYGREEN-LIKE (SGRL) promotes abiotic stress-induced leaf yellowing during vegetative growth. *FEBS Lett.* 588, 3830–3837. doi: 10.1016/j.febslet.2014.09.018

San Miguel, A., Faure, M., Ravanel, P., and Raveton, M. (2012). Biological responses of maize (*Zea mays*) plants exposed to chlorobenzenes. Case study of monochloro-, 1, 4-dichloro-and 1, 2, 4-trichloro-benzenes. *Ecotoxicology* 21, 315–324. doi: 10.1007/s10646-011-0792-0

Santos, C. V. (2004). Regulation of chlorophyll biosynthesis and degradation by salt stress in sunflower leaves. *Sci. Hort.* 103, 93–99. doi: 10.1016/j.scienta.2004.04.009

Schüssler, M. D., Alexandersson, E., Bienert, G. P., Kichey, T., Laursen, K. H., Johanson, U., et al. (2008). The effects of the loss of TIP1; 1 and TIP1; 2 aquaporins in *Arabidopsis thaliana*. *Plant J.* 56, 756–767. doi: 10.1111/j.1365-313X.2008.03632.x

Sedlak, J., and Lindsay, R. H. (1968). Estimation of total, protein bound and non-protein sulfhydryl groups in tissue with Ellmann's reagent. *Anal. Biochem.* 25, 192–205. doi: 10.1016/0003-2697(68)90092-4

Seifrtova, M., Halesova, T., Sulcova, K., Riddellova, K., and Erban, T. (2017). Distributions of imidacloprid, imidacloprid-olefin and imidacloprid-urea in green plant tissues and roots of rapeseed (*Brassica napus*) from artificially contaminated potting soil. *Pest Manag. Sci.* 73, 1010–1016. doi: 10.1002/ps.4418

Sen, G., Eryilmaz, I. E., and Ozakca, D. (2014). The effect of aluminium-stress and exogenous spermidine on chlorophyll degradation, glutathione reductase activity and the photosystem II D1 protein gene (psbA) transcript level in lichen *Xanthoria parietina*. *Phytochemistry* 98, 54–59. doi: 10.1016/j.phytochem.2013.11.021

Shahzad, B., Tanveer, M., Che, Z., Rehman, A., Cheema, S. A., Sharma, A., et al. (2018). Role of 24-epibrassinolide (EBL) in mediating heavy metal and pesticide induced oxidative stress in plants: a review. *Ecotoxicol. Environ. Saf.* 147, 935–944. doi: 10.1016/j.ecoenv.2017.09.066

Shan, X., Zhang, Y., Peng, W., Wang, Z., and Xie, D. (2009). Molecular mechanism for jasmonate-induction of anthocyanin accumulation in *Arabidopsis*. *J. Exp. Bot.* 60, 3849–3860. doi: 10.1093/jxb/erp223

Sharma, A., Kumar, V., Bhardwaj, R., and Thukral, A. K. (2017a). Seed pre-soaking with 24-epibrassinolide reduces the imidacloprid pesticide residues in green pods of *Brassica juncea* L. *Toxicol. Environ. Chem.* 99, 95–103. doi: 10.1080/02772248.2016.1146955

Sharma, A., Kumar, V., Kanwar, M. K., Thukral, A. K., and Bhardwaj, R. (2017b). Ameliorating imidacloprid induced oxidative stress by 24-epibrassinolide in *Brassica juncea* L. *Russ. J. Plant Physiol.* 64, 509–517. doi: 10.1134/S1021443717040124

Sharma, A., Thakur, S., Kumar, V., Kesavan, A. K., Thukral, A. K., and Bhardwaj, R. (2017c). 24-epibrassinolide stimulates imidacloprid detoxification by modulating the gene expression of *Brassica juncea* L. *BMC Plant Biol.* 17:56. doi: 10.1186/s12870-017-1003-9

Sharma, A., Kumar, V., Kumar, R., Shahzad, B., Thukral, A. K., and Bhardwaj, R. (2018). Brassinosteroid-mediated pesticide detoxification in plants: a mini-review. *Cogn. Food Agric.* 4:1436212. doi: 10.1080/23311932.2018.1436212

Sharma, A., Bhardwaj, R., Kumar, V., and Thukral, A. K. (2016a). GC-MS studies reveal stimulated pesticide detoxification by brassinolide application in *Brassica juncea* L. plants. *Environ. Sci. Pollut. Res.* 23, 14518–14525. doi: 10.1007/s11356-016-6650-0

Sharma, A., Kumar, V., Singh, R., Thukral, A. K., and Bhardwaj, R. (2016b). Effect of seed pre-soaking with 24-epibrassinolide on growth and photosynthetic parameters of *Brassica juncea* L. in imidacloprid soil. *Ecotoxicol. Environ. Saf.* 133, 195–201. doi: 10.1016/j.ecoenv.2016.07.008

Sharma, A., Kumar, V., Thukral, A. K., and Bhardwaj, R. (2016c). Epibrassinolide-imidacloprid interaction enhances non-enzymatic antioxidants in *Brassica juncea* L. *Ind. J. Plant Physiol.* 21, 70–75. doi: 10.1007/s40502-016-0203-x

Sharma, A., Thakur, S., Kumar, V., Kanwar, M. K., Kesavan, A. K., Thukral, A. K., et al. (2016d). Pre-sowing seed treatment with 24-epibrassinolide ameliorates pesticide stress in *Brassica juncea* L. through the modulation of stress markers. *Front. Plant Sci.* 7:1569. doi: 10.3389/fpls.2016.01569

Singleton, V. L., and Rossi, J. A. (1965). Colorimetry of total phenolics with phosphomolybdic-phosphotungstic acid reagents. *Am. J. Enol. Vitic.* 16, 144–158.

Sirhindi, G., Mir, M. A., Abd-Allah, E. F., Ahmad, P., and Gucel, S. (2016). Jasmonic acid modulates the physio-biochemical attributes, antioxidant enzyme activity, and gene expression in Glycine max under nickel toxicity. *Front. Plant Sci.* 7:591. doi: 10.3389/fpls.2016.00591

Smelt, J. H., Scorza, R. P., Hendriks, R. F., and Boesten, J. J. (2003). "Preferential transport of imidacloprid in a cracking clay soil," in *Proceedings of the XII Symposium on Pesticide Chemistry. Pesticide in Air, Plant, Soil & Water System*, Piacenza, 319–326.

Stamm, M. D., Hengmoss, T. M., Baxendale, F. P., Siegfried, B. D., Blankenship, E. E., and Nauen, R. (2016). Uptake and translocation of imidacloprid, clothianidin and flupyradifurone in seed-treated soybeans. *Pest Manag. Sci.* 72, 1099–1109. doi: 10.1002/ps.4152

Stoner, K. A., and Eitzer, B. D. (2012). Movement of soil-applied imidacloprid and thiamethoxam into nectar and pollen of squash (*Cucurbita pepo*). *PLoS One* 7:e39114. doi: 10.1371/journal.pone.0039114

Szarka, A., Tomasskovics, B., and Bánhegyi, G. (2012). The ascorbate-glutathione-α-tocopherol triad in abiotic stress response. *Int. J. Mol. Sci.* 13, 4458–4483. doi: 10.3390/ijms13044458

Tapparo, A., Marton, D., Giorio, C., Zanella, A., Soldà, L., Marzaro, M., et al. (2012). Assessment of the environmental exposure of honeybees to particulate matter containing neonicotinoid insecticides coming from corn coated seeds. *Environ. Sci. Technol.* 46, 2592–2599. doi: 10.1021/es2035152

Tohge, T., Nishiyama, Y., Hirai, M. Y., Yano, M., Nakajima, J. I., Awazuhara, M., et al. (2005). Functional genomics by integrated analysis of metabolome and transcriptome of *Arabidopsis* plants over-expressing an MYB transcription factor. *Plant J.* 42, 218–235. doi: 10.1111/j.1365-313X.2005.02371.x

Vardhini, B. V., and Anjum, N. A. (2015). Brassinosteroids make plant life easier under abiotic stresses mainly by modulating major components of antioxidant defense system. *Front. Environ. Sci.* 2:67. doi: 10.3389/fenvs.2014.00067

Wang, Q., Que, X., Zheng, R., Pang, Z., Li, C., and Xiao, B. (2015). Phytotoxicity assessment of atrazine on growth and physiology of three emergent plants. *Environ. Sci. Pollut. Res.* 22, 9646–9657. doi: 10.1007/s11356-015-4104-8

Wasternack, C., and Hause, B. (2013). Jasmonates: biosynthesis, perception, signal transduction and action in plant stress response, growth and development. An update to the 2007 review in Annals of Botany. *Ann. Bot.* 111, 1021–1058. doi: 10.1093/aob/mct067

Whitehorn, P. R., O'Connor, S., Wackers, F. L., and Goulson, D. (2012). Neonicotinoid pesticide reduces bumble bee colony growth and queen production. *Science* 336, 351–352. doi: 10.1126/science.1215025

Wilkins, R. (2000). Leaching and persistence of imidacloprid and diuron in a citrus crop in Valencia. *Fresenius Environ. Bull.* 9, 638–645.

Wolucka, B. A., Goossens, A., and Inzé, D. (2005). Methyl jasmonate stimulates the de novo biosynthesis of vitamin C in plant cell suspensions. *J. Exp. Bot.* 56, 2527–2538. doi: 10.1093/jxb/eri246

Wu, G. L., Cui, J., Tao, L., and Yang, H. (2010). Fluroxypyr triggers oxidative damage by producing superoxide and hydrogen peroxide in rice (*Oryza sativa*). *Ecotoxicology* 19, 124–132. doi: 10.1007/s10646-009-0396-0

Xia, X. J., Huang, Y. Y., Wang, L., Huang, L. F., Yu, Y. L., Zhou, Y. H., et al. (2006). Pesticides-induced depression of photosynthesis was alleviated by 24-epibrassinolide pretreatment in *Cucumis sativus* L. *Pestic. Biochem. Physiol.* 86, 42–48. doi: 10.1016/j.pestbp.2006.01.005

Zeng, F., Chen, S., Miao, Y., Wu, F., and Zhang, G. (2008). Changes of organic acid exudation and rhizosphere pH in rice plants under chromium stress. *Environ. Pollut.* 155, 284–289. doi: 10.1016/j.envpol.2007.11.019

Zhang, Q., Zhao, M., Qian, H., Lu, T., Zhang, Q., and Liu, W. (2012). Enantioselective damage of diclofop acid mediated by oxidative stress and acetyl-CoA carboxylase in nontarget plant *Arabidopsis thaliana*. *Environ. Sci. Technol.* 46, 8405–8412. doi: 10.1021/es300049q

Zhang, W., Curtin, C., Kikuchi, M., and Franco, C. (2002). Integration of jasmonic acid and light irradiation for enhancement of anthocyanin biosynthesis in *Vitis vinifera* suspension cultures. *Plant Sci.* 162, 459–468. doi: 10.1016/S0168-9452(01)00586-6

Zhou, Y., Xia, X., Yu, G., Wang, J., Wu, J., Wang, M., et al. (2015). Brassinosteroids play a critical role in the regulation of pesticide metabolism in crop plants. *Sci. Rep.* 5:9018. doi: 10.1038/srep09018

Zhu, X., Chen, J., Xie, Z., Gao, J., Ren, G., Gao, S., et al. (2015). Jasmonic acid promotes degreening via MYC 2/3/4-and ANAC 019/055/072-mediated regulation of major chlorophyll catabolic genes. *Plant J.* 84, 597–610. doi: 10.1111/tpj.13030

Conflict of Interest Statement: The authors declare that the research was conducted in the absence of any commercial or financial relationships that could be construed as a potential conflict of interest.

frontiers in Plant Science

ORIGINAL RESEARCH
published: 13 November 2018
doi: 10.3389/fpls.2018.01601

Response of Wheat to a Multiple Species Microbial Inoculant Compared to Fertilizer Application

Salmabi K. Assainar[1,2], Lynette K. Abbott[1,2], Bede S. Mickan[1,2,3], Andrew S. Whiteley[1,2], Kadambot H. M. Siddique[1,2] and Zakaria M. Solaiman[1,2]*

[1] UWA School of Agriculture and Environment, The University of Western Australia, Perth, WA, Australia, [2] The UWA Institute of Agriculture, The University of Western Australia, Perth, WA, Australia, [3] Richgro Garden Products, Jandakot, WA, Australia

OPEN ACCESS

Edited by:
Youssef Rouphael, Università degli Studi di Napoli Federico II, Italy

Reviewed by:
Philipp Franken, Leibniz-Institut für Gemüse- und Zierpflanzenbau (IGZ), Germany
Rodica Pena, Georg-August-Universität Göttingen, Germany

***Correspondence:**
Salmabi K. Assainar
salmabi.kayakkeelassainar@research.uwa.edu.au

Specialty section:
This article was submitted to Plant Microbe Interactions, a section of the journal Frontiers in Plant Science

Received: *27 July 2018*
Accepted: *17 October 2018*
Published: *13 November 2018*

Citation:
Assainar SK, Abbott LK, Mickan BS, Whiteley AS, Siddique KHM and Solaiman ZM (2018) Response of Wheat to a Multiple Species Microbial Inoculant Compared to Fertilizer Application. Front. Plant Sci. 9:1601. doi: 10.3389/fpls.2018.01601

Microbial inoculants, including those formed from multiple species, may have dual functions as biostimulants and/or biocontrol agents, and claimed agricultural benefits are instrumental for regulatory categorisation. Biostimulants include commercial products containing substances or microorganisms that stimulate plant growth. Biostimulant microbes can be involved in a range of processes that affect N and P transformations in soil and thus influence nutrient availability, and N and P fertilizers can influence soil microbial diversity and function. A glasshouse experiment was conducted to investigate the effect of a multiple species microbial inoculant relative to a rock-based mineral fertilizer and a chemical fertilizer on wheat growth and yield, and on microbial diversity in the rhizosphere. The microbial inoculant was compared to the mineral fertilizer (equivalent to 5.6 kg N ha^{-1} and 5.6 kg P ha^{-1}), and to the chemical fertilizer applied at three rates equivalent to: (i) 7.3 kg N ha^{-1} and 8.4 kg P ha^{-1} as recommended for on-farm use, (ii) 5.6 kg N ha^{-1} and 6.5 kg P ha^{-1} which matched the N in the mineral fertilizer, and (iii) 4.9 kg N ha^{-1} and 5.6 kg P ha^{-1} which matched P content in the mineral fertilizer. Despite an early reduction in plant growth, the microbial inoculant treatment increased shoot growth at maturity compared to the control. Similarly, grain yield was higher after application of the microbial inoculant when compared to control, and it was similar to that of plants receiving the fertilizer treatments. Using 16S rRNA sequencing, the microbial inoculant and fertilizer treatments were shown to influence the diversity of rhizosphere bacteria. The microbial inoculant increased the relative abundance of the phylum *Actinobacteria*. At tillering, the proportion of roots colonized by arbuscular mycorrhizal (AM) fungi increased with the microbial inoculant and mineral fertilizer treatments, but decreased with the chemical fertilizer treatments. At maturity, there were no treatment effects on the proportion of wheat roots colonized by AM fungi. Overall, the multiple species microbial inoculant had beneficial effects in terms of wheat yield relative to the commercial mineral and chemical fertilizers applied at the level recommended for on-farm use in south-western Australia.

Keywords: biostimulants, mineral fertilizer, chemical fertilizer, grain yield, harvest index, abiotic stress

INTRODUCTION

Biostimulants are derived from a wide range of materials including live microbial cultures, extracts of microbes, animal or plant origin, soil organic compounds (humic and fulvic acids), industrial by-products and chemicals, and synthetic molecules (Saa et al., 2015). Biostimulants can stimulate plant growth and yield even when used in small amounts, but not to the same extent as traditional fertilizer (Fleming et al., 2014). Various rhizosphere microorganisms synthesize plant growth-promoters, siderophores and antibiotics, hence, plant-microbe symbioses can reduce dependency on nitrogen (N) and phosphorus (P) fertilizers (Ahemad and Kibret, 2014). Some plant responses to biostimulants cannot be explained by current understanding, and this represents both a challenge and an opportunity (Brown and Saa, 2015). The global market for biostimulants is projected to increase by 12% per year and reach more than US$2.2 billion by 2018 (Calvo et al., 2014).

Soil microbial communities are extremely diverse and perform key functions, including the cycling of carbon (C), nutrients and water, maintaining soil productivity, dissolution of rock minerals, and remediation of contaminants (Schmitz et al., 2015). There is potential to increase the effectiveness of poorly soluble rock phosphate by managing soil biological processes, with some microorganisms contributing to the dissolution of ground rock fertilizers through the release of organic ligands, H^+ ions and organic acids into the soil (Barker et al., 1997; Hinsinger et al., 2001; Richardson, 2001). In nutrient-deficient soils, microorganisms may preferentially colonize ground rock fertilizers if they contain growth-limiting nutrients. For example, Roger and Bennett (2004) showed that in a P-limiting environment, microorganisms selectively colonized the surface of minerals containing P. The effectiveness of plant access to P in rock phosphate can be improved by the activities of arbuscular mycorrhizal (AM) fungi (Barrow et al., 1977; Pairunan et al., 1980). Soil microorganisms are vital constituents of the rhizosphere, and they play key roles in P cycling (Richardson, 1994). AM fungi are members of the Glomeromycota, a key component of the soil microbiota which form the most common and widespread terrestrial plant symbioses. They are obligate symbiotic soil fungi, and they form intimate associations with approximately 80% of terrestrial plant species including the majority of crops (Smith and Read, 2008). AM fungi have been shown to benefit crop productivity due to their contribution to plant nutrition, soil structure and other ecosystem services (Smith and Read, 2008). The predominant function of AM fungi is attributed to increased host plant P uptake as a consequence of their high-affinity P uptake mechanism (Smith and Smith, 2012). Ground rock addition to soil is likely to affect microbial communities through indirect effects on physicochemical transformations (Hinsinger et al., 1996; Gillmann et al., 2002; Loganathan et al., 2002; Priyono and Gilkes, 2004) and by increasing plant growth (Sanz and Rowell, 1988; Coroneos et al., 1996; Bakken et al., 2000).

The two most-reported mechanisms by which microorganisms solubilise P are the production of organic acids (Goldstein, 1996) and the production of phosphatases (Rodriguez et al., 2006). Organic acids transform insoluble phosphate forms into soluble forms through their hydroxyl groups. These groups chelate the cations bound to phosphate, thereby facilitating the release of phosphate ions (Rodriguez and Fraga, 1999). In nutrient-poor soils, such as the highly weathered soils in south-western Australia, minerals containing limiting concentrations of nutrients may influence the soil microbial community structure (Carson et al., 2012). Phosphate solubilisation takes place through various microbial processes/mechanisms including organic acid production and proton extrusion as a result of the combined effect of a pH decrease and organic acid production (He and Zhu, 1988; Surange et al., 1995; Dutton and Evans, 1996; Nahas, 1996; Deubel and Merbach, 2005; Fankem et al., 2006).

Rhizosphere microorganisms, including plant growth promoting rhizobacteria (PGPRs) have been investigated for their effects on plant growth (Adesemoye et al., 2008). One proposed mechanism by which PGPRs can affect nutrient uptake is by enhancing growth and development of roots, leading to larger root systems with the greater surface area and more root hairs, which are then able to access more nutrients (Biswas et al., 2000a; Adesemoye et al., 2008). These studies have included comparisons with plant-microbe interactions and fertilizer use efficiency (Adesemoye et al., 2008).

We compared the efficacy of a multiple species microbial inoculant to a rock-based mineral fertilizer and a more traditional chemical fertilizer for its capacity to increase wheat yield in an agricultural soil that was moderately deficient in N and P for the growth of wheat. We hypothesized that the microbial inoculant would be as effective as the mineral fertilizer on growth, yield and nutrient uptake of wheat based on its potential to supply N and P through mineralization of organic matter. The microbial inoculant was expected to be less effective than the chemical fertilizer. Mechanisms underlying these hypotheses could be associated with activities of rhizosphere bacterial communities and AM fungi whereby (1) the introduction of the microbial inoculant would augment the existing rhizosphere bacterial community, and (2) the application of the chemical fertilizer would reduce mycorrhizal colonization of wheat roots but the microbial inoculant and mineral fertilizer would not affect colonization.

MATERIALS AND METHODS

Experimental Design

A multiple species microbial inoculant and two fertilizers (a rock-based mineral fertilizer, and a chemical fertilizer) were applied to soil before sowing wheat seeds. A control treatment had neither microbial inoculant nor fertilizer added to the soil which was collected from an agricultural field and was moderately N and P deficient for growing wheat. There were four replicates of each treatment. The experiment was arranged in a completely randomized design. Plants were harvested at tillering (7 weeks) and maturity (12 weeks).

Six treatments were (**Table 1**): (i) a control that did not receive any amendments; (ii) a multiple species microbial inoculant (1 g powder/pot applied), (iii) MF: a rock-mineral fertilizer at

TABLE 1 | Composition of treatments applied at the commencement of the experiment.

Treatment	Composition	Application rate
Control	Unamended	Nil
Microbial inoculant (Microbes)	A multiple species microbial inoculant is a talc-based formulation containing (per g) isolates of *Agrobacterium (1 × 10'9), Azotobacter (1.2 × 10'9), Azospirillum (1.1 × 10'9), Bacillus (112 × 10'9), Pseudomonas (2.3 × 10'9), Streptomyces (1 × 10'9), Trichoderma (8 × 10'9),* and *Rhizophagus irregularis (75 spores).*	Microbial inoculant was provided by Australian Mineral Fertilizer Pty Ltd. as a powder form and applied at the rate of 1g pot^{-1}
Mineral fertilizer (MF)	Mineral-based fertilizer (from Australian Mineral Fertilizer Pty Ltd.), consists of a proprietary combination of fine mineral ores, such as micas, alkali feldspars, soft rock phosphate, dolomite, basalt, granite and crystalline silica, that are blended with various sulfates (ammonium, potassium, manganese, copper and zinc) containing nutrients (in %, w/w) N-7.5, P 7.5, K-4.5, S-8.0, Mg-0.9, Fe-2.6, Si-6.7, Mn-0.4, Zn-0.043, Cu-0.043, B-0.0017	Equivalent to 5.6 kg N and 5.6 kg P ha^{-1}; (1g pot^{-1})
Chemical fertilizer -1 75 kg ha^{-1} (CF-1)	Gusto Gold from Summit Fertilizers - fully granulated compound fertilizer with all nutrients in each granule as in % (w/w) N-10.2, P-13.1, K-12.0, S -7.2, Cu-0.09, Zn-0.13	Equivalent to 7.3 kg N and 8.4 kg P ha^{-1} which includes recommended on-farm equivalent rates of N and P respectively; (1g pot^{-1})
Chemical fertilizer - 2 55 kg ha^{-1} (CF-2)	Gusto Gold from Summit Fertilizers- fully granulated compound fertilizer with all nutrients in each granule as in % (w/w) N-10.2, P-13.1, K-12.0, S -7.2, Cu-0.09, Zn-0.13	Equivalent to 5.6 kg N and 6.5 kg P ha^{-1} which matched the N in the mineral fertilizer; (0.735g pot^{-1})
Chemical fertilizer – 3 43 kg ha^{-1} (CF-3)	Gusto Gold – Summit Fertilizers- fully granulated compound fertilizer with all nutrients in each granule as in % (w/w) N-10.2, P-13.1, K-12.0, S -7.2, Cu-0.09, Zn-0.13	Equivalent to 4.9 kg N and 5.6 kg P ha^{-1} which matched the P in the mineral fertilizer; (0.574g pot^{-1})

75 kg ha^{-1} (equivalent to 5.6 kg N ha^{-1} and 5.6 kg P ha^{-1}), (iv) CF-1: 75 kg ha^{-1} of a traditional soluble chemical fertilizer (equivalent to 7.3 kg N ha^{-1} and 8.4 kg P ha^{-1}, applied at the recommended on-farm), (v) CF-2: 55 kg ha^{-1} of the same chemical fertilizer (equivalent to 5.6 kg N ha^{-1} and 6.5 kg P ha^{-1} that matched the N concentration in the mineral fertilizer), and (vi) CF-3: 43 kg ha^{-1} of the same chemical fertilizer (equivalent to 4.9 kg N ha^{-1} and 5.6 kg P ha^{-1}, which matched the P concentration in the mineral fertilizer).

Soil Properties and Plant Growth Conditions

Field soil at 0–10 cm depth was collected from an agricultural field at Dowerin, Western Australia (latitude 31.22^0S, longitude 117.02^0E). The soil was a moderately nutrient-deficient, loamy sand with the following properties: pH (1:5, soil/water) 5.7, EC: 0.133 dS m^{-1}, soil bulk density 1.5 g cm^{-3}, 10.8g organic C kg^{-1}, total N: 0.9 g kg^{-1}, C:N ratio 12.2gkg^{-1}, NH_4^+ -N 2mg Kg^{-1}, NO_3 –N 29mg Kg^{-1} and 24 mg available Colwell P kg^{-1}. The soil was sieved to 2 mm immediately after collection and stored at 4°C prior to potting in 2L undrained pots. We measured the above parameters by following standard methods (Rayment and Lyons, 2010).

The microbial inoculant and fertilizers were added separately to the soil according to the treatments (**Table 1**). The microbial inoculant powder was homogenously mixed into the top 3 cm soil. Fertilizers were also homogenously mixed into the top 3 cm soil in the respective treatments. The agricultural soil had been previously cropped with wheat and contained a community of naturally occurring AM fungi (*Rhizophagus irregularis)*. Wheat (*Triticum aestivum* L. cultivar: Wyalkatchem) was sown as it is widely grown in south-western Australia. Seeds were carefully selected for uniformity. Four seeds were sown in each pot, and the seedlings thinned to two per pot after emergence. The soil in each pot was watered and maintained at 70% (w/w basis) field capacity.

Harvesting and Nutrient Analysis

Plant growth was assessed as plant height, number of tillers (**Table 2**), total biomass, grain quantity, 1000 grain weight, grain yield and nutrient concentration from shoot and grains. The number of generative tillers was counted just before flowering. Shoot and root fresh and dry weights were measured at tillering (7 weeks) and at maturity (12 weeks). Shoots were dried at 70°C in a forced-air oven for at least 72 h, weighed and finely ground. N was assessed using combustion analysis in an Elementar (vario Marco CNS; Elementar, Germany). Subsamples were digested in 5:0.5 HNO_3-$HClO_4$ (Simmons, 1975, 1978). P and K were measured using a Perkin Elmer Optima 5300dv instrument. Shoot N, P and K uptake were calculated as the product of shoot dry mass yield and the concentration of individual macro-element.

The number of grains per ear was determined at maturity and 1000 grain weight was calculated (**Table 2**). Harvest index was calculated as the dry matter of grain yield divided by the sum of dry matter of grain yield and straw yield (Metho and Hammes, 2000). Wheat grains were ground prior to chemical analysis, and N, P and K uptake in grains were calculated as the product of grain dry mass yield and the concentration of individual macro-element.

Soil DNA Extraction, PCR Amplification, and Sequencing

At each harvest, roots were carefully removed from the soil and shaken to remove loosely adhering soil. The more tightly

TABLE 2 | Grain yield, harvest index, grain number per pot, and 1000 grain weight at maturity in the six treatments: control, microbial inoculant (Microbes), mineral fertilizer (MF), and three rates of chemical fertilizer [CF-1 (75 kg ha^{-1}), CF-2 (55 kg ha^{-1}) and CF-3 (43 kg ha^{-1})].

Treatment	Grain Yield (g pot^{-1})	Harvest index (%)	Grain number (pot^{-1})	1000 Grain weight (g)	Tiller number
Control	3.80 ± 0.14a	86 ± 0.1c	81 ± 3.1a	47.13 ± 1.02a	4.0 ± 0a
Microbes	4.41 ± 0.18b	85 ± 0.5bc	92 ± 6.3a	49.03 ± 0.93a	3.3 ± 0.14a
MF	4.70 ± 0.16b	78 ± 0.2a	150 ± 2.5bc	47.09 ± 1.66a	6.5 ± 0c
CF-1	4.66 ± 0.14b	76 ± 0.4a	169 ± 5.1c	46.62 ± 0.14a	6.4 ± 0.31c
CF-2	4.41 ± 0.03ab	78 ± 0.9a	139 ± 8.4b	44.12 ± 0.35a	6.4 ± 0.24c
CF-3	4.25 ± 0.07ab	83 ± 0.5b	95 ± 2.6a	44.79 ± 1.76a	5.3 ± 0.14b
$LSD_{0.05}$	0.404	1.6	15.3	3.84	0.539
P-value	0.002	<0.001	<.001	0.147	<.001

Means followed by the same letter within a column are not significantly different according to $LSD_{0.05}$.

adhering rhizosphere soil (Mickan et al., 2017) was collected and used for subsequent analysis of rhizosphere bacteria. Rhizosphere soil was transferred to a −20°C freezer prior to DNA extraction as described previously (Mickan et al., 2017). Paired end sequencing on the Illumina MiSeq was performed using the primer set of 27F-519R (Lane et al., 1985; Lane, 1991).

Bioinformatics

Paired-end reads were assembled by aligning the forward and reverse reads using PEAR (version 0.9.5) (Zhang et al., 2014). Primers were identified, and trimmed sequences were processed using Quantitative Insights into Microbial Ecology (QIIME 1.8) (Caporaso et al., 2010) USEARCH (Edgar, 2010; Edgar et al., 2011) (version 8.0.1623) and UPARSE software. Using USEARCH tools sequences were quality filtered, and full-length duplicate sequences removed and sorted by abundance. Singletons or unique reads in the data set were discarded. Sequences were clustered following chimera filtering using the "RDP_Gold" database as a reference, Reads were mapped back to OTUs with a minimum identity of 97% to obtain the number of reads in each OUT. Using QIIME, taxonomy was assigned using the Greengenes database5 (Version 13_8, Aug 2013).

Arbuscular Mycorrhizal (AM) Colonization in Roots

Roots were washed well with tap water to remove any remaining adhering soil particles, blotted dry with tissue paper, weighed, cut into 1 cm segments and mixed thoroughly. A 1g subsample of roots was taken and stained prior to assessment of AM fungal colonization (Abbott and Robson, 1981). After random dispersion of the stained roots in a 9-cm diameter Petri plate with gridlines, the percentage of root length colonized by AM fungi and meter of root length colonized were determined using the gridline intersect method (Giovannetti and Mosse, 1980) and estimated the root length (Newman, 1966).

Soil Analysis at Harvest

EC and pH were measured (1:5 soil/water ratio) using a probe inserted into the water and 0.01M $CaCl_2$, respectively. Soil pH was determined with a glass electrode (pH probe) using a soil-to-water ratio of 1:5 mixture and with $CaCl_2$ as well. The soil EC was measured in water at 1:5 (w/v) ratios. Soil EC was also measured in $CaCl_2$ at 1:5 (w/v) ratios.

Statistical Analysis

One-way analysis of variance (ANOVA) was carried out using Genstat 18th edition (64 bit) software for wheat growth, mycorrhizal colonization, basic soil data and wheat nutrient data for shoots and grain. Main effects were compared using the least significant difference (LSD) for multiple comparison tests, where $P \leq 0.05$ was considered the threshold value for significance. The effect of treatments on wheat biomass, grain yield and components of yield were investigated using ANOVA.

To assess bacterial community assemblages and alpha (α) diversity, a two-way ANOVA was applied to test for the effects of 'Fertilizer' and 'Harvest time' treatments on bacterial relative abundance and alpha diversity. To assess the OTU level community data, a non-metric multidimensional scaling (NMDS) plot of soil bacterial community at tillering and maturity, and fertilizer treatments using Bray-Curtis dissimilarity matrix method (97% similarity). A permutational multivariate analysis of variance (PERMANOVA) was used to test the significant difference between taxonomic bacterial (OTU level) data (beta diversity) and treatments (fertilizer and harvest time) using 999 permutations in the 'vegan' package (Oksanen et al., 2013).

RESULTS

Plant Growth and Yield

There were significant differences between the microbial inoculant, mineral and chemical fertilizer treatments (P<0.001), with the greatest effects on shoot and root biomass in the fertilizer treatments relative to the microbial inoculant and the untreated control at tillering and maturity (**Figures 1A,B**).

Shoot and root dry weights were unaffected by the application of the microbial inoculant ($P < 0.05$; **Figures 1A,B**). At tillering, shoot dry weight increased with the application of mineral and chemical fertilizer (**Figure 1A**). Total shoot dry weight accumulation increased with increasing levels of chemical fertilizer, especially at maturity (**Figure 1A**). The lower chemical fertilizer rate had little effect on plant growth, while the higher

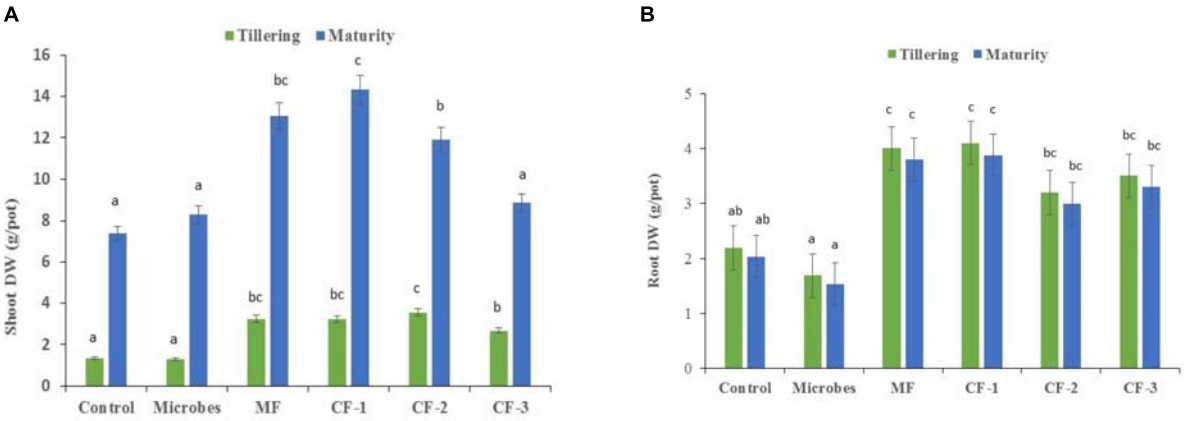


FIGURE 1 | (A) Shoot dry weight and **(B)** root dry weight at tillering and maturity in the six treatments: control, microbial inoculant (Microbes), mineral fertilizer (MF), and three rates of chemical fertilizer [CF-1 (75 kg ha^{-1}), CF-2 (55 kg ha^{-1}), and CF-3 (43 kg ha^{-1})]. Mean data followed by a similar letter(s) are not statistically significant within each sampling time.

fertilizer rate increased shoot growth (**Figure 1A**). A similar trend was observed for root dry weight, except with the CF-2 (55 kg ha^{-1}) treatment (**Figure 1B**).

The microbial inoculant produced less shoot and root dry weight at tillering and maturity than did the mineral and chemical fertilizers when applied at equivalent rates (**Figures 1A,B**). At maturity, the microbial inoculant produced similar shoot dry weight to the lowest rate of chemical fertilizer (43 kg ha^{-1}), but less than the two higher rates (55 and 75 kg ha^{-1}), and lower root dry weight than the three chemical fertilizer treatments (**Figure 1B**).

The microbial inoculant increased grain yield relative to the control ($P < 0.05$, **Table 2**). Harvest index was not affected by the microbial inoculant ($P > 0.05$, **Table 2**) but was decreased with all fertilizer treatments relative to the control. Thousand grain weight was not affected by any of the treatments ($P > 0.05$, **Table 2**). The mineral fertilizer and chemical fertilizer treatments generally increased grain number (**Table 2**); the microbial inoculant produced the higher grain yield than the control, but it did not differ from any of the fertilizer treatments (**Table 2**).

Nutrient Concentration and Uptake

The microbial inoculant and mineral fertilizer treatments had no effect on shoot N concentration at tillering and maturity, except for an increase with 75 kg ha^{-1} chemical fertilizer at tillering but not at maturity (**Table 3**). The shoot P concentration of wheat treated with the microbial inoculant was similar to that of the control at tillering but was higher with the mineral and chemical fertilizer treatments. At maturity, there was no difference in P concentration of shoots treated with the microbial inoculant and chemical fertilizer compared with the control, but it was lower for the mineral fertilizer (**Table 3**). Wheat treated with the microbial inoculant had a similar shoot K concentration to that of all other treatments at tillering, but at maturity, it was lower than the two higher rates of chemical fertilizer (55 and 75 kg ha^{-1}).

The N, P, and K uptake by shoots were significantly influenced by treatments both at tillering and maturity (**Supplementary Table S1**; $p<0.001$). At both tillering and maturity, wheat inoculated with the microbial inoculant had similar N, P, and K uptake to the control plants, but lower than the fertilized plants, except for P and K uptake which was similar to 43 kg/ha chemical fertilizer (**Supplementary Table S1**).

Wheat inoculated with the multiple species microbial inoculant had similar grain N concentration compared to all other treatments, but it was reduced with all fertilizer treatments compared to control (**Supplementary Table S2**). Grain P concentration was higher with the mineral and chemical fertilizer at 75 kg ha^{-1}, but there was no effect of the microbial inoculant. Grain K concentration was the same for the microbial inoculant and the control but lower with the fertilizer treatments (**Supplementary Table S2**).

Application of the microbial inoculant did not increase grain N uptake. P uptake in grain with the microbial inoculant was same as that of the control and chemical fertilizer applied at both 43 and 55 kg ha^{-1} (**Supplementary Table S2**). Microbial inoculant application increased K uptake in wheat grain compared to all fertilizer treatments (**Supplementary Table S2**).

Soil pH

Although the overall variation in soil pH was moderate, ranging from 4.5 to 5.8, there was a significant difference in pH between soils receiving the microbial inoculant and mineral fertilizer treatments ($P = 0.05$, **Supplementary Table S3**). Application of the microbial inoculant significantly increased soil pH (measured in water and $CaCl_2$) at maturity compared to all other fertilizer treatments.

AM Fungal Colonization

Mycorrhizal colonization was recorded at both tillering and maturity (**Figure 2A**). Colonization (%) increased with application of the microbial inoculant compared to all chemical fertilizer treatments at tillering but not at maturity (**Figure 2A**). The mineral fertilizer treatments had the highest length of root colonized by AM fungi at tillering relative to all other

TABLE 3 | Shoot N, P, and K concentrations at tillering and maturity in the six treatments: control, microbial inoculant (Microbes), mineral fertilizer (MF), and three rates of chemical fertilizer [CF-1 (75 kg ha^{-1}), CF-2 (55 kg ha^{-1}), and CF-3 (43 kg ha^{-1})].

Treatment	Tillering			Maturity		
	N (%)	*P* (%)	*K* (%)	*N* (%)	*P* (%)	*K* (%)
Control	2.19 ± 0.02a	0.36 ± 0.02ab	3.79 ± 07ab	0.35 ± 0.01a	0.31 ± 0.01b	1.9 ± 0.09ab
Microbes	2.40 ± 0.08a	0.34 ± 0.03a	3.95 ± 0.09ab	0.35 ± 0.04a	0.25 ± 0.03ab	1.6 ± 0.17a
MF	2.58 ± 0.17a	0.46 ± 0.02b	4.00 ± 0.19b	0.40 ± 0.02a	0.17 ± 0.01a	2.1 ± 0.09ab
CF-1	3.15 ± 0.16b	0.62 ± 0.02c	4.38 ± 0.21b	0.40 ± 0.02a	0.30 ± 0.02b	2.4 ± 0.12b
CF-2	2.57 ± 0.10a	0.61 ± 0.02c	4.13 ± 0.17b	0.37 ± 0.02a	0.26 ± 0.02b	2.3 ± 0.13b
CF-3	2.33 ± 0.08a	0.58 ± 0.01c	3.31 ± 0.06a	0.35 ± 0.03a	0.28 ± 0.02b	1.9 ± 0.09ab
$LSD_{0.05}$	0.354	0.078	0.44	0.077	0.056	0.356
P-value	<0.001	<0.001	0.002	0.472	<0.001	0.002

Means followed by the same letter within a column are not significantly different according to LSD $_{0.05}$.

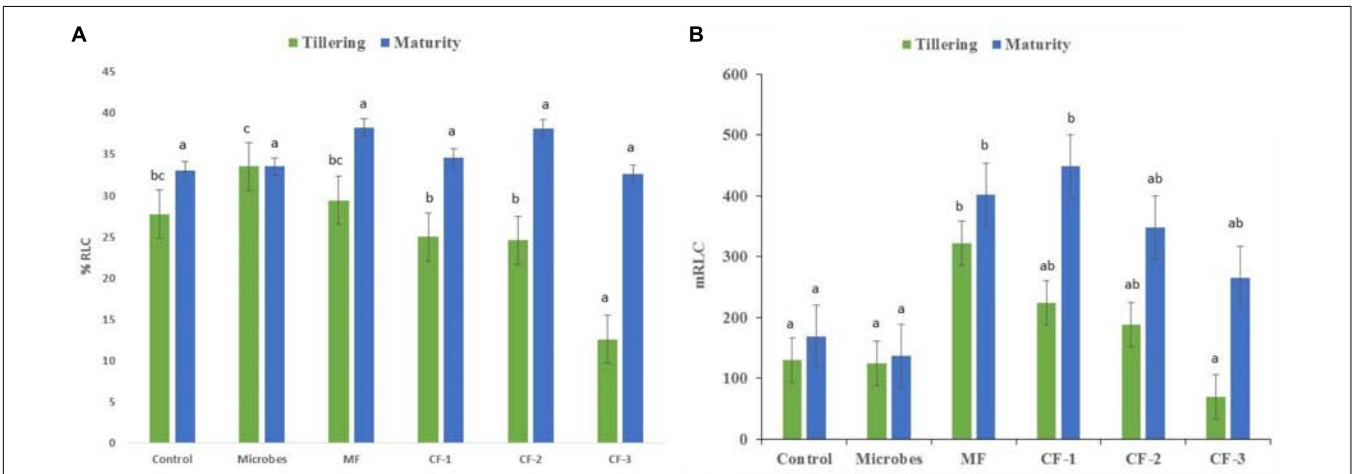


FIGURE 2 | (A) The percentage of root length colonized by mycorrhiza (% RLC) and **(B)** root length colonized by mycorrhiza (m RLC) at tillering and maturity in the six treatments: control, microbial inoculant (Microbes), mineral fertilizer (MF), and three rates of chemical fertilizer [CF-1 (75 kg ha^{-1}), CF-2 (55 kg ha^{-1}), and CF-3 (43 kg ha^{-1})]. Mean data followed by a similar letter(s) are not statistically significant within each sampling time.

treatments except the chemical fertilizer at 55 and 75 kg ha^{-1}, and was greatest at maturity for chemical fertilizer applied at 75 kg ha^{-1} (**Figure 2B**) compared to the microbial inoculant.

Soil Bacterial Community Composition and Diversity

The bacterial phyla *Proteobacteria* (36%) and *Actinobacteria* (35%) were dominant in all rhizosphere soil for treatments (**Figure 3**). Alpha diversity showed that the fertilizer treatments altered Evenness ($P < 0.001$) and OTU richness ($P = 0.03$) as well as the diversity index of Inverse Simpson ($P = 0.005$) and Fisher ($P < 0.001$) (**Supplementary Table S4**). There were no significant effects of treatments at maturity. However, there were interactions between fertilizer and harvesting time for OTU richness ($P < 0.001$) and Fisher ($P = 0.042$).

The relative abundance at phylum level varied with the fertilizer treatments for *Actinobacteria* ($P < 0.001$), *Proteobacteria* ($P < 0.001$), *Chloroflexi* ($P < 0.001$), *Planctomycetes* ($P < 0.001$), *Firmicutes* ($P < 0.001$), and *Bacteroidetes* ($P < 0.001$). The relative abundance of *Actinobacteria* ($P = 0.016$), *Proteobacteria* ($P = 0.001$), and *Firmicutes* ($P < 0.001$) varied with harvesting time. There were interactions between fertilizer and harvest time for *Firmicutes* ($P < 0.001$) and for *TM7* ($P = 0.001$) (**Supplementary Table S5** and **Figure 4**).

Impact of Fertilizer Treatments and Harvesting Time on Bacterial OTU Community Composition

A NMDS plot was used to visualize the community assemblages at the OTU level. There was distinct clustering of all treatments, although the microbial inoculant and the mineral fertilizer treatments had similar community assemblages (**Figure 5**). PERMANOVA revealed that the fertilizer treatments significantly altered the community composition at the OTU level ($P < 0.001$), and it also changed with time of harvest ($P = 0.003$). There was an interaction between fertilizer and harvest time ($P < 0.001$) (**Supplementary Table S6**).

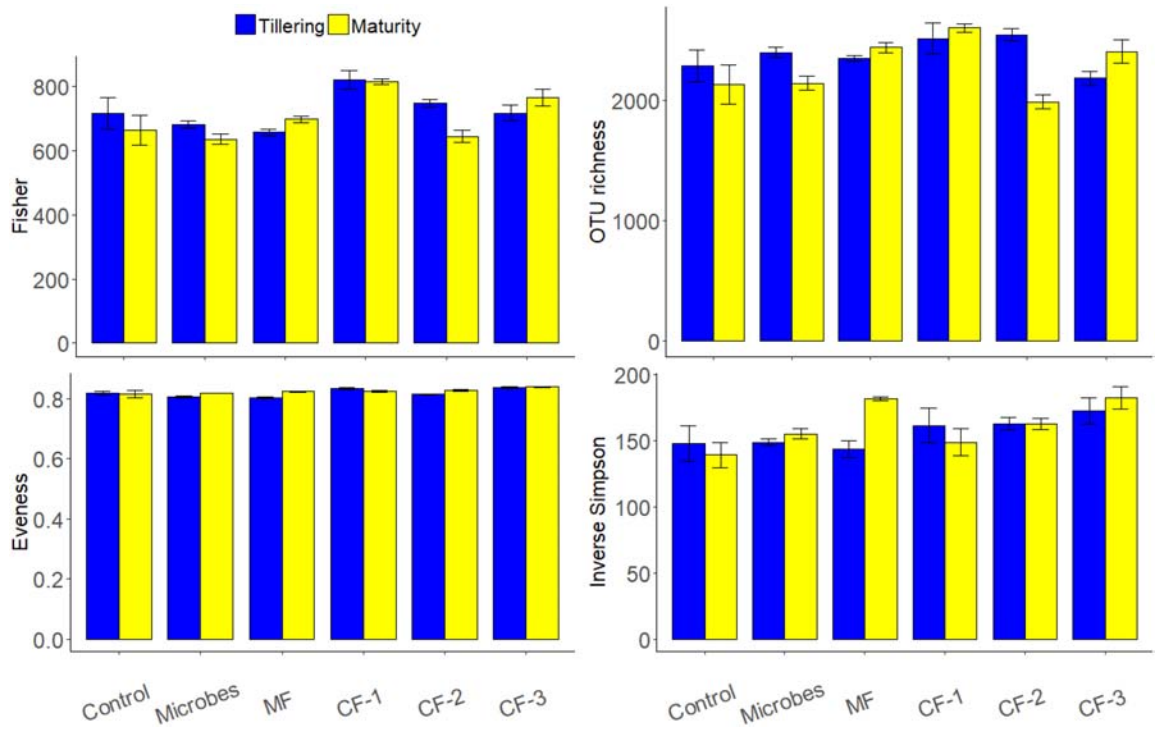


FIGURE 3 | Alpha diversity indices based on OTU composition (97% similarity) on the effect of 'fertilizer treatments,' 'harvests' (tillering and maturity), and their interaction for the six treatments: control, microbial inoculant (Microbes), mineral fertilizer (MF), and three rates of chemical fertilizer [CF-1 (75 kg ha^{-1}), CF-2 (55 kg ha^{-1}), and CF-3 (43 kg ha^{-1})].

DISCUSSION

We hypothesized that a mixed microbial inoculant would be as effective as a mineral fertilizer applied at the recommended level for wheat growth and yield in the agricultural soil used. Indeed, the microbial inoculant significantly increased grain yield without increasing shoot growth of wheat.

The wheat response to N and P fertilizer (a conventional chemical fertilizer) and the low soil organic matter content in the soil indicated that the mineralization potential of this soil was far below the N and P requirements of the wheat crop. Previous studies with different microbial inoculants have shown increased plant height and spikelets per spike in various crops (e.g., Khalid et al., 1997; Biswas et al., 2000b). Several studies reported increased seed P content associated with the application of phosphate-solubilising microorganisms (Kucey, 1987; Mehana and Wahid, 2002; Zaidi et al., 2004). However, the microbial inoculant used in our study did not affect P concentration but did increase N and K concentrations ($P < 0.001$).

The mineral fertilizer was expected to be less effective than the chemical fertilizer when applied at equivalent levels of N and P because of differences in solubility of these nutrients. Unexpectedly, for grain yield, the mineral fertilizer was as effective as all of the chemical fertilizer treatments. For shoot growth, the mineral fertilizer was as effective as the chemical fertilizer when applied at equivalent levels of N and P at tillering, and when applied at equivalent levels of N at maturity.

It was expected that the mechanisms underlying potential benefits of the microbial inoculant in this study would be linked to soil bacterial diversity in the rhizosphere and root colonization by AM fungi. The microbial inoculant was expected to complement the existing bacterial community in the rhizosphere, but relatively minor changes in the bacterial community composition were recorded at maturity. In relation to mycorrhizal colonization, it was expected that the microbial inoculant and mineral fertilizer would not influence colonization of the wheat roots by AM fungi, but the chemical fertilizer would reduce the extent by which roots were colonized by AM fungi. However, mycorrhizal colonization of roots increased with application of the microbial inoculant at an early stage of plant growth (tillering) where benefits to the plant could be expected. In contrast, as expected, the chemical fertilizer decreased mycorrhizal colonization, which could reduce potential benefits compared to plants with higher AM fungal colonization in the microbial inoculant treatment. Low nutrient availability stresses plants, which leads to the development of longer and more active roots with mycorrhizas that scavenge for nutrients (Hui-Lian, 2001).

The microbial inoculant used in our study contained a group of beneficial microorganisms that promoted plant

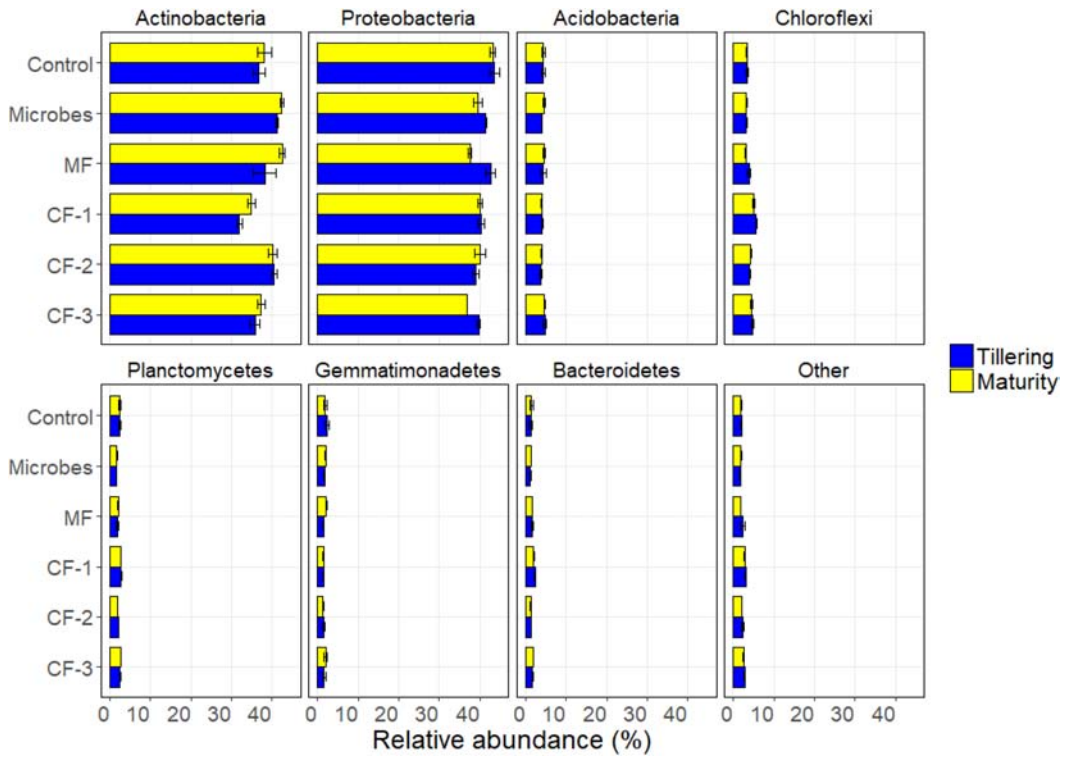


FIGURE 4 | Relative abundance of soil bacteria at phylum resolution in the six treatments: control, microbial inoculant (Microbes), mineral fertilizer (MF), and three rates of chemical fertilizer [CF-1 (75 kg ha^{-1}), CF-2 (55 kg ha^{-1}), and CF-3 (43 kg ha^{-1})].

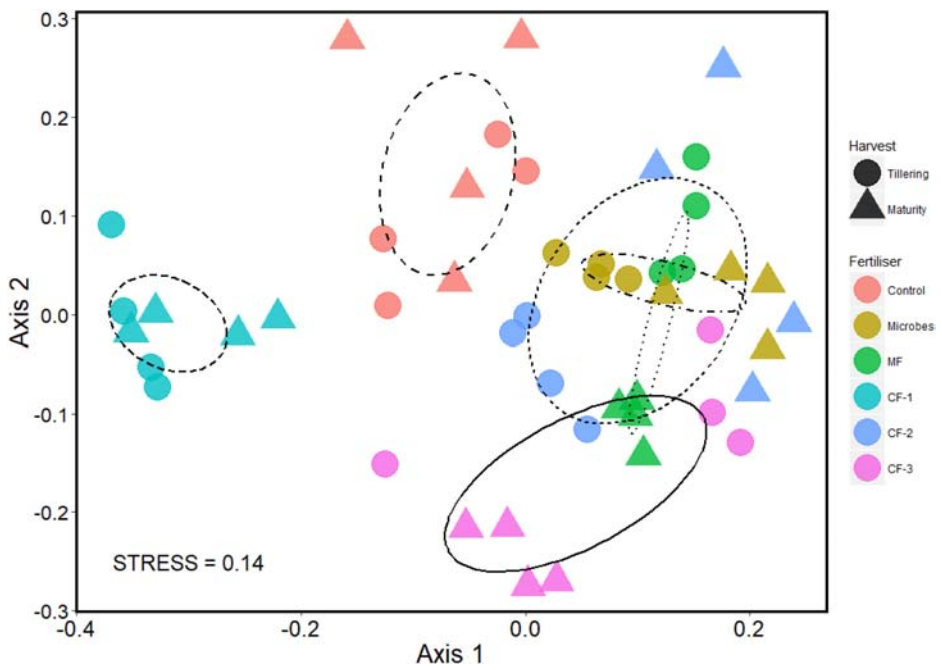


FIGURE 5 | Non-metric multidimensional scaling (NMDS) plot of OTU community assemblage analysis based on 97% similarity OTU abundance data (square root transformed), using 999 permutations in the six treatments: control, microbial inoculant (Microbes), mineral fertilizer (MF), and three rates of chemical fertilizer[CF-1 (75 kg ha^{-1}), CF-2 (55 kg ha^{-1}), and CF-3 (43 kg ha^{-1})].

growth and increased grain yield in the absence of chemical fertilizer. Based on previous studies, this could be attributed to increased photosynthetic capacity and nutrient availability (Hui-Lian, 2001). Overall, the application of fertilizer to this soil had little effect on the bacterial community composition, with increases in relative abundance of only two of the top 25 most-abundant OTUs (*Proteobacteria* and *Actinobacteria*). Farmers have anecdotally reported increases in plant growth, yield and grain quality following application of a range of microbial inoculants. Further studies are needed to examine the mechanisms of how microbial inoculants influence plant growth to elucidate issues confronted in the technology of microbial inoculant development.

CONCLUSION

The multiple species microbial inoculant stimulated grain yield of wheat to the same level as did the fertilizers applied to the moderately N and P deficient soil used in this experiment. While microbial inoculation is unlikely to significantly change the abundance and composition of indigenous microbial communities, the localized intervention via inoculation has the potential to contribute to nutrient cycling in the wheat rhizosphere. Combinations of microbial inoculants and fertilizers of different elemental solubility should be investigated to identify alternative strategies for increasing profitability and sustainability of crop production, with greater emphasis placed on the role of beneficial soil microorganisms.

AUTHOR CONTRIBUTIONS

SA conducted the experiment, analyzed the data, and wrote the manuscript. LA, KS, and ZS supervised the work, helped to develop the experiments, revised the manuscript, and contributed to the writing. AW has revised the manuscript and contributed to the writing. BM helped in bioinformatics and interpretation of results.

FUNDING

The University of Western Australia funded this research through the University Postgraduate Awards program to SA and the Australian Research Council Linkage Grant (LP140100046) with the industry partner Australian Mineral Fertilizers Pty Ltd.

SUPPLEMENTARY MATERIAL

The Supplementary Material for this article can be found online at: https://www.frontiersin.org/articles/10.3389/fpls.2018.01601/full#supplementary-material

REFERENCES

Abbott, K. L., and Robson, A. (1981). Infectivity and effectiveness of vesicular arbuscular mycorrhizal fungi. *Aust. J. Agric. Res.* 32, 631–639. doi: 10.1071/AR9810631

Adesemoye, A. Q., Torbert, H. A., and Kloepper, J. W. (2008). Enhanced plant nutrient use efficiency with PGPR and AMF in an integrated nutrient management system. *Can. J. Microbiol.* 54, 876–886. doi: 10.1139/W08-081

Ahemad, M., and Kibret, M. (2014). Mechanisms and applications of plant growth promoting rhizobacteria: current perspective. *J. King Saud Uni – Sci.* 26, 1–20. doi: 10.1016/j.jksus.2013.05.001

Bakken, A. K., Gautneb, H., Sveistrup, T., and Myhr, K. (2000). Crushed rocks and mine tailings applied as K fertilisers on grassland. *Nutr. Cycl. Agroecosys.* 56, 53–57. doi: 10.1023/A:1009709914578

Barker, W. W., Welch, S. A., and Banfield, J. F. (1997). Biogeochemical weathering of silicate minerals. *Rev. Mineral. Geochem.* 35, 391–428.

Barrow, N. J., Malajczuk, N., and Shaw, T. C. (1977). A direct test of the ability of vesicular-arbuscular mycorrhiza to help plants take up fixed soil phosphate. *New Phytol.* 78, 269–276. doi: 10.1111/j.1469-8137.1977.tb04830.x

Biswas, J. C., Ladha, J. K., and Dazzo, F. B. (2000a). Rhizobia inoculation improves nutrient uptake and growth of lowland rice. *Soil Sci. Soc. Am. J.* 64, 1644–1650. doi: 10.2136/sssaj2000.6451644x

Biswas, J. C., Ladha, J. K., Dazzo, F. B., Yanni, Y. G., and Rolfe, B. G. (2000b). Rhizobial inoculation influences seedling vigor and yield of rice. *Agron. J.* 92, 880–886. doi: 10.2134/agronj2000.925880x

Brown, P., and Saa, S. (2015). Biostimulants in agriculture. *Front. Plant Sci.* 6:671. doi: 10.3389/fpls.2015.00671

Calvo, V. P., Nelson, L., and Kloepper, J. (2014). Agricultural uses of plant biostimulants. *Plant Soil* 383, 3–41. doi: 10.1007/s11104-014-2131-8

Caporaso, J. G., Kuczynski, J., Stombaugh, J., Bittinger, K., Bushman, F. D., Costello, E. K., et al. (2010). QIIME allows analysis of high-throughput community sequencing data. *Nat. Methods* 7, 335–336. doi: 10.1038/nmeth.f.303

Carson, J., Harley, A., Abbott, L. K., and Gleeson. (2012). *Adding Value to Organic Pasture: Microbes and Minerals*. Kingston, ACT: Rural Industries Research and Development Corporation.

Coroneos, C., Hinsinger, P., and Gilkes, R. J. (1996). Granite powder as a source of potassium for plants: a glasshouse bioassay comparing two pasture species. *Fert. Res.* 45, 143–152. doi: 10.1007/BF00790664

Deubel, A. and Merbach, W. (2005). "Influence of microorganisms on phosphorus bioavailability in soils," in *Microorganisms in Soils: Roles in Genesis and Functions*, eds F. Buscot and A. Varma (Berlin: Springer-Verlag), 62.

Dutton, V. M., and Evans, C. S. (1996). Oxalate production by fungi: its role in pathogenicity and ecology in the soil environment. *Can. J. Microbiol.* 42, 881–895. doi: 10.1139/m96-114

Edgar, R. C. (2010). Search and clustering orders of magnitude faster than BLAST. *Bioinformatics* 26, 2460–2461. doi: 10.1093/bioinformatics/btq461

Edgar, R. C., Haas, B. J., Clemente, J. C., Quince, C., and Knight, R. (2011). UCHIME improves sensitivity and speed of chimera detection. *Bioinformatics* 27, 2194–2200. doi: 10.1093/bioinformatics/btr381

Fankem, H., Nwaga, D., Deubel, A., Dieng, L., Merbach, W., and Etoa, F. X. (2006). Occurrence and functioning of phosphate solubilizing microorganisms from oil palm tree (*Elaeis guineensis*) rhizosphere in Cameroon. *Afr. J. Biotech.* 5, 2450–2460.

Fleming, C., Sharma, S. H., Selby, C. H., Rao, J. R., and Trevor, M. (2014). Plant biostimulantsl: a review on the processing of macroalgae and use of extracts for crop management to reduce abiotic and biotic stresses. *J. Appl. Phycol.* 26, 465–490. doi: 10.1007/s10811-013-0101-9

Gillmann, G. P., Burkett, D. C., and Coventry, R. J. (2002). Amending highly weathered soils with finely ground basalt rock. *Appl. Geochem.* 17, 987–1001. doi: 10.1016/S0883-2927(02)00078-1

Giovannetti, M., and Mosse, B. (1980). An evaluation of techniques for measuring vesicular mycorrhizal colonization in roots. *New Phytol.* 84, 489–500. doi: 10.1111/j.1469-8137.1980.tb04556.x

Goldstein, A. H. (1996). "Involvement of the quinoprotein glucose dehydrogenase in the solubilisation of exogenous phosphates by Gram-negative bacteria,"

in *Phosphate in Microorganisms: Cellular and Molecular Biology,* eds A. Torriani-Gorini, E. Yagi, and S. Silver (Washington, DC: ASM Press), 197–203.

He, Z. L., and Zhu. J. (1988). Microbial utilization and transformation of phosphate adsorbed by variable charged minerals. *Soil Biol. Biochem.* 30, 917–923. doi: 10.1016/S0038-0717(97)00188-0

Hinsinger, P., Barros, O. N. F., Benedetti, M. F., Noack, Y., and Callot, G. (2001). Plant-induced weathering of a basaltic rock: experimental evidence. *Geochim. Cosmochim. Acta* 65, 137–152. doi: 10.1016/S0016-7037(00)00524-X

Hinsinger, P., Bolland, M. D. A., and Gilkes, R. J. (1996). Silicate rock powder: effect on selected chemical properties of a range of soils from Western Australia and on plant growth as assessed in a glasshouse experiment. *Fert. Res.* 45, 69–79. doi: 10.1007/BF00749883

Hui-Lian, X. (2001). Effects of a microbial inoculant and organic fertilizers on the growth, photosynthesis and yield of sweet corn. *J. Crop. Prod.* 3, 183–214. doi: 10.1300/J144v03n01_16

Khalid, A., Arshad, M., Zahir, Z. A., and Khaliq, A. (1997). potential of plant growth promoting rhizobacteria for enhancing wheat yield. *J. Anim. Plant Sci.* 7, 53–56.

Kucey, R. M. (1987). Increased phosphorus uptake by wheat and field beans inoculated with a phosphorus-solubilizing *Penicillium bilaji* Strain and with vesicular-arbuscular mycorrhizal fungi. *Appl. Environ. Microbiol.* 53, 2699–2703.

Lane, D. J. (1991). "Nucleic acid techniques in bacterial systematics," in *16S/23S rRNA Sequencing*, eds E. Stackebrandt and M. Goodfellow (New York, NY: Wiley), 115–175.

Lane, D. J., Pace, B., Olsen, G. J., Stahl, D. A., Sogin, M. L., and Pace, N. R. (1985). Rapid determination of 16S ribosomal RNA sequences for phylogenetic analyses. *Proc. Natl. Acad. Sci. U.S.A.* 82, 6955–6959. doi: 10.1073/pnas.82.20.6955

Loganathan, P., Hedley, M. J., Bolan, N. S., and Currie, L. D. (2002). Field evaluation of the liming value of two phosphate rocks and their partially acidulated products after 16 years of annual application to grazed pasture. *Nutr. Cycle Agroecosys.* 72, 287–297. doi: 10.1007/s10705-005-4277-5

Mehana, T. A., and Wahid, O. A. A. (2002). Associative effect of phosphate dissolving fungi, *Rhizobium* and phosphate fertilizer on some soil properties, yield components and the phosphorus and nitrogen concentration and uptake by *Vicia faba* L. under field conditions. *Pak. J. Biol. Sci.* 5, 1226–1231. doi: 10.3923/pjbs.2002.1226.1231

Metho, L. A., and Hammes, P. S. (2000). The harvest index of individual ears of four South African wheat (*Triticum aestivum* L.) cultivars. *South Afr. J. Plant Soil* 17, 144–146. doi: 10.1080/02571862.2000.10634887

Mickan, B., Hart, M. M., Solaiman, Z. M., Jenkins, S. N., Siddique, K. H. M., and Abbott, L. K. (2017). Molecular divergence of fungal communities in soil, roots and hyphae highlight the importance of sampling strategies. *Rhizosphere* 4, 104–111. doi: 10.1016/j.rhisph.2017.09.003

Nahas, E. (1996). Factors determining rock phosphate solubilization by microorganism isolated from soil. *World J. Microb. Biotechnol.* 12, 18–23. doi: 10.1007/BF00327716

Newman, E. I. (1966). A method of estimating the total length of a root in a smaple. *J. Appl. Ecol.* 3, 139–145. doi: 10.2307/2401670

Oksanen, J., Blanchet, F. G., Kindt, R., Legendre, P., Minchin, P. R., O'Hara, R. B., et al. (2013). *Vegan: Community Ecology Package.* Available at: https://cran.r-project.org/web/packages/vegan/index.html

Pairunan, A. K., Robson, A. D., and Abbott, L. K. (1980). The effectiveness of vesicular-arbuscular mycorrhizas in increasing growth and phosphorus uptake of subterranean clover from phosphorus sources of different solubilities. *New Phytol.* 84, 327–338. doi: 10.1111/j.1469-8137.1980.tb04433.x

Priyono, J., and Gilkes, R. (2004). Dissolution of milled-silicate rock fertilisers in the soil. *Aus. J. Soil Res.* 42, 441–448. doi: 10.1071/SR03138

Rayment, G. E., Lyons, D. J. (2010). *Soil Chemical Methods*. Melbourne, VIC: Australasia CSIRO Publishing

Richardson, A. E. (1994). *Soil Microorganisms and Phosphorus Availability in Soil Biota: Management in Sustainable Farming Systems,* eds C. E. Pankhurst. Melbourne, VIC: CSIRO, 50–62.

Richardson, A. E. (2001). Prospects for using soil microorganisms to improve the acquisition of phosphorus by plants. *Aus. J. Plant. Physiol.* 28, 897–906.

Rodriguez, H., and Fraga, R. (1999). Phosphate solubilizing bacteria and their role in plant growth promotion. *Biotechnol. Adv.* 17, 319–339. doi: 10.1016/S0734-9750(99)00014-2

Rodriguez, H., Gonzalez, T., and Bashan, Y. (2006). Genetics of phosphate solubilization and its potential applications for improving plant growth – Promoting bacteria. *Plant Soil* 287, 15–21. doi: 10.1007/s11104-006-9056-9

Roger, J. R., and Bennett, P. C. (2004). Mineral stimulation of subsurface microorganisms: release of limiting nutrients from silicates. *Chem. Geol.* 203, 91–108. doi: 10.1016/j.chemgeo.2003.09.001

Saa, S., Rio, A. O., Castro, S., and Brown, P. H. (2015). Foliar application of microbial and plant based biostimulants increases growth and potassium uptake in almond (*Prunus dulcis* [Mill.] D.A,Webb). *Front. Plant. Sci.* 87:6. doi: 10.3389/fpls.2015.00087

Sanz, S. J. I., and Rowell, D. L. (1988). The use of feldspars as potassium fertilizers in the savannah of Colombia. *Fert. Res.* 17, 71–83. doi: 10.1007/BF01050458

Schmitz, O. J., Buchkowski, R. W., Burghardt, K. T., and Donihue, C. M. (2015). Functional traits and trait-mediated interactions: connecting community-level interactions with ecosystem functioning. *Adv. Ecol. Res.* 52, 319–343. doi: 10.1016/bs.aecr.2015.01.003

Simmons, W. J. (1975). Background absorption error in determination of copper in plants by flame atomic absorption spectrometry. *Anal. Chem.* 50, 870–873. doi: 10.1021/ac50029a014

Simmons, W. J. (1978). Determination of low concentrations of cobalt in small samples of plant material by flameless atomic absorption spectrophotometry. *Anal. Chem.* 47, 2015–2018. doi: 10.1021/ac60362a013

Smith, S. E., and Smith, F. A. (2012). Fresh perspectives on the roles of arbuscular mycorrhizal fungi in plant nutrition and growth. *Mycologia* 104, 1–13. doi: 10.3852/11-229

Smith, S. E., and Read, D. J. (eds). (2008) *Mycorrhizal Symbiosis*, 3rd Edn. Cambridge: Academic press.

Surange, S., Wollum, A. G., Kumar, N., and Nautiyal, C. S. (1995). Characterization of *Rhizobium* from root nodules of leguminous trees growing in alkaline soils. *Can. J. Microbiol.* 43, 891–894. doi: 10.1139/m97-130

Zaidi, A., Khan, M. S., and Aamil, M. (2004). Bioassociative effect of rhizospheric microorganisms on growth, yield, and nutrient uptake of greengram. *J. Plant Nutr.* 27, 601–612. doi: 10.1081/PLN-120030370

Zhang, J., Kobert, K., Flouri, T., and Stamatakis, A. (2014). PEAR: a fast and accurate illumina paired-end reAd mergeR. *Bioinformatics* 30, 614–620. doi: 10.1093/bioinformatics/btt593

Conflict of Interest Statement: The authors declare that the research was conducted in the absence of any commercial or financial relationships that could be construed as a potential conflict of interest.

frontiers in Plant Science

MINI REVIEW
published: 13 November 2018
doi: 10.3389/fpls.2018.01655

Synergistic Biostimulatory Action: Designing the Next Generation of Plant Biostimulants for Sustainable Agriculture

Youssef Rouphael[1]* *and Giuseppe Colla*[2]

[1] Department of Agricultural Sciences, University of Naples Federico II, Portici, Italy, [2] Department of Agriculture and Forest Sciences, University of Tuscia, Viterbo, Italy

Over the past 10 years, interest in plant biostimulants (PBs) has been on the rise compelled by the growing interest of scientists, extension specialists, private industry, and growers in integrating these products in the array of environmentally friendly tools that secure improved crop performance and yield stability. Based on the new EU regulation PBs are defined through claimed agronomic effects, such as improvement of nutrient use efficiency, tolerance to abiotic stressors and crop quality. This definition entails diverse organic and inorganic substances and/or microorganisms such as humic acids, protein hydrolysates, seaweed extracts, mycorrhizal fungi, and N-fixing bacteria. The current mini-review provides an overview of the direct (stimulatory on C and N metabolism) and indirect (enhancing nutrient uptake and modulating root morphology) mechanisms by which microbial and non-microbial PBs improve nutrient efficiency, plant performance, and physiological status, resilience to environmental stressors and stimulate plant microbiomes. The scientific advances underlying synergistic and additive effects of microbial and non-microbial PBs are compiled and discussed for the first time. The review identifies several perspectives for future research between the scientific community and private industry to design and develop a second generation of PBs products (biostimulant 2.0) with specific biostimulatory action to render agriculture more sustainable and resilient.

Keywords: biostimulant 2.0, humic acids, microbial inoculants, microbiome, protein hydrolysate, physiological mechanisms, seaweed extracts, synergistic properties

OPEN ACCESS

Edited by:
Paul Christiaan Struik, Wageningen University & Research, Netherlands

Reviewed by:
Giovanni Povero, Valagro SpA, Italy
Aad Termorshuizen, Aad Termorshuizen Consultancy, Netherlands

***Correspondence:**
Youssef Rouphael
youssef.rouphael@unina.it; joerouphael@yahoo.com

Specialty section:
This article was submitted to Crop and Product Physiology, a section of the journal Frontiers in Plant Science

Received: *24 September 2018*
Accepted: *25 October 2018*
Published: *13 November 2018*

Citation:
Rouphael Y and Colla G (2018) Synergistic Biostimulatory Action: Designing the Next Generation of Plant Biostimulants for Sustainable Agriculture. Front. Plant Sci. 9:1655. doi: 10.3389/fpls.2018.01655

DEFINING PLANT BIOSTIMULANTS: ACTION, COMPOSITION, REGULATIONS

According to the United Nations estimates, the global population will expand from approximately 7.5 billion presently to more than 9.7 billion by 2050, compelling modern agriculture to become increasingly more efficient by producing more food in an eco-friendly and sustainable way. An innovative technology addressing these important challenges involves the development of novel plant biostimulants (PBs) and effective methods for their application (Calvo et al., 2014; Bulgari et al., 2015; Yakhin et al., 2017). The global biostimulant market is estimated today at

about $ 2.0 billion, expected to reach $ 3.0 billion by 2021 at an annual growth rate of 10–12% (Dunhamtrimmer.com, 2018). Based on the Marketsandmarkets.com (2017) database, Europe is the largest PBs market accounting for 34% of the worldwide market share, followed by the North American and Asian-Pacific biostimulant markets which account roughly for 23 and 22%, respectively, of the global market. The main factors driving the rapid growth of the biostimulants market have been associated with: (i) the growing availability of novel biostimulant products addressing specific agronomic needs; (ii) the need to promote a more efficient and effective use of synthetic chemicals and mineral fertilizers; (iii) the increasing frequency of adverse environmental conditions for crop growth and productivity.

Plant biostimulants are natural compounds that trigger physiological and molecular processes modulating crop yield and quality, though their primary function is neither to supply nutrients (fertilizers) nor to protect plants against soilborne or foliar pests and pathogens (Plant Protection Products) (du Jardin, 2015). Therefore, PBs were initially defined by *what they are not*. The term "biostimulant" was first proposed by Zhang and Schmidt (1997) to denote "*materials that, in minute quantities, promote plant growth*." The PBs referred to in the former article were humic substances and macro-algal extracts whose stimulatory effect on plants was essentially suggesting a hormonal mode of action.

The term was subsequently adopted by many researchers, regulators, and extension specialists to denote "Substance(s) and/or micro-organisms whose function is to stimulate natural processes that enhance nutrient uptake, nutrient use efficiency, tolerance to abiotic stress, and crop quality" [European Biostimulant Industry Council (EBIC)][1]. From a regulatory point of view, there is no agreement globally over the definition of PBs and many EU and non-EU countries lack a specific legal framework (Yakhin et al., 2017; Caradonia et al., 2018; Rouphael et al., 2018). Recently the EU decided to reshape the existing Fertilizers Regulations to promote internal market operations for fertilizing products and also to establish a common legal framework for PBs, currently fragmented across Member States (Caradonia et al., 2018; Rouphael et al., 2018). Under the new regulation, "plant biostimulants will be CE marked as fertilizing products stimulating plant nutrition processes independently of the products' nutrient content with the sole aim of improving one or more of the following characteristics of the plant **and the plant rhizosphere or phyllosphere**: nutrient use efficiency (NUE), tolerance to abiotic stress, crop quality, **availability of confined nutrients in the soil and rhizosphere, humification and degradation of organic compounds in the soil**" (In **bold**: amendments adopted by the European Parliament on October 24, 2017, still to be discussed with the European Council and the Commission; European Commission, 2016). PBs are thus to be defined on the basis of claimed effects, in other words "by the plant response they elicit rather than by their makeup," since the category entails diverse organic and inorganic substances and/or microorganisms such as humic acids, protein hydrolysates, seaweed extracts, mycorrhizal fungi, and N-fixing bacteria (du Jardin, 2015; Rouphael et al., 2018).

Significant advancements have been made in studying and unraveling the mode of action(s) of PBs, thanks to the omics science, in particular genomic and transcriptomic tools, as well as through high-throughput phenotyping technologies (Calvo et al., 2014; Lucini et al., 2015; Povero et al., 2016; Bulgari et al., 2017; Rouphael et al., 2018). In addition, over the past 10 years, private industries have been investing in increasing the effectiveness of their formulations through blends of microbial and non-microbial PBs. However, the approach used for the development of these mixtures is mainly empirical without solid scientific evidence of interactive effects (i.e., antagonism, additive, or synergistic) between their microbial and non-microbial components. This approach rests on the premise that the more biostimulants are combined in a mixture the better that will work; however, *more isn't always better*. The identification of synergistic properties among PBs based on reasonable scientific hypotheses and sound experimental approaches (testing PBs alone and in combination), rather than a *try-and-see approach*, can be pivotal for developing novel and target-specific biostimulant products able to increase NUE and improve plant resilience to environmental stressors.

Taking this background into consideration, limited published data is available concerning the interaction between microbial and non-microbial PBs. The current mini-review article examines the mode of actions/mechanisms by which microbial and non-microbial PBs affect nutrient uptake efficiency, plant performance, and tolerance for abiotic stressors. Subsequently, the scientific advances addressing the synergistic and additive effects among microbial and non-microbial PBs are reviewed and discussed. Finally, the current mini-review identifies the challenges ahead and the future direction of research to develop and exploit a second generation of effective biostimulants rendering agriculture more sustainable and resilient.

MECHANISMS IMPLICATED IN PLANT BIOSTIMULATORY EFFECTS ON CROP PHYSIOLOGY AND AGRONOMIC PERFORMANCE

Organic Non-microbial Plant Biostimulants

Based on the latest draft of the European Commission (2016), organic non-microbial PBs include natural substances such as humic acids (HA), protein hydrolysates (PH), and seaweed extracts (SWE), with the first two categories commanding half of the market share, whereas the SWE segment amounts to 37% of the total market.

Humic substances such as humic and fulvic acids are natural organic molecules originating from the biological and chemical transformations of dead organic matter (Nardi et al., 2007; Canellas et al., 2015). Humic substances are generally applied as soil drench and in some cases (fulvic acids) through foliar application (Halpern et al., 2015). Humic substances have been

[1] www.biostimulants.eu

perceived for long as primordial components of soil fertility and structure, acting on chemical, physical as well as biological properties of soils (du Jardin, 2015). The biostimulation action of HAs on soil nutrient availability and uptake has been attributed to several mechanisms affecting soil processes and plant physiology including: (i) improving soil structure, (ii) increasing cation exchange capacity and neutralizing soil pH, (iii) improving solubility of phosphorus by interfering with Ca-phosphate precipitation and also by increasing the availability of micronutrients by preventing leaching, (iv) improving lateral root induction and hair growth due to the auxin-like activity, which triggers plasma membrane H^+-ATPase activity, and (v) stimulating nitrate assimilation through the upregulation of the target enzymes (NR, GDH, and GER) involved in this process (Pinton et al., 1999; Delgado et al., 2002; García-Mina et al., 2004; Schmidt et al., 2007; Halpern et al., 2015; Zandonadi et al., 2016; De Pascale et al., 2017). The biostimulatory action of HAs is highly influenced by soil fertility conditions, HAs being more effective under soil conditions of poor fertility and low organic matter content (du Jardin, 2015). Variability in the effects of HAs is also due to the source of humic substances, with higher plant performance obtained in response to HAs extracted from humidified organic matter (e.g., peat), composts and vermicomposts rather than those coming from fossil humus (du Jardin, 2015).

In addition to the indirect and direct effects of HAs on plant metabolism and physiology, several studies demonstrated their biostimulatory activity in terms of stress protection particularly against salinity and drought (Türkmen et al., 2004; Paksoy et al., 2010; Aydin et al., 2012; García et al., 2012; Petrozza et al., 2014). Presumed mechanisms involved in salt and drought tolerance are: (i) reducing hydrogen peroxide and lipid peroxidation, (ii) increasing proline content, (iii) differential regulation of gene expression, and (iv) improving root growth as well as the chemical, microbiological and physical properties of soil (Calvo et al., 2014; Battacharyya et al., 2015).

According to Colla et al. (2015a, 2017b) animal- as well as plant-based PH represent an important category of organic non-microbial PBs, having as main components a mixture of free amino acids, oligo- and polypeptides which act as *signaling molecules*. PHs are mainly applied as foliar spray and to a lesser extent as a substrate drench and as seed treatment (Colla et al., 2015a). In several greenhouse and open-field studies, PHs demonstrated an important role as PBs by triggering physiological and molecular processes that stimulate growth and productivity thus mitigating the impact of several abiotic stressors on crops (Colla et al., 2017b). Direct effects behind the biostimulation activity and abiotic stress tolerance of PHs include: (i) triggering of key enzymes involved in N assimilation (NR, NiR, GS, and GOCAT) and C metabolism (citrate synthase, malate, and isocitrate dehydrogenase), (ii) heightened auxin- and gibberellin-like activities, and (iii) increase in antioxidant enzyme activity, pigment biosynthesis, and production of secondary metabolites (Schiavon et al., 2008; Ertani et al., 2009, 2013, 2017; Rouphael et al., 2017a, 2018; Sestili et al., 2018). In addition to the direct effect of PHs, indirect effects on crop performance and nutritional status have been also demonstrated when PHs were applied as foliar spray or as substrate drench (Colla et al., 2017b). In fact, the application of PHs has enhanced nutrient uptake by increasing the effective volume of soil exploited by the root system, through their effects on root system architecture, in particular the increase in root hair diameter, density and length (Colla et al., 2014, Colla et al., 2017b). Moreover, in a recent review Colla et al. (2017b) reported that PHs can also affect plant microbiomes residing in both rhizosphere and phyllosphere, thus improving plant performance by altering development and physiological processes, resulting in higher water and nutrient uptake as well as enhanced resilience against major environmental threats.

Seaweeds are brown, green, and red macroalgae, available on the biostimulant market as powder, granular form and as liquid extracts and may be applied as foliar sprays or side-dressed near the root. The major components of commercial SWE are polysaccharides, followed by phenolics, vitamins precursors, osmolytes (mannitol), phytohormones, and hormone-like compounds (Battacharyya et al., 2015). Brown macroalgae, with *Ascophyllum*, *Ecklonia*, *Fucus*, *Laminaria*, and *Sargassum* as main genera, are widely used in crops as PBs for their plant-growth promoting benefits, abiotic stress resistance, and improved postharvest quality and shelf-life (Vernieri et al., 2006; Khan et al., 2009; Craigie, 2011; Rouphael et al., 2017b). The beneficial effects of SWE may be attributed to several growth enhancing mechanisms like (i) physiological (delayed senescence) and biochemical changes (increased micronutrients), (ii) improved WUE (improved stomatal conductance and root-to-shoot ratio), (iii) differential regulation of genes (*CBF3*, *SOS*, *RD22*), and (iv) rhizosphere effects (increased activity of rhizobacteria and mycorrhizae) (Battacharyya et al., 2015). Although significant advancements have been shedding light on the modes of action/mechanisms of the organic non-microbial PBs, additional research is needed to optimize the use of PBs including the standardization of their raw materials, characteristics, extraction methods as well as identifying the optimal application time, dose and mode for each species and set of environmental conditions.

Microbial Plant Biostimulants

The use of microbial-based biostimulants such as plant growth promoting rhizobacteria (PGPR) of strains belonging to the genera *Azospirillum*, *Azotobacter*, and *Rhizobium* spp. as well as mycorrhizal fungi are highly considered as promising means not only to secure yield stability under low-input conditions (i.e., N and/or P deficiency) but also as a tool to solving some environmental constraints (Rouphael et al., 2015; Ruzzi and Aroca, 2015). In fact, several studies (Lace et al., 2015; Ruzzi and Aroca, 2015; Fiorentino et al., 2018) demonstrated that PGPR and endophytic fungi including mycorrhizal fungi can modulate quantitatively and qualitatively the rhizosphere microbial population with positive impact on the soil ecosystem.

The phytostimulation effect of PGPR and mycorrhizal fungi under both optimal and suboptimal conditions could be attributed to several direct and indirect mechanisms including: (i) improved uptake and translocation of nutrients including N and P and micronutrients (Fe, Zn, and Mn), (ii) more vigorous

root system apparatus (higher root biomass, surface area, and number of lateral roots) especially in crops having a taproot system (e.g., carrot) or a shallow root apparatus (e.g., onion), (iii) improved water relations and photosynthetic capacity, (iv) stronger antioxidative defense system, (v) regulation of plant hormones (auxins, ABA, cytokinins, ethylene, and gibberellins), (vi) promotion of nutrient transporters (NRT1.1, NRT2, NAR2.2, AMT, Pht1, and PT2-1) activity, and (vii) production of enzymes (phosphatases) and/or excretion of low- (amino acids, sugars, organic acids, and phenolics) and high-molecular weight organic compounds (mucilage and proteins) in the rhizosphere (Hayat et al., 2010; Candido et al., 2013, 2015; Colla et al., 2015a,b; Rouphael et al., 2015; Saia et al., 2015; De Pascale et al., 2017; Bitterlich et al., 2018).

EXPLOITING SYNERGISTIC INTERACTIONS AMONG PLANT BIOSTIMULANTS: MOVING TOWARD THE NEXT GENERATION OF BIOSTIMULANTS

The agricultural sector's pursuit of decreased reliance on organic and inorganic fertilizers by improving NUE and mitigating the negative impact of environmental stress factors and soil degradation (biological, chemical, and physical) on crop growth and productivity. Biostimulants have the capacity to improve NUE and reduce abiotic stress on crops, and these are claims supporting their placing on the market. In fact, Colla et al. (2017a) demonstrated that under the same fertilization program (rates and time of application) the application of PBs improved the NUE and thus the yield of greenhouse fresh tomato by 6.6–11.0%. Similarly, an endophytic fungal consortium inoculum boosted the marketable yield of open-field zucchini squash and lettuce by 14 and 70%, respectively, compared to non-inoculated plants under the same fertilization regime (Colla et al., 2015b). Thus, application of microbial and non-microbial PBs could be considered an efficient approach to boost yield without raising the rate of applied nutrients (i.e., higher NUE). Crops are also faced with multiple/combined abiotic constraints, particularly drought, salinity, and heat. These are the ones forecasted to escalate most according to climate change models and challenge yield stability (Mittler, 2006; Suzuki et al., 2014). Thus, research on the potential synergistic effects among PBs should be at the core of future efforts in addressing global food security, complemented by sustainable and optimized use of nutrients.

In terms of efficacy, there are three types of interactions implicating microbial and/or non-microbial PBs: they can be antagonistic, additive or synergistic based on their effective action. In antagonistic interactions the overall effect of the PBs applied is less than the additive effect of the PBs applied independently. This type of interaction is normally associated to the antagonistic non-target action of several *Trichoderma* spp. through mycoparasitism on the mycorrhizal fungi mycelium (McAllister et al., 1994; Martinez et al., 2004; De Jaeger et al., 2010). For instance, De Jaeger et al. (2011) reported a sharp decrease in P uptake in mycorrhized plants because of the disruption of the hyphae continuity of *Rhizophagus intraradices* by *T. harzianum*. In the case of additive interaction, the applied PBs have a similar type of effect on the plants, hence their combined effect equals the sum of their independent effects. Finally, synergistic interaction is observed when the combined effect of the applied PBs exceeds their additive effects when applied independently under the same conditions.

In recent years, limited experimental studies testing the additive and/or synergistic effects of various PBs categories, have demonstrated that combinations of non-microbial PBs or microbial inoculants with HA, SWE, or PH give more reproducible benefits to plant growth and production (Borges Baldotto et al., 2010; Bettoni et al., 2014; Nikbakht et al., 2014;

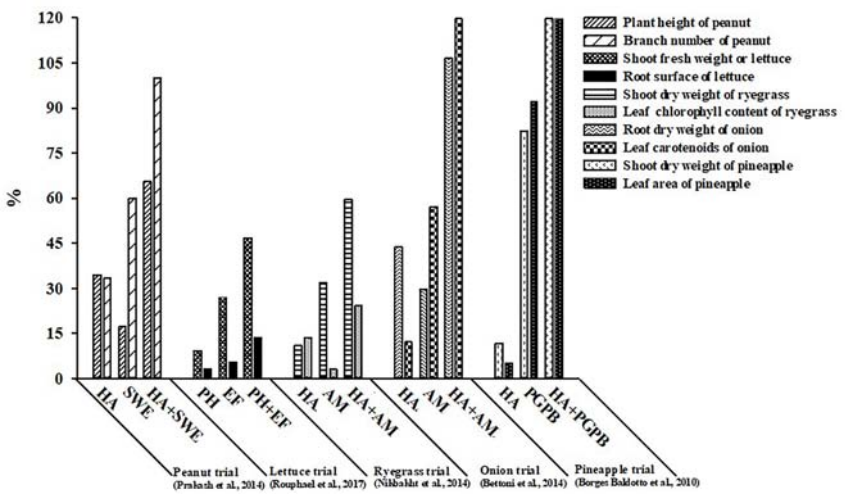


FIGURE 1 | The relative effect of the various categories of non-microbial and microbial plant biostimulants, separately or in synergistic combination, on morphological and biochemical traits of open-field and protected cultivation crops (peanut, lettuce, perennial ryegrass, onion, and pineapple).

Prakash et al., 2014; Rouphael et al., 2017a). For instance, groundnut sprayed at biweekly intervals with HA or SWE increased plant height and branching by 34.5 and 33% (for HA) and 17.2 and 60.0% (for SWE), respectively, in comparison to the untreated control treatment, whereas the applications of both PBs together (SWE:HA) exhibited a synergistic interaction with higher increase (65.0 and 100%, respectively) compared to the sum of the independent biostimulant effects (**Figure 1**; Prakash et al., 2014). In the same study, improvement in plant growth was associated to the stimulation of N uptake and chlorophyll biosynthesis which may have improved the photosynthetic activity, triggering the translocation of photosynthates to the sinks. The application of HA 1 day after transferring seedlings or the application of liquid mycorrhizal inoculum (*R. intraradices*) to the roots 2 days after transplanting resulted in a significant increase in onion root dry weight and leaf carotenoids by 43.9 and 12.1% (for HA) and by 29.6 and 57.1% (for mycorrhizal fungi), respectively, whereas the application of both HA and mycorrhizal fungi induced a synergistic effect with the highest accumulation of the two parameters measured (106.7 and 123.6%) (**Figure 1**; Bettoni et al., 2014). The presumed mode of action involved in the stimulation of crop performance was linked to enhanced nutrient availability driven by the synergistic action of HA and mycorrhizal fungi applied in combination. Similarly, in perennial ryegrass the combination of pre-sowing the substrate with mycorrhizal fungi (*R. intraradices*) and HA spray applications at 30-day intervals was more effective in enhancing root biomass and chlorophyll biosynthesis than either application alone (**Figure 1**; Nikbakht et al., 2014). Dipping the roots of micropropagated pineapple plantlets for 24 h before planting in a suspension of vermicompost derived-HA followed by application of HA to the basal leaf axils of plants at 14-day intervals and/or dipping of the roots before planting in a PGPB cell suspension for 30 min, increased the shoot dry weight and the leaf area of pineapple during the vitro acclimatization stage when applied separately but more so when applied in synergistic combination (**Figure 1**). Finally, Rouphael et al. (2017a) reported that the combination of an endophytic fungal consortium (*R. irregulare* BEG72 and *T. atroviride* MUCL45632) with weekly substrate drench applications of a plant-derived PH was more effective than microbial or non-microbial biostimulant applications alone in improving crop productivity (**Figure 1**). The beneficial effects of the combined biostimulants were associated with increased chlorophyll biosynthesis, the capability of maintaining higher photochemical activity in PSII, and also with a superior nutritional status of the leaf tissues.

CONCLUSION AND CHALLENGES AHEAD

The use of PBs in agriculture has greatly increased in the last 10 years, mostly due to their *multifaceted properties*. Significant advancements have been made in studying the physiological and biochemical mechanisms of PBs owing to the "omics" sciences and recently to the high-throughput phenotyping technologies. Nonetheless, additional research is required for confronting a number of open questions, such as: (1) which molecular mechanisms underlie the observed biostimulatory action? (2) what is the optimal method, time, rate of application and phenological stage for improving plant performance and resilience to stress and to what extend the plant species/cultivar, environment and management practices applied may affect these effects? (3) how effectively can the PBs modulate the microbial population quantitatively and qualitatively when applied as foliar spray, substrate drench or seed treatment? (4) how long do the PBs effects persist subsequently to their foliar application and how do factors such as leaf cuticle morphology and stomatal aperture interact with the different components of PBs and the target species in impacting leaf permeability and thus the efficacy of the product? and (5) what are the physiological and molecular mechanisms behind the synergistic properties among PBs and how can they be accounted for in developing novel and specific biostimulant products? While presently there seem to be more questions than answers, the findings of the few researchers that have attempted to unravel the complex biostimulation action behind PBs, particularly the synergistic properties, suggest that additional investment in research interaction between the scientific community and the private industry is required in order to develop a second generation of PBs products (biostimulant 2.0) with specific biostimulation action.

AUTHOR CONTRIBUTIONS

YR and GC had the original idea of Synergistic Biostimulatory Action and were both involved in writing the article.

REFERENCES

Aydin, A., Kant, C., and Turan, M. (2012). Humic acid application alleviate salinity stress of bean (*Phaseolus vulgaris* L.) plants decreasing membrane leakage. *Afr. J. Agric. Res.* 7, 1073–1086. doi: 10.5897/ajar10.274

Battacharyya, D., Babgohari, M. Z., Rathor, P., and Prithiviraj, B. (2015). Seaweed extracts as biostimulants in horticulture. *Sci. Hortic.* 196, 39–48. doi: 10.1016/j.scienta.2015.09.012

Bettoni, M. M., Mogor, A. F., Pauletti, V., and Goicoechea, N. (2014). Growth and metabolism of onion seedlings as affected by the application of humic substances, mycorrhizal inoculation and elevated CO2. *Sci. Hortic.* 180, 227–235. doi: 10.1016/j.scienta.2014.10.037

Bitterlich, M., Rouphael, Y., Graefe, J., and Franken, P. (2018). Arbuscular mycorrhizas: a promising component of plant production systems provided favorable conditions for their growth. *Front. Plant Sci.* 9:1329. doi: 10.3389/fpls.2018.01329

Borges Baldotto, L. E., Altoé Baldotto, M., Canellas, L. P., Smith, R. B., and Olivares, F. L. (2010). Growth promotion of pineapple "Vitória" by humic acids and *Burkholderia* spp. During acclimatization. *Rev. Bras. Ciênc. Solo* 34, 1593–1600. doi: 10.1590/s0100-06832010000500012

Bulgari, R., Cocetta, G., Trivellini, A., Vernieri, P., and Ferrante, A. (2015). Biostimulants and crop responses: a review. *Biol. Agric. Hortic.* 31, 1–17. doi: 10.1080/01448765.2014.964649

Bulgari, R., Morgutti, S., Cocetta, G., Negrini, N., Farris, S., Calcante, A., et al. (2017). Evaluation of borage extracts as potential biostimulant using a

phenomic, agronomic, physiological, and biochemical approach. *Front. Plant Sci.* 8:935. doi: 10.3389/fpls.2017.00935

Calvo, P., Nelson, L., and Kloepper, J. W. (2014). Agricultural uses of plant biostimulants. *Plant Soil* 383, 3–41. doi: 10.1007/s11104-014-2131-8

Candido, V., Campanelli, G., D'Addabbo, T., Castronuovo, D., Renco, M., and Camele, I. (2013). Growth and yield promoting effect of artificial mycorrhization combined with different fertiliser rates on field-grown tomato. *Ital. J. Agron.* 8, 168–174. doi: 10.4081/ija.2013.e22

Candido, V., Campanelli, G. D., Addabbo, T., Castronuovo, D., Perniola, M., and Camele, I. (2015). Growth and yield promoting effect of artificial mycorrhization on field tomato at different irrigation regimes. *Sci. Hortic.* 187, 35–43. doi: 10.1016/j.scienta.2015.02.033

Canellas, L. P., Olivares, F. L., Aguiar, N. O., Jones, D. L., Nebbioso, A., Mazzei, P., et al. (2015). Humic and fulvic acids as biostimulants in horticulture. *Sci. Hortic.* 196, 15–27. doi: 10.1016/j.scienta.2015.09.013

Caradonia, F., Battaglia, V., Righi, L., Pascali, G., and La Torre, A. (2018). Plant biostimulant regulatory framework: prospects in europe and current situation at international level. *J. Plant Growth Regul.* 1–11. doi: 10.1007/s00344-018-9853-4

Colla, G., Cardarelli, M., Bonini, P., and Rouphael, Y. (2017a). Foliar applications of protein hydrolysate, plant and seaweed extracts increase yield but differentially modulate fruit quality of greenhouse tomato. *HortScience* 52, 1214–1220. doi: 10.21273/hortsci12200-17

Colla, G., Hoagland, L., Ruzzi, M., Cardarelli, M., Bonini, P., Canaguier, R., et al. (2017b). Biostimulant action of protein hydrolysates: unraveling their effects on plant physiology and microbiome. *Front. Plant Sci.* 8:2202. doi: 10.3389/fpls.2017.02202

Colla, G., Nardi, S., Cardarelli, M., Ertani, A., Lucini, L., Canaguier, R., et al. (2015a). Protein hydrolysates as biostimulants in horticulture. *Sci. Hortic.* 196, 28–38. doi: 10.1016/j.scienta.2015.08.037

Colla, G., Rouphael, Y., Di Mattia, E., El-Nakhel, C., and Cardarelli, M. (2015b). Co-inoculation of *Glomus intraradices* and *Trichoderma atroviride* acts as a biostimulant to promote growth, yield and nutrient uptake of vegetable crops. *J. Sci. Food Agric.* 95, 1706–1715. doi: 10.1002/jsfa.6875

Colla, G., Rouphael, Y., Canaguier, R., Svecova, E., and Cardarelli, M. (2014). Biostimulant action of a plant-derived protein hydrolysate produced through enzymatic hydrolysis. *Front. Plant Sci.* 5:448. doi: 10.3389/fpls.2014.00448

Craigie, J. S. (2011). Seaweed extract stimuli in plant science and agricolture. *J. Appl. Phycol.* 23, 371–393. doi: 10.1007/s10811-010-9560-4

De Jaeger, N., de la Providencia, I. E., Rouhier, H., and Declerck, S. (2011). Co-entrapment of *Trichoderma harzianum* and *Glomus* sp. Within alginate beads: impact on the arbuscular mycorrhizal fungi life cycle. *J. Appl. Microbiol.* 111, 125–135. doi: 10.1111/j.1365-2672.2011.05035.x

De Jaeger, N., Declerck, S., and de la Providencia, I. E. (2010). Mycoparasitism of arbuscular mycorrhizal fungi: a pathway for the entry of saprotrophic fungi into roots. *FEMS Microbiol. Ecol.* 73, 312–322. doi: 10.1111/j.1574-6941.2010.00903.x

De Pascale, S., Rouphael, Y., and Colla, G. (2017). Plant biostimulants: innovative tool for enhancing plant nutrition in organic farming. *Eur. J. Hortic. Sci.* 82, 277–285. doi: 10.17660/eJHS.2017/82.6.2

Delgado, A., Madrid, A., Kassem, S., Andreu, L., and del Campillo, M. C. (2002). Phosphorus fertilizer recovery from calcareous soils amended with humic and fulvic acids. *Plant Soil* 245, 277–286. doi: 10.1023/A:1020445710584

du Jardin, P. (2015). Plant biostimulants: definition, concept, main categories and regulation. *Sci. Hortic.* 196, 3–14. doi: 10.1016/j.scienta.2015.09.021

Dunhamtrimmer.com. (2018). Available at: http://dunhamtrimmer.com/products-services/

Ertani, A., Cavani, L., Pizzeghello, D., Brandellero, E., Altissimo, A., Ciavatta, C., et al. (2009). Biostimulant activities of two protein hydrolysates on the growth and nitrogen metabolism in maize seedlings. *J. Plant. Nutr. Soil Sci.* 172, 237–244. doi: 10.1002/jpln.200800174

Ertani, A., Schiavon, M., Muscolo, A., and Nardi, S. (2013). Alfalfa plant-derived biostimulant stimulate short-term growth of salt stressed *Zea mays* L. plants. *Plant Soil* 364, 145–158. doi: 10.1007/s11104-012-1335-z

Ertani, A., Schiavon, M., and Nardi, S. (2017). Transcriptome-wide identification of differentially expressed genes in *Solanum lycopersicon* L. in response to an alfalfa-protein hydrolysate using microarrays. *Front. Plant Sci.* 8:1159. doi: 10.3389/fpls.2017.01159

European Commission (2016). *Proposal for a Regulation Laying Down Rules on the Making Available on the Market of CE Marked Fertilizing Products and Amending Regulations (EC)1069/2009 and (EC)1107/2009.COM(2016)*. Brussels: European Commission, 157.

Fiorentino, N., Ventorino, V., Woo, S. L., Pepe, O., De Rosa, A., Gioia, L., et al. (2018). Trichoderma-based biostimulants modulate rhizosphere microbial populations and improve N uptake efficiency, yield and nutritional quality of leafy vegetables. *Front. Plant Sci.* 9:743. doi: 10.3389/fpls.2018.00743

García, A. C., Berbara, R. L. L., Farías, L. P., Guridi Izquierdo, F., Lázaro Hernández, L., Hernandez Campos, R., et al. (2012). Humic acids of vermicompost as an ecological pathway to increase resistance of rice seedlings to water stress. *Afr. J. Biotechnol.* 11, 3125–3134. doi: 10.5897/ajb11.1960

García-Mina, J. M., Antolin, M. C., and Sanchez-Diaz, M. (2004). Metal–humic complexes and plant micronutrient uptake: a study based on different plant species cultivated in diverse soil types. *Plant Soil* 258, 57–68. doi: 10.1023/b:plso.0000016509.56780.40

Halpern, M., Bar-Tal, A., Ofek, M., Minz, D., Muller, T., and Yermiyahu, U. (2015). The use of biostimulants for enhancing nutrient uptake. *Adv. Agron.* 130, 141–174. doi: 10.1016/bs.agron.2014.10.001

Hayat, R., Ali, S., Amara, U., Khalid, R., and Ahmed, I. (2010). Soil beneficial bacteria and their role in plant growth promotion: a review. *Ann. Microbiol.* 60, 579–598. doi: 10.1007/s13213-010-0117-1

Khan, W., Rayirath, U. P., Subramanian, S., Jithesh, M. N., Rayorath, P., Hodges, D. M., et al. (2009). Seaweed extracts as biostimulants of plant growth and development. *J. Plant Growth Regul.* 28, 386–399. doi: 10.1007/s00344-009-9103-x

Lace, B., Genre, A., Woo, S., Faccio, A., Lorito, M., and Bonfante, P. (2015). Gate crashing arbuscular mycorrhizas: in vivo imaging shows the extensive colonization of both symbionts by *Trichoderma atroviride*. *Environ. Microbiol. Rep.* 7, 64–77. doi: 10.1111/1758-2229.12221

Lucini, L., Rouphael, Y., Cardarelli, M., Canaguier, R., Kumar, P., and Colla, G. (2015). The effect of a plant-derived protein hydrolysate on metabolic profiling and crop performance of lettuce grown under saline conditions. *Sci. Hortic.* 182, 124–133. doi: 10.1016/j.scienta.2014.11.022

Marketsandmarkets.com (2017). *Biostimulants Market by Active Ingredient (Humic Substances, Seaweed, Microbials, Trace Minerals, Vitamins & Amino Acids), Crop Type (Row Crops, Fruits & Vegetables, Turf & Ornamentals), Formulation, Application Method, and Region—Global Forecast to 2022*. Pune: MarketsandMarkets^{TM} INC.

Martinez, A., Obertello, M., Pardo, A., Ocampo, J. A., and Godeas, A. (2004). Interactions between *Trichoderma pseudokoningii* strains and the arbuscular mycorrhizal fungi *Glomus mosseae* and *Gigaspora rosea*. *Mycorrhiza* 14, 79–84. doi: 10.1007/s00572-003-0240-y

McAllister, C. B., Garcıa-Romera, I., Godeas, A., and Ocampo, J. A. (1994). Interactions between *Trichoderma koningii*, *Fusarium solani* and *Glomus mosseae*: effects on plant growth, arbuscular mycorrhizas and the saprophyte inoculants. *Soil Biol. Biochem.* 26, 1363–1367. doi: 10.1016/0038-0717(94)90218-6

Mittler, R. (2006). Abiotic stress, the field environment and stress combination. *Trends Plant Sci.* 11, 15–19. doi: 10.1016/j.tplants.2005.11.002

Nardi, S., Muscolo, A., Vaccaro, S., Baiano, S., Spaccini, R., and Piccolo, A. (2007). Relationship between molecular characteristics of soil humic fractions and glycolytic pathway and krebs cycle in maize seedlings. *Soil Biol. Biochem.* 39, 3138–3146. doi: 10.1016/j.soilbio.2007.07.006

Nikbakht, A., Pessarakli, M., Daneshvar-Hakimi-Maibodi, N., and Kafi, M. (2014). Perennial ryegrass growth responses to mycorrhizal infection and humic acid treatments. *Agron. J.* 106, 585–595. doi: 10.2134/agronj2013.0275

Paksoy, M., Türkmen, Ö, and Dursun, A. (2010). Effects of potassium and humic acid on emergence, growth and nutrient contents of okra (*Abelmoschus esculentus* L.) seedling under saline soil conditions. *Afr. J. Biotechnol.* 9, 5343–5346.

Petrozza, A., Santaniello, A., Summerer, S., Di Tommaso, G., Di Tommaso, D., Paparelli, E., et al. (2014). Physiological responses to Megafol® treatments in tomato plants under drought stress: a phenomic and molecular approach. *Sci. Hortic.* 174, 185–192. doi: 10.1016/j.scienta.2014.05.023

Pinton, R., Cesco, S., Iacolettig, G., Astolfi, S., and Varanini, Z. (1999). Modulation of NO_3- uptake by water-extractable humic substances: involvement of root

plasma membrane H + ATPase. *Plant Soil* 215, 155–161. doi: 10.1023/A:1004752531903

Povero, G., Mejia, J. F., Di Tommaso, D., Piaggesi, A., and Warrior, P. (2016). A systematic approach to discover and characterize natural plant biostimulants. *Front. Plant Sci.* 7:435. doi: 10.3389/fpls.2016.00435

Prakash, P., Mehdi, S., Saikia, S., Narendrakumar, G., Thirugnanasambandam, G., and Stanley Abraham, L. (2014). Production, formulation and application of seaweed liquid fertilizer using humic acid on growth of *Arachis hypogaea*. *Biosci. Biotechnol. Res. Asia* 11, 1515–1519. doi: 10.13005/bbra/1546

Rouphael, Y., Cardarelli, M., Bonini, P., and Colla, G. (2017a). Synergistic action of a microbial-based biostimulant and a plant derived-protein hydrolysate enhances lettuce tolerance to alkalinity and salinity. *Front. Plant Sci.* 8:131. doi: 10.3389/fpls.2017.00131

Rouphael, Y., De Micco, V., Arena, C., Raimondi, G., Colla, G., and De Pascale, S. (2017b). Effect of *Ecklonia maxima* seaweed extract on yield, mineral composition, gas exchange and leaf anatomy of zucchini squash grown under saline conditions. *J. Appl. Phycol.* 29, 459–470. doi: 10.1007/s10811-016-0937-x

Rouphael, Y., Franken, P., Schneider, C., Schwarz, D., Giovannetti, M., Agnolucci, M., et al. (2015). Arbuscular mycorrhizal fungi act as biostimulants in horticultural crops. *Sci. Hortic.* 196, 91–108. doi: 10.1016/j.scienta.2015.09.002

Rouphael, Y., Spíchal, L., Panzarová, K., Casa, R., and Colla, G. (2018). High-throughput plant phenotyping for developing novel biostimulants: from lab to field or from field to lab? *Front. Plant Sci.* 9:1197. doi: 10.3389/fpls.2018.01197

Ruzzi, M., and Aroca, R. (2015). Plant growth-promoting rhizobacteria act as biostimulants in horticulture. *Sci. Hortic.* 196, 124–134. doi: 10.1016/j.scienta.2015.08.042

Saia, S., Rappa, V., Ruisi, P., Abenavoli, M. R., Sunseri, F., Giambalvo, D., et al. (2015). Soil inoculation with symbiotic microorganisms promotes plant growth and nutrient transporter genes expression in durum wheat. *Front. Plant Sci.* 6:815. doi: 10.3389/fpls.2015.00815

Schiavon, M., Ertani, A., and Nardi, S. (2008). Effects of an alfalfa protein hydrolysate on the gene expression and activity of enzymes of TCA cycle and N metabolism in *Zea mays* L. *J. Agric. Food Chem.* 56, 11800–11808. doi: 10.1021/jf802362g

Schmidt, W., Santi, S., Pinton, R., and Varanini, Z. (2007). Water-extractable humic substances alter root development and epidermal cell pattern in *Arabidopsis*. *Plant Soil* 300, 259–267. doi: 10.1007/s11104-007-9411-5

Sestili, F., Rouphael, Y., Cardarelli, M., Pucci, A., Bonini, P., Canaguier, R., et al. (2018). Protein hydrolysate stimulates growth and N uptake in tomato coupled with N-dependent gene expression involved in N assimilation. *Front. Plant Sci.* 9:1233. doi: 10.3389/fpls.2018.01233

Suzuki, N., Rivero, R. M., Shulaev, V., Blumwald, E., and Mittler, R. (2014). Abiotic and biotic stress combinations. *New Phytol.* 203, 32–43. doi: 10.1111/nph.12797

Türkmen, Ö, Dursun, A., Turan, M., and Erdinc, C. (2004). Calcium and humic acid affect seed germination, growth and nutrient content of tomato (*Lycopersicon esculentum* L.) seedlings under saline soil conditions. *Acta Agric. Scand. Sect. B Soil Plant Sci.* 54, 168–174. doi: 10.1080/09064710310022014

Vernieri, P., Borghesi, E., Tognoni, F., Serra, G., Ferrante, A., and Piaggesi, A. (2006). Use of biostimulants for reducing nutrient solution concentration in floating system. *Acta Hortic.* 718, 477–484. doi: 10.17660/ActaHortic.2006.718.55

Yakhin, O. I., Lubyanov, A. A., Yakhin, I. A., and Brown, P. H. (2017). Biostimulants in plant science: a global perspective. *Front. Plant Sci.* 7:2049. doi: 10.3389/fpls.2016.02049

Zandonadi, D. B., Santos, M. P., Caixeta, L. S., Marinho, E. B., Peres, L. E. P., and Façanha, A. R. (2016). Plant proton pumps as markers of biostimulant action. *Sci. Agric.* 73, 24–28. doi: 10.1590/0103-9016-2015-0076

Zhang, X., and Schmidt, R. E. (1997). The impact of growth regulators on alpha-tocopherol status of water-stressed *Poa pratensis* L. *Int. Turfgrass Soc. Res. J.* 8, 1364–1373.

Conflict of Interest Statement: The authors declare that the research was conducted in the absence of any commercial or financial relationships that could be construed as a potential conflict of interest.

frontiers in Plant Science

OPINION
published: 04 December 2018
doi: 10.3389/fpls.2018.01801

Microbial Consortia: Promising Probiotics as Plant Biostimulants for Sustainable Agriculture

Sheridan L. Woo[1,2,3]* *and Olimpia Pepe*[3,4,5]*

[1] Department of Pharmacy, University of Naples Federico II, Naples, Italy, [2] National Research Council, Institute for Sustainable Plant Protection, Portici, Italy, [3] Task Force on Microbiome Studies, University of Naples Federico II, Naples, Italy, [4] Department of Agricultural Sciences, University of Naples Federico II, Portici, Italy, [5] CIRAM-Interdepartmental Center for Environmental Research, University of Naples Federico II, Naples, Italy

Keywords: beneficial microbes, plant growth promotion, *Trichoderma*, *Azotobacter*, improved crop production

OPEN ACCESS

Edited by:
Giuseppe Colla,
Università degli Studi della Tuscia, Italy

Reviewed by:
Andrea Ertani,
Università degli Studi di Padova, Italy
Claudio Altomare,
Italian National Research Council, Italy
Enrique Monte,
University of Salamanca, Spain

***Correspondence:**
Sheridan L. Woo
woo@unina.it
Olimpia Pepe
olipepe@unina.it

Specialty section:
This article was submitted to Crop and Product Physiology, a section of the journal Frontiers in Plant Science

Received: *30 September 2018*
Accepted: *19 November 2018*
Published: *04 December 2018*

Citation:
Woo SL and Pepe O (2018) Microbial Consortia: Promising Probiotics as Plant Biostimulants for Sustainable Agriculture. Front. Plant Sci. 9:1801. doi: 10.3389/fpls.2018.01801

PLANT "BIO"-STIMULANTS

Plant biostimulants are applied to improve crop production and nutritional quality of agrifood products. They are often included in agricultural management practices aimed at reducing chemical inputs, increasing productivity and recovering the natural equilibrium in agro-ecosystems.

The widely accepted definition of plant biostimulants (see EBIC, 2013; du Jardin, 2015) is: substance(s) and/or micro-organisms whose function when applied to plants or the soil rhizosphere stimulates the natural processes to enhance/benefit nutrient uptake and efficiency, tolerance to abiotic stress, and crop quality. Typically, biostimulants do not have a direct action against pests.

Commercial formulations may contain a mix of: humic and fulvic acids, amino acids, seaweeds or plant extracts, natural poly- and oligo-mers, chemical elements (Al, Co, Na, Se, and Si), beneficial fungi or bacteria (du Jardin, 2015; Yakhin et al., 2017). Not all listed components are "biological," which makes the term "bio"-stimulant somewhat ambiguous. The "bio" designation may be attributed to the living organism components, and their natural substances. Instead, the non-organic factors can be considered as positive effectors of the "biological" processes that regulate the plant physiology, metabolism, morphology and interactions within the agroecosystem.

REGULATORY LEGISLATION—PLANT PROTECTION PRODUCTS VS. PLANT FERTILIZERS

The registration of agricultural products in Europe follows two distinct legislation pathways: Plant Protection Products (PPPs) or Fertilizers. PPPs, including microbes and chemicals, as defined in Regulation (EC)[1], protect plants or plant products against harmful organisms, influence the life process of plants (i.e., affect plant growth, but are not nutrients), preserve plant products, destroy undesired plants or their parts. The PPP registration process is cumbersome and often not suitable for plant biostimulants (du Jardin, 2015), for which companies seek permission for their use as Fertilizers (see Regulation (EC)[2], that would also reduce time and expenses required for product registration.

[1]Regulation (EC) No 1107/2009 of the European Parliament and of the Council of 21 October 2009 Concerning the Placing of Plant Protection Products on the Market and Repealing Council Directives 79/117/EEC and 91/414/EEC. Available online at: https://eur-lex.europa.eu/legal-content/EN/TXT/?uri=celex%3A32009R1107 (Latest consolidated version: 02/08/2018)
[2]Regulation (EC) No 2003/2003 of the European Parliament and of the Council of 13 October 2003 Relating to Fertilisers (Text With EEA Relevance). Available online at: https://eur-lex.europa.eu/legal-content/EN/TXT/?qid=1537797652760&uri=CELEX:32003R2003 (Latest consolidated version: 01/07/2017).

To date, regulatory processes designed for plant biostimulants have not been established. Official definitions and the basic principles for new legislation are still being discussed both in the EU and the U.S.A (du Jardin, 2015). To this end, it is important to consider the inclusion in the registration pipeline of Plant Growth Promoting Microbes (PGPM): microbial individuals and consortia, their bioactive compounds, and potential multi-component mixtures—as they are important components of many successful plant biostimulant products.

Numerous microorganisms, such as *Trichoderma* spp., are registered as PPPs and classified as Microbial Biological Control Agents (MBCA; Woo et al., 2014). Although single strains are enlisted as biopesticides, many are also known to have properties that result in plant growth promotion and other beneficial effects (Lorito and Woo, 2015), typically not indicated in the registered product disclaimer. Conversely, there are plant biostimulants such as arbuscular mycorrhizal fungi (AMF; Rouphael et al., 2015), that are also capable of inducing systemic resistance conferring crop protection to disease and pest attack (Cameron et al., 2013). This means that there is an urgent need to create a new registration track for microbes or microbial consortia with multiple plant beneficial functions (e.g., MBCA and PGPM) in order to regulate the use of effective agricultural products that are "all inclusive" (e.g., biostimulant, biofertilizer, biopesticide).

PLANT GROWTH PROMOTING MICROBES (PGPM) IN BENEFICIAL MICROBIAL CONSORTIA

Important examples of positive plant-microbe interactions associated to plant growth promotion include PGP rhizobacteria: non-pathogenic *Pseudomonas* and *Bacillus*, *Azotobacter*, *Serratia*, *Azospirillum* capable of improving nutrient availability in soil, plant nutrient uptake and assimilation, as well as supporting nitrogen cycling (Raaijmakers et al., 2009; Berg et al., 2014; Lugtenberg, 2015).

PGPM of fungal origins are widely applied, but less recognized in the literature. The best documented example is that of the mycorrhizal fungi (AMF, VAM) including *Gigaspora, Funneliformis* or *Rhizophagus* (*Glomus)*, and *Laccaria*, that are root obligate biotrophs able to establish mutualistic symbiosis with >80% of vascular plant species (Pringle et al., 2009; Rouphael et al., 2015). They are involved in carbon exchange, and augment the capacity of the plant to absorb water plus nutrients, thus counteracting negative effects of biotic and abiotic stresses. Another case is the fungus *Trichoderma*. It is an active ingredient in hundreds of agricultural products commercialized worldwide (Woo et al., 2014), it has multiple beneficial effects on plants (Harman et al., 2004), and used extensively in biological and integrated pest management (Lorito and Woo, 2015).

Many recent studies demonstrate the potential as plant biostimulants of microbial consortia, rhizobacteria, and rhizofungi, that function as an agricultural probiotics (de Vries and Wallenstein, 2017; Wallenstein, 2017; Kong et al., 2018). The present work describes an example of two prospective microbes and their qualities as consortium components.

Trichoderma: the Evolving MBCA With Multiple Plant Beneficial Effects

Numerous strains of *Trichoderma* are successful MBCA of various plant pathogens. Initially, the biopesticidal activity was considered as the only benefit, but eventually these MBCA were demonstrated to be effective biofertilizers, biostimulants, bio-enhancers of crop resistance to both biotic and abiotic stresses (Harman et al., 2004; Fontenelle et al., 2011; Lorito and Woo, 2015). In fact, scientific evidence demonstrated that the PGP effect could be the result of a true symbiotic interaction (Harman et al., 2004; Vinale et al., 2008; Shoresh et al., 2010; Studholme et al., 2013; Lorito and Woo, 2015).

In certain conditions, *Trichoderma* may activate a state of alert in the plant (i.e., priming), thus producing a ready response to pathogen attack, which eventually anticipates the establishment of a Systemic Acquired Resistance (SAR) and/or Induced Systemic Resistance (ISR; Rubio et al., 2014; Hossain et al., 2017; Martínez-Medina et al., 2017; Manganiello et al., 2018). Furthermore, results from laboratory and field tests with *Trichoderma*, performed on a variety of crops, have shown a reduction in symptoms caused by abiotic diseases (e.g., water, salt, nutrients) following treatments (Mastouri et al., 2012; Brotman et al., 2013; Sofo et al., 2014; Fiorentino et al., 2018).

Improvement in plant development is typically noted with increased seed germination, above- and below-ground plant parts, chlorophyll content and yield, size and/or number of flowers and/or fruits (Harman et al., 2004; Hermosa et al., 2012; Studholme et al., 2013; Mendoza-Mendoza et al., 2018). In particular, modifications to the roots increases the area of absorption, improving nutrient uptake and translocation, then the efficient use of NPK and micronutrients attributes to enhanced plant biomass (Samolski et al., 2012). The PGP effect is attributed to the role of *Trichoderma* in the solubilization of phosphate and micronutrients (Altomare et al., 1999), mediated by the release of siderophores and secondary metabolites (Vinale et al., 2009, 2013, 2014; Spaepen, 2015), or modifications in ethylene and auxin (Hermosa et al., 2013; Contreras-Cornejo et al., 2015) that stimulate plant development.

Trichoderma spp. produce over 250 metabolic products including cell wall degrading enzymes, peptides, secondary metabolites and other proteins (Sivasithamparam and Ghisalberti, 1998; Harman et al., 2004; Morán-Diez et al., 2009; Lorito et al., 2010; Keswani et al., 2014; Ruocco et al., 2015). Many of these compounds are bioactive and can affect the plant response to other microbes, by improving defense mechanisms, while stimulating plant growth and development, especially at the root level (Sivasthamparam and Ghisalberti, 1998; Vinale et al., 2009, 2013; Lombardi et al., 2018). Synergistic effects on biocontrol have been found in many combinations of

diverse strains, metabolites, mixtures of bioactive compounds, originating from *Trichoderma* as well as other microbes or plants, which suggests a wealth of possibilities for developing a new generation of biostimulants.

Azotobacter: Rhizocompetent Stress Tolerant N_2 Free-Living Bacteria

Azotobacter includes free-living species that directly influence nutrition in agroecosystems through nitrogen fixation, thus increasing the soil level of this vital element for plants. The bacterium has the ability to form heat and desiccation-resistant cysts, providing inoculant with a long shelf-life (Inamdar et al., 2000) and tolerance to drought and salinity stress (Vacheron et al., 2013; Berg et al., 2014; Viscardi et al., 2016). In its resistant form, *Azotobacter* can withstand biotic and abiotic stresses while positively interacting with other microrganisms and plants in agroecosystems (Babalola, 2010; Ahmad et al., 2011; Berendsen et al., 2012; Bhattacharyya and Jha, 2012; Gaiero et al., 2013; Philippot et al., 2013). Numerous commercial biofertilizer products contain *Azotobacter* as active ingredients, often in association with fungi, actinomycetes as well as other bacteria (e.g., bacilli; EBIC, 2013).

The ability of beneficial *Azotobacter* strains to secrete plant growth promoting and regulating substances such as phytohormones, vitamins, and antifungal metabolites have been studied. Phosphate solubilization (Hariprasad and Niranjana, 2009; Rojas-Tapias et al., 2012; Wani et al., 2013) and Fe mobilization (Rizvi and Khan, 2018) have been demonstrated *in vitro* and in soil, also under abiotic stress conditions (Viscardi et al., 2016; Van Oosten et al., 2018).

Furthermore, the *Azotobacter*-mediated synthesis of superoxide dismutase (SOD), catalase (CAT), proline, and high levels of 1-aminocyclopropane-1-carboxylate (ACC) activity (Glick, 2014) can influence plant health and bring benefits to a wide variety of crops such as tomato (Viscardi et al., 2016), maize (Rojas-Tapias et al., 2012), rice, wheat, and sorghum (Inamdar et al., 2000; Di Stasio et al., 2017; Van Oosten et al., 2018). Barra et al. (2016) confirmed the importance of ACC deaminase (ACCd) activity and indole-3-acetic acid (IAA) production for the alleviation of salt stress in plants treated with rhizo-competent stress tolerant *Azotobacter* strains. Similarly, a model proposed by Hermosa et al. (2012) indicated that the ACCd and IAAs produced by *Trichoderma* also regulated the equilibrium between plant growth and defense.

AGRICULTURAL PROBIOTICS: MICROBIAL CONSORTIA TO ENHANCE PGP EFFICACY

Recently, a new approach to "rhizosphere engineering" proposes the addition of effective microbial inoculants to emulate the structured biological networks in native soils, thus stimulating the recovery of functional, beneficial microbial groups positively linked to soil fertility (Ruzzi and Aroca, 2015; Shi et al., 2016; Wallenstein, 2017; Stringlis et al., 2018), and replenishing the natural microbiome reduced by crop domestication (Leff et al., 2016; Perez-Jaramillo et al., 2016). These treatments may activate nitrogen fixation, phosphate solubilization, siderophore, phytohormone, and exopolysaccharide production known to enhance growth while protecting the plant from abiotic stresses, e.g., extreme temperature, pH, salinity, drought (Ashraf et al., 2004; Compant et al., 2005; Gopalakrishnan et al., 2015; Viscardi et al., 2016; Van Oosten et al., 2017), plus heavy metal, and pesticide pollution (Ventorino et al., 2014). Even though knowledge is limited on the survival of the microbial inoculants, the ability of rhizosphere competent bacteria and fungi to establish close associations with the native microbiota and soil fauna has been sufficiently demonstrated (Hardoim et al., 2015; Bonanomi et al., 2017, 2018; de Vries and Wallenstein, 2017). The synthetic bacteria-fungi consortia have the potential to establish novel microbial communities (Ahmad et al., 2011; Berg et al., 2014; du Jardin, 2015; Lugtenberg, 2015), while co-applications of different microbes may activate new PGP effects not obtained by using single species (Wargo and Hogan, 2006).

Plant microbiome engineering requires the identification and culturing of potential PGPMs, deep analysis/selection of the various components, evaluation of the compatibility between microorganisms, determination of the cause and effects in the native agroecosystem, development of adequate formulation recipes and distribution technology, plus provision of technical support to end-users (Berendsen et al., 2012; Berg et al., 2014; Lugtenberg, 2015; Yakhin et al., 2017; Kong et al., 2018). To this end, the extensive studies on *Trichoderma* and *Azotobacter* suggest that these fungi and bacteria could be functionally complementary in a PGP consortium, although the effects on the resident rhizosphere microbiota have not been sufficiently elucidated. Furthermore, the *Trichoderma-Azotobacter* consortia could be integrated with botanical and inorganic compounds, seaweeds, polymers, animal-derived products to develop truly effective, and reliable beneficial plant products. 'Omics studies can reveal basic mechanisms regulating these complex interactions and provide new knowledge concentrated on the mechanisms that could be relevant for improving the next generation of plant biostimulants (Bell et al., 2015; Soni et al., 2017; Fiorentino et al., 2018; Ventorino et al., 2018).

The global biopesticide market is continuously growing due to changing agricultural legislations and regulations, increased demand for biological/organic products, conversions from conventional to integrated pest management (IPM), and organic farming systems (Woo et al., 2014; Lugtenberg, 2015). Similarly, a steady growth is observed in the biofertilizer market (about 10% per year; EBIC, 2013). The new frontier for plant biostimulants should profit from the beneficial associations of microorganisms and compounds, by building on a deeper understanding of plant-microbe interactions developed by Nature. New microbial consortium can be designed, e.g., *Trichoderma* plus *Azotobacter*, as agricultural probiotics suitable for sustaining the agroecosystem while improving the quantity and quality of yield.

AUTHOR CONTRIBUTIONS

SW and OP conceived the concepts and wrote the manuscript in collaboration.

FUNDING

JGI Community Scientific Program 2016, Proposal 1966-CSP 2016; Project ProBio - IZSM (Portici), n. D01 6309, 14/12/2016; Project SIX - B.U. Regione Puglia, n. 111, 29/9/2016, Cod. C; Project LINFA - MIUR-PON, n. 03PE_00026_1, 09/04/2014; KENYA-AID, n. 10306/CEFA/KEN, 18/05/2017.

ACKNOWLEDGMENTS

We thank Valeria Ventorino, Francesco Vinale, Nadia Lombardi, and Matteo Lorito for editorial assistance and comments.

REFERENCES

Ahmad, I., Khan, M. S. A., Aqil, F., and Singh, M. (2011). "Microbial applications in agriculture and the environment: a broad perspective," in *Microbes and Microbial Technology: Agricultural and Environmental Applications*, eds I. Ahmad, F. Ahmad, and J. Pichtel (New York, NY: Springer), 1–27.

Altomare, C., Norvell, W. A., Bjorkman, T., and Harman, G. E. (1999). Solubilization of phosphates and micronutrients by the plant-growth-promoting and biocontrol fungus *Trichoderma harzianum* Rifai 1295-22. *Appl. Environ. Microbiol.* 65, 2926–2933.

Ashraf, M., Hasnain, S., Berge, O., and Mahmood, T. (2004). Inoculating wheat seedlings with exopolysaccharide-producing bacteria restricts sodium uptake and stimulates plant growth under salt stress. *Biol. Fertil. Soils* 40, 157–162. doi: 10.1007/s00374-004-0766-y

Babalola, O. O. (2010). Beneficial bacteria of agricultural importance. *Biotechnol. Lett.* 32, 1559–1570. doi: 10.1007/s10529-010-0347-0

Barra, P. J., Inostroza, N. G., Acuña, J. J., Mora, M. L., Crowley, D. E., and Jorquera, M. A. (2016). Formulation of bacterial consortia from avocado (*Persea americana* Mill.) and their effect on growth, biomass and superoxide dismutase activity of wheat seedlings under salt stress. *Appl. Soil Ecol.* 102, 80–91. doi: 10.1016/j.apsoil.2016.02.014

Bell, C. W., Asao, S., Calderon, F., Wolk, B., and Wallenstein, M. D. (2015). Plant nitrogen uptake drives rhizosphere bacterial community assembly during plant growth. *Soil Biol. Biochem.* 85, 170–182. doi: 10.1016/j.soilbio.2015.03.006

Berendsen, R. L., Pieterse, C. M., and Bakker, P. A. (2012). The rhizosphere microbiome and plant health. *Trends Plant Sci.* 17, 478–486. doi: 10.1016/j.tplants.2012.04.001

Berg, G., Grube, M., Schloter, M., and Smalla, K. (2014). Unraveling the plant microbiome: looking back and future perspectives. *Front. Microbiol.* 5:148. doi: 10.3389/fmicb.2014.00148

Bhattacharyya, P. N., and Jha, D. K. (2012). Plant growth-promoting rhizobacteria (PGPR): emergence in agriculture. *World J. Microbiol. Biotechnol.* 28, 1327–1350. doi: 10.1007/s11274-011-0979-9

Bonanomi, G., Ippolito, F., Cesarano, G., Vinale, F., Lombardi, N., Crasto, A., et al. (2017). Biochar chemistry defined by13C-CPMAS NMR explains opposite effects on soilborne microbes and crop plants. *Appl. Soil Ecol.* 124, 351–361. doi: 10.1016/j.apsoil.2017.11.027

Bonanomi, G., Lorito, M., Vinale, F., and Woo, S. L. (2018). Organic amendments, beneficial microbes, and soil microbiota: toward a unified framework for disease suppression. *Annu. Rev. Phytopathol.* 56, 1–20. doi: 10.1146/annurev-phyto-080615-100046

Brotman, Y., Landau, U., Cuadros-Inostroza, Á., Tohge, T., Fernie, A. R., Chet, I., et al. (2013). *Trichoderma*-plant root colonization: escaping early plant defense responses and activation of the antioxidant machinery for saline stress tolerance. *PLoS Pathog.* 9:e1003221. doi: 10.1371/journal.ppat.1003221

Cameron, D. D., Neal, A. L., van Wees, S. C., and Ton, J. (2013). Mycorrhiza-induced resistance: more than the sum of its parts? *Trends Plant Sci.* 18:539–545. doi: 10.1016/j.tplants..06.004

Compant, S., Duffy, B., Nowak, J., Clément, C., and Barka, E. A. (2005). Use of plant growth-promoting bacteria for biocontrol of plant diseases: principles, mechanisms of action, and future prospects. *Appl. Environ. Microbiol.* 71, 4951–4959. doi: 10.1128/AEM.71.9.4951–4959.2005

Contreras-Cornejo, H. A., López-Bucio, J. S., Méndez-Bravo, A., Macías-Rodríguez, L., Ramos-Vega, M., Guevara-García, Á. A., et al. (2015). Mitogen-activated protein kinase 6 and ethylene and auxin signaling pathways are involved in *Arabidopsis* root-system architecture alterations by *Trichoderma atroviride*. *Mol. Plant-Microbe Interact.* 28, 701–710. doi: 10.1094/MPMI-01-15-0005-R

de Vries, F. T., and Wallenstein, M. D. (2017). Below-ground connections underlying above-ground food production: a framework for optimising ecological connections in the rhizosphere. *J. Ecol.* 105, 913–920. doi: 10.1111/1365-2745.12783

Di Stasio, E., Maggio, A., Ventorino, V., Pepe, O., Raimondi, G., and De Pascale, S. (2017). Free-living (N_2)-fixing bacteria as potential enhancers of tomato growth under salt stress. *Acta Hortic.* 1164, 151–156. doi: 10.17660/ActaHortic.2017.1164.19

du Jardin, P. (2015). Plant biostimulants: definition, concept, main categories and regulation. *Sci. Hortic.* 196, 3–14. doi: 10.1016/j.scienta.2015.09.021

EIBC (European Biostimulants Industry Council) (2013). *Promoting the Biostimulant Industry and the Role of Plant Biostimulants in Making Agriculture More Sustainable*. Available online at: www.biostimulants.eu/

Fiorentino, N., Ventorino, V., Woo, S. L., Pepe, O., De Rosa, A., Gioia, L., et al. (2018). *Trichoderma*-based biostimulants modulate rhizosphere microbial populations and improve N uptake efficiency, yield, and nutritional quality of leafy vegetables. *Front. Plant Sci.* 9:743. doi: 10.3389/fpls.2018.00743.

Fontenelle, A. D. B., Guzzo, S. D., Lucon, C. M. M., and Harakava, R. (2011). Growth promotion and induction of resistance in tomato plant against *Xanthomonas euvesicatoria* and *Alternaria solani* by *Trichoderma* spp. *Crop Prot.* 30, 1492–1500. doi: 10.1016/j.cropro.2011.07.019

Gaiero, J. R., McCall, C. A., Thompson, K. A., Dayu, N. J., Best, A. S., and Dunfield, K. E. (2013). Inside the root microbiome: bacterial root endophytes and plant growth promotion. *Am. J. Bot.* 100, 1738–1750. doi: 10.3732/ajb.1200572

Glick, B. R. (2014). Bacteria with ACC deaminase can promote plant growth and help to feed the world. *Microbiol. Res.* 169, 30–39. doi: 10.1016/j.micres.2013.09.009

Gopalakrishnan, S., Sathya, A., Vijayabharathi, R., Varshney, R. K., Gowda, C. L. L., and Krishnamurthy, L. (2015). Plant growth promoting rhizobia: challenges and opportunities. *3 Biotech* 5, 355–377. doi: 10.1007/s13205-014-0241-x

Hardoim, P. R., van Overbeek, L. S., Berg, G., Pirttil,ä, A. M., Compant, S., Campisano, A., et al. (2015). The hidden world within plants: ecological and evolutionary considerations for defining functioning of microbial endophytes. *Microbiol. Mol. Biol. Rev.* 79, 293–320. doi: 10.1128/MMBR.00050-14

Hariprasad, P., and Niranjana, S. R. (2009). Isolation and characterization of phosphate solubilizing rhizobacteria to improve plant health of tomato. *Plant Soil* 316, 13–24. doi:10.1007/s11104-008-9754-6

Harman, G. E., Howell, C. R., Viterbo, A., Chet, I., and Lorito, M. (2004). *Trichoderma* species - opportunistic, avirulent plant symbionts. *Nat. Rev. Microbiol.* 2, 43–56. doi: 10.1038/nrmicro797

Hermosa, R., Rubio, M. B., Cardoza, R. E., Nicolás, C., Monte, E., and Gutiérrez, S. (2013). The contribution of *Trichoderma* to balancing the costs of plant growth and defense. *Int. Microbiol.* 16, 69–80. doi: 10.2436/20.1501.01.181

Hermosa, R., Viterbo, A., Chet, I., and Monte, E. (2012). Plant-beneficial effects of *Trichoderma* and of its genes. *Microbiology* 158, 17–25. doi: 10.1099/mic.0.052274-0

Hossain, M. M., Sultana, F., and Islam, S. (2017). "Plant Growth-Promoting Fungi (PGPF): Phytostimulation and induced systemic resistance," in *Plant-Microbe Interactions in Agro-Ecological Perspectives, Volume 2: Microbial Interactions*

and Agro-Ecological Impacts, eds D. Singh, H. Singh, and R. Prabha (Singapore: Springer), 135–191. doi: 10.1007/978-981-10-6593-4

Inamdar, S., Kanitkar, R. U., and Watve, M. G. (2000). Longevity of *Azotobacter* cysts and a model for optimization of cyst density in liquid bioinoculants. *Curr. Sci.* 78, 719–721.

Keswani, C., Mishra, S., Sarma, B. K., Singh, S. P., and Singh, H. B. (2014). Unraveling the efficient applications of secondary metabolites of various *Trichoderma* spp. *Appl. Microbiol. Biotechnol.* 98, 533–544. doi: 10.1007/s00253-013-5344-5

Kong, W., Meldgin, D. R., Collins, J. J., and Lu, T. (2018). Designing microbial consortia with defined social interactions. *Nat. Chem. Biol.* 14, 821–829. doi: 10.1038/s41589-018-0091-7

Leff, J. W., Lynch, R. C., Kane, N. C., and Fierer, N. (2016). Plant domestication and the assembly of bacterial and fungal communities associated with strains of the common sunflower, *Helianthus annuus*. *New Phytol.* 214, 412–423. doi: 10.1111/nph.14323

Lombardi, N., Vitale, S., Turrà, D., Reverberi, M., Fanelli, C., Vinale, F., et al. (2018). Root exudates of stressed plants stimulate and attract *Trichoderma* soil fungi. *Mol. Plant-Microbe Interact.* 31, 982–994. doi: 10.1094/MPMI-12-17-0310-R

Lorito, M., and Woo, S. L. (2015). "*Trichoderma*: a multi-purpose tool for integrated pest management," in *Principles of Plant-Microbe Interactions*, ed B. Lugtenberg (Cham: Springer International Publishing), 345–353. doi: 10.1007/978-3-319-08575-3_36

Lorito, M., Woo, S. L., Harman, G. E., and Monte, E. (2010). Translational research on *Trichoderma*: from 'omics to the field. *Annu. Rev. Phytopathol.* 48, 395–417. doi: 10.1146/annurev-phyto-073009-114314

Lugtenberg, B. (2015). *Principles of Plant-Microbe Interactions: Microbes for Sustainable Agriculture*. Cham: Springer International Publishing, p. 448.

Manganiello, G., Sacco, A., Ercolano, M. R., Vinale, F., Lanzuise, S., Pascale, A., et al. (2018). Modulation of tomato response to *Rhizoctonia solani* by *Trichoderma harzianum* and its secondary metabolite harzianic acid. *Front. Microbiol.* 9:1966. doi: 10.3389/fmicb.2018.01966

Martínez-Medina, A., Van Wees, S., and Pieterse, C. M. (2017). Airborne signals by *Trichoderma* fungi stimulate iron uptake responses in roots resulting in priming of jasmonic acid-dependent defences in shoots of *Arabidopsis thaliana* and *Solanum lycopersicum*. *Plant Cell Environ.* 40, 2691–2705. doi: 10.1111/pce.13016

Mastouri, F., Björkman, T., and Harman, G. E. (2012). *Trichoderma harzianum* enhances antioxidant defense of tomato seedlings and resistance to water defecit. *Mol. Plant-Microbe Interact.* 9, 1264–1271. doi: 10.1094/MPMI-09-11-0240

Mendoza-Mendoza, A., Zaid, R., Lawry, R., Hermosa, R., Monte, E., Horwitz, B. A., et al. (2018). Molecular dialogues between *Trichoderma* and roots: role of the fungal secretome. *Fungal Biol. Rev.* 32, 62–85. doi: 10.1016/j.fbr.2017.12.001

Morán-Diez, E., Hermosa, R., Ambrosino, P., Cardoza, R. E., Gutiérrez, S., Lorito, M., et al. (2009). The ThPG1 endopolygalacturonase is required for the *Trichoderma harzianum*–plant beneficial interaction. *Mol. Plant-Microbe Interac.* 22, 1021–1031. doi: 10.1094/MPMI-22-8-1021

Perez-Jaramillo, J. E., Mendes, R., and Raaijmakers, J. M. (2016). Impact of plant domestication on rhizosphere microbiome assembly and functions. *Plant Mol. Biol.* 90, 635–644. doi: 10.1007/s11103-015-0337-7

Philippot, L., Raaijmakers, J. M., Lemanceau, P., and van der Putten, W. H. (2013). Going back to the roots: the microbial ecology of the rhizosphere. *Nat. Rev. Microbiol.* 11, 789–799. doi: 10.1038/nrmicro3109

Pringle, A., J. D., Bever, M., Gardes, J. L., Parrent, M. C., and Rillig, and, J. N., Klironomos, (2009). Mycorrhizal symbioses and plant invasions. *Annu. Rev. Ecol. Syst.* 40, 699–715. doi: 10.1146/annurev.ecolsys.39.110707.173454

Raaijmakers, J. M., Paulitz, T. C., Steinberg, C., Alabouvette, C., and Moënne-Loccoz, Y. (2009). The rhizosphere: a playground and battlefield for soilborne pathogens and beneficial microorganisms. *Plant Soil* 321, 341–361. doi: 10.1007/s11104-008-9568-6

Rizvi, A., and Khan, M. S. (2018). Heavy metal induced oxidative damage and root morphology alterations of maize (*Zea mays* L.) plants and stress mitigation by metal tolerant nitrogen fixing *Azotobacter chroococcum*. *Ecotoxicol. Environ. Saf.* 157, 9–20. doi: 10.1016/j.ecoenv.2018.03.063

Rojas-Tapias, D., Moreno-Galván, A., Pardo-Díaz, S., Obando, M., Rivera, D., and Bonilla, R. (2012). Effect of inoculation with plant growth-promoting bacteria (PGPB) on amelioration of saline stress in maize (*Zea mays*). *Appl. Soil Ecol.* 61, 264–272. doi: 10.1016/j.apsoil.2012.01.006

Rouphael, Y. P., Franken, C., Schneider, D., Schwarz, M., Giovannetti, M., Agnolucci, S., et al. (2015). Arbuscular mycorrhizal fungi act as biostimulants in horticultural crops. *Sci. Hortic.* 196, 91–108. doi: 10.1016/j.scienta.2015.09.002

Rubio, B. M., Quijada, N. M., Pérez, E., Domínguez, S., Monte, E., and Hermosa, R. (2014). Identifying beneficial qualities of *Trichoderma parareesei* for plants. *Appl. Environ. Microbiol* 6, 1864–1873. doi: 10.1128/AEM.03375-13

Ruocco, M., Lanzuise, S., Lombardi, N., Woo, S. L., Vinale, F., Marra, R., et al. (2015). Multiple roles and effects of a novel *Trichoderma* hydrophobin. *Mol. Plant-Microbe Interact.* 28, 167–179. doi: 10.1094/MPMI-07-14-0194-R

Ruzzi, M., and Aroca, R. (2015). Plant growth-promoting rhizobacteria act as biostimulants in horticulture. *Sci. Hortic.* 196, 124–134. doi: 10.1016/j.scienta.2015.08.042

Samolski, I., Rincón, A. M., Pinzón, L. M., Viterbo, A., and Monte, E. (2012). The qid74 gene from *Trichoderma harzianum* has a role in root architecture and plant biofertilization. *Microbiology* 158, 129–138. doi: 10.1099/mic.0.053140-0

Shi, S., Nuccio, E. E., Shi, Z. J., He, Z., Zhou, J., and Firestone, M. K. (2016). The interconnected rhizosphere: high network complexity dominates rhizosphere assemblages. *Ecol. Lett.* 19, 926–936. doi: 10.1111/ele.12630

Shoresh, M., Harman, G. E., and Mastouri, F. (2010). Induced systemic resistance and plant responses to fungal biocontrol agents. *Annu. Rev. Phytopathol.* 48, 21–43. doi: 10.1146/annurev-phyto-073009-114450

Sivasithamparam, K., and Ghisalberti, E. L. (1998). "Secondary metabolism in *Trichoderma* and *Gliocladium*," in *Trichoderma and Gliocladium. Volume 1: Basic Biology, Taxonomy and Genetics*, eds G. E. Harman and C. P. Kubicek (London: Taylor and Francis Ltd.), 139–191.

Sofo, A., Nuzzaci, M., Vitti, A., Tataranni, G., and Scopa, A. (2014). "Control of biotic and abiotic stresses in cultivated plants by the use of biostimulant microorganisms," in *Improvement of Crops in the Era of Climatic Changes*, eds P. Ahmad, M. Wani, M. Azooz, and L. S. Tran (New York, NY: Springer), 107–117. doi: 10.1007/978-1-4614-8830-9_5

Soni, R., Kumar, V., Suyal, D. C., Jain, L., and Goel, R. (2017). "Metagenomics of plant rhizosphere microbiome," in *Understanding Host-Microbiome Interactions - An Omics Approach*, eds R. Singh, R. Kothari, P. Koringa, and S. Singh (Singapore: Springer), 193–205. doi: 10.1007/978-981-10-5050-3_12.

Spaepen, S. (2015). "Plant hormones produced by microbes," in *Principles of Plant-Microbe Interactions*, ed B. Lugtenberg (Cham: Springer International Publishing), 247–256. doi: 10.1007/978-3-319-08575-3_26

Stringlis, I. A., Zhang, H., Pieterse, C. M. J., Bolton, M. D., and de Jonge, R. (2018). Microbial small molecules - weapons of plant subversion. *Nat. Prod. Rep.* 35, 410–433. doi: 10.1039/c7np00062f

Studholme, D. J., Harris, B., Le Cocq, K., Winsbury, R., Perera, V., Ryder, L., et al. (2013). Investigating the beneficial traits of *Trichoderma hamatum* GD12 for sustainable agriculture – insights from genomics. *Front. Plant Sci.* 4:258. doi: 10.3389/fpls.2013.00258

Vacheron, J., Desbrosse, G., Bouffaud, M. L., Touraine, B., Moënne-Loccoz, Y., Muller, D., et al. (2013). Plant growth-promoting rhizobacteria and root system functioning. *Front. Plant Sci.* 4:356. doi: 10.3389/fpls.2013.00356.

Van Oosten, M. J., Di Stasio, E., Cirillo, V., Silletti, S., Ventorino, V., Pepe, O., et al. (2018). Root inoculation with *Azotobacter chroococcum* 76A enhances tomato plants adaptation to salt stress under low N conditions. *BMC Plant Biol.* 18:205. doi: 10.1186/s12870-018-1411-5

Van Oosten, M. J., Pepe, O., De Pascale, S., Silletti, S., and Maggio, A. (2017). The role of biostimulants and bioeffectors as alleviators of abiotic stress in crop plants. *Chem. Biol. Technol. Agric.* 4:5. doi: 10.1186/s40538-017-0089-5

Ventorino, V., Pascale, A., Adamo, P., Rocco, C., Fiorentino, N., Mori, M., et al. (2018). Comparative assessment of autochthonous bacterial and fungal communities and microbial biomarkers of polluted agricultural soils of the Terra dei Fuochi. *Sci. Rep.* 8:14281. doi: 10.1038/s41598-018-32688-5

Ventorino, V., Sannino, S., Piccolo, A., Cafaro, V., Carotenuto, R., and Pepe, O. (2014). *Methylobacterium populi* VP2: plant growth-promoting bacterium isolated from a highly polluted environment for polycyclic aromatic hydrocarbon (PAH) biodegradation. *Sci. World J.* 2014, 1–11. doi: 10.1155/2014/931793

Vinale, F., Flematti, G., Sivasithamparam, K., Lorito, M., Marra, R., Skelton, B. W., et al. (2009). Harzianic acid, an antifungal and plant growth promoting

metabolite from *Trichoderma harzianum*. *J. Nat. Prod.* 72, 2032–2035. doi: 10.1021/np900548p

Vinale, F., Manganiello, G., Nigro, M., Mazzei, P., Piccolo, A., Pascale, A., et al. (2014). A novel fungal metabolite with beneficial properties for agricultural applications. *Molecules* 19, 9760–9772. doi: 10.3390/molecules19079760

Vinale, F., Nigro, M., Sivasithamparam, K., Flematti, G., Ghisalberti, E. L., Ruocco, M., et al. (2013). Harzianic acid: a novel siderophore from *Trichoderma harzianum*. *FEMS Microbiol. Lett.* 347, 123–129. doi: 10.1111/1574-6968.12231

Vinale, F., Sivasithamparam, K., Ghisalberti, E. L., Marra, R., Barbetti, M. J., Li, H., et al. (2008). A novel role for *Trichoderma* secondary metabolites in the interactions with plants. *Physiol. Mol. Plant Pathol.* 72, 80–86. doi: 10.1016/j.pmpp.2008.05.005

Viscardi, S., Ventorino, V., Duran, P., Maggio, A., De Pascale, S., Mora, M. L., et al. (2016). Assessment of plant growth promoting activities and abiotic stress tolerance of *Azotobacter chroococcum* strains for a potential use in sustainable agriculture. *J. Soil Sci. Plant Nutr.* 16, 848–863. doi: 10.4067/S0718-95162016005000060

Wallenstein, M. D. (2017). Managing and manipulating the rhizosphere microbiome for plant health: a systems approach. *Rhizosphere* 3, 230–232. doi: 10.1016/j.rhisph.2017.04.004

Wani, S. A., Chand, S., and Ali, T. (2013). Potential use of *Azotobacter chroococcum* in crop production: an overview. *Curr. Agr. Res. J.* 1, 35–38. doi: 10.12944/CARJ.1.1.04

Wargo, M. J., and Hogan, D. A. (2006). Fungal-bacterial interactions: a mixed bag of mingling microbes. *Curr. Opin. Microbiol.* 9, 359–364. doi:10.1016/j.mib.2006.06.001

Woo, S. L., Ruocco, M., Vinale, F., Nigro, M., Marra, R., Lombardi, N., et al. (2014). *Trichoderma*-based products and their widespread use in agriculture. *Open Mycol. J.* 8, 71–126. doi: 10.2174/1874437001408010071

Yakhin, O. I., Lubyanov, A. A., Yakhin, I. A., and Brown, P. H. (2017). Biostimulants in plant science: a global perspective. *Front. Plant Sci.* 7:2049. doi: 10.3389/fpls.2016.02049

Conflict of Interest Statement: The authors declare that the research was conducted in the absence of any commercial or financial relationships that could be construed as a potential conflict of interest.

frontiers in Microbiology

ORIGINAL RESEARCH
published: 05 December 2018
doi: 10.3389/fmicb.2018.02985

Enriching Beneficial Microbial Diversity of Indoor Plants and Their Surrounding Built Environment With Biostimulants

Alexander Mahnert, Marika Haratani, Maria Schmuck and Gabriele Berg*

Institute of Environmental Biotechnology, Graz University of Technology, Graz, Austria

Microbial diversity is suggested as the key for plant and human health. However, how microbial diversity can be enriched is largely unknown but of great interest for health issues. Biostimulants offer the way to directly augment our main living areas by the healthy microbiome of indoor plants. Here, we investigated shifts of the microbiome on leaves of spider plants (*Chlorophytum comosum*) and its surrounding abiotic surfaces in the built environment after irrigation with a vermicompost-based biostimulant for 12 weeks. The biostimulant could not only promote plant growth, but changed the composition of the microbiome and abundance of intact microbial cells on plant leaves and even stronger on abiotic surfaces in close vicinity under constant conditions of the microclimate. Biostimulant treatments stabilized microbial diversity and resulted in an increase of *Bacteroidetes* and a surprising transient emerge of new phyla, e.g., *Verrucomicrobia*, *Acidobacteria,* and *Thaumarchaeota.* The proportion of potentially beneficial microorganisms like *Brevibacillus, Actinoallomurus, Paenibacillus, Sphaerisporangium* increased relatively; microbial diversity was stabilized, and the built environment became more plant-like. Detected metabolites like indole-3-acetic acid in the biostimulant were potentially contributed by species of *Pseudomonas*. Overall, effects of the biostimulant on the composition of the microbiome could be predicted with an accuracy of 87%. This study shows the potential of biostimulants not only for the plant itself, but also for other living holobionts like humans in the surrounding environment.

Keywords: indoor plants, built environment, biostimulants, vermicompost, microbiome, 16S rRNA gene amplicon analysis, qPCR, LC-MS

OPEN ACCESS

Edited by:
Giuseppe Colla,
Università degli Studi della Tuscia, Italy

Reviewed by:
Marc Viñas,
Institut de Recerca i Tecnologia Agroalimentàries (IRTA), Spain
Angela Sessitsch,
Austrian Institute of Technology (AIT), Austria
Collin M. Timm,
Johns Hopkins University, United States

****Correspondence:***
Alexander Mahnert
alexander.mahnert@gmail.com

Specialty section:
This article was submitted to Plant Microbe Interactions, a section of the journal Frontiers in Microbiology

Received: *14 August 2018*
Accepted: *19 November 2018*
Published: *05 December 2018*

Citation:
Mahnert A, Haratani M, Schmuck M and Berg G (2018) Enriching Beneficial Microbial Diversity of Indoor Plants and Their Surrounding Built Environment With Biostimulants. Front. Microbiol. 9:2985. doi: 10.3389/fmicb.2018.02985

INTRODUCTION

Plants are apart from humans and animals often part of indoor environments and provide a sustainable but underexploited solution to enhance indoor air quality (Brilli et al., 2018), and serve as an important source for microbial communities (Berg et al., 2014; Mahnert et al., 2015). Plants themselves possess a unique microbiome, and their functional interplay determines health and productivity of the plant holobiont (Bulgarelli et al., 2013; Vandenkoornhuyse et al., 2015). The plant microbiome varies between different locations on the plant (Turner et al., 2013). For example, the rhizosphere is rich in nutrients derived from root exudates, and represents a relatively stable and protected interface to the surrounding soil (Philippot et al., 2013).

The phyllosphere, which represents the air-plant interface, is nutrient-poor and its environment is more dynamic and affected by abiotic factors from the surrounding outdoor environment (Turner et al., 2013). Nevertheless, both microenvironments (rhizosphere and phyllosphere) and their inhabiting microorganisms are connected by the endosphere (Berg et al., 2005; Hardoim et al., 2015). This was also shown in the study of (Badri et al., 2013), where application of soil microbes to the roots resulted in a direct increase in metabolism of the corresponding plant leaves. This phenomenon and the observations that plants can alter microbial abundance and diversity within the built environment (Mahnert et al., 2015) suggest that it might be possible to not only influence the microbiome of a plant, but also their environment by increasing and stabilizing the existing microbial community with them. While the impact of them on the rhizosphere microbiome is well studied (Erlacher et al., 2014; Kröber et al., 2014), less is known about their impact on the phyllosphere and on the environmental microbiome.

Based on the beneficial plant-microbe interactions and mode of action, beneficial microorganisms can be used as biofertilizers, plant strengtheners, biostimulants, and biopesticides (Berg, 2009). Biostimulants have broad applications ranging from enhancing nutrition efficiency, over crop quality till abiotic stress tolerance as reviewed by du Jardin (2015). They can include diverse formulations of compounds, substances and microorganisms that are applied to plants or soils to improve plants vigor, yields, quality and tolerance of abiotic stresses. The mode of action of biostimulants has been associated to direct effects by stimulation of enzyme activities and hormonal activities and also indirectly by improvement of soil nutrient availability. Moreover, a modification of natural microbial communities is suggested, but was never shown. However, the detailed molecular, cellular and physiological mechanisms underlying plant-biostimulant interactions under different environment and management strategies remain largely unknown. Although, the concept of biostimulants based on the principle that biological function can be positively modulated through application of molecules, or mixtures of molecules (Yakhin et al., 2017), an understanding of the mechanism is important for consistent effects. Our hypothesis was that biostimulants have a positive impact on plant growth and performance due to their ability to stabilize the whole plant microbiome, and beyond that of the surrounding microbiome.

In the frame of this study we applied the model biostimulant "bio-guss universal compost tea" (GARTENleben GmbH, Austria) on the house plant *Chlorophytum comosum* (Thunb.) Jacques (spider plant), a common indoor plant in homes and offices around the world, which showed meliorations of indoor air (Sriprapat et al., 2014) and the potential to change microbial diversity and abundance in its surroundings (Mahnert et al., 2015). The applied biostimulant consists of dried organic compost soil and plant residues in the form of tea-bags, which can be used for steeping plant irrigation waters and should act as a natural fertilizer with its own set of natural microorganisms[1]. The main ingredient is vermicompost produced by earthworms, which is a humus-like, nutrient and microorganism-rich compost (Lim et al., 2015). We developed a specific experimental design to test the effect of the biostimulant (**Figure 1**), and analyzed comparatively the microbiome by 16S rRNA gene profiling (diversity), qPCR (abundance), and HPLC-MS (metabolite profiling) to understand changes in microbial diversity and abundance in plant soil, on plant leaves and surrounding abiotic surfaces in the presence of the biostimulant its microbiota and metabolites.

[1]http://www.gartenleben.at/bioguss

MATERIALS AND METHODS

Experimental and Sampling Design

For the experimental set-up 4 different plant systems were established (see **Figure 1**). Three of them contained plants, which were grown under gnotobiotic conditions and one was grown with natural seeds and soil. The biostimulant was added to the common plants and to one of the gnotobiotic plants. The remaining two functioned as control systems, which were irrigated with tab- and sterile water. All plants were grown for 12 weeks in a desiccator to minimize environmental influences and prevent microbial contaminations. The desiccators (Bartelt GmbH, Graz, Austria) were cleaned with water and detergent (Shower Cleaner, Bluestar, Germany), dried at room temperature and then closed to avoid further contamination. Afterwards they were dry-heated at 170°C for 24 h for sterilization and to degrade any remaining DNA. All sampling devices made from glass or metal (e.g., Erlenmeyer flasks, spatulas, tweezers etc.) and alpha-wipes (TX1009 Alpha-Wipe, ITW Texwipe, VWR, Austria) were heat-sterilized for 24 h at 170°C to degrade also DNA contaminants. The remaining plastic devices (e.g., 50 ml tubes) were UV-sterilized for 20–30 min under a laminar flow. All sampling devices were sterilized again before each sample collection.

Propagation of Seeds and Plants

Four different desiccator systems (incubation chambers) were prepared according to **Figure 1**. The whole set-up comprised three plants, which were grown from surface-sterilized seeds in autoclaved soil and one plant grown under natural conditions. The gnotobiotic plants were watered with the biostimulant, tap water or sterile ultrapure water. The plants growing in natural soil were only treated with the biostimulant. Seeds of *Chlorophytum comosum* were provided by the botanical garden of Graz. For the gnotobiotic cultivation, the soil (Profi-Substrat, Gramoflor, Vechta, Germany) was autoclaved two times in an interval of 3 days and the seeds were surface sterilized using 2% sodium hypochlorite solution (Carl Roth GmbH & Co. KG, Karlsruhe, Germany) for 5 min. Afterwards the seeds were rinsed six times with sterile dH_2O. The natural soil and seeds remained untreated. The pre-growth of the gnotobiotic *C. comosum* plants started on September 23rd, 2016 and the natural seeds were sown on September 26th, 2016 in tiny plastic boxes (9 cm × 10 cm^2). The gnotobiotic plants were transferred to bigger plastic boxes (15 cm × 15 cm) on December 12th, 2016. During the whole

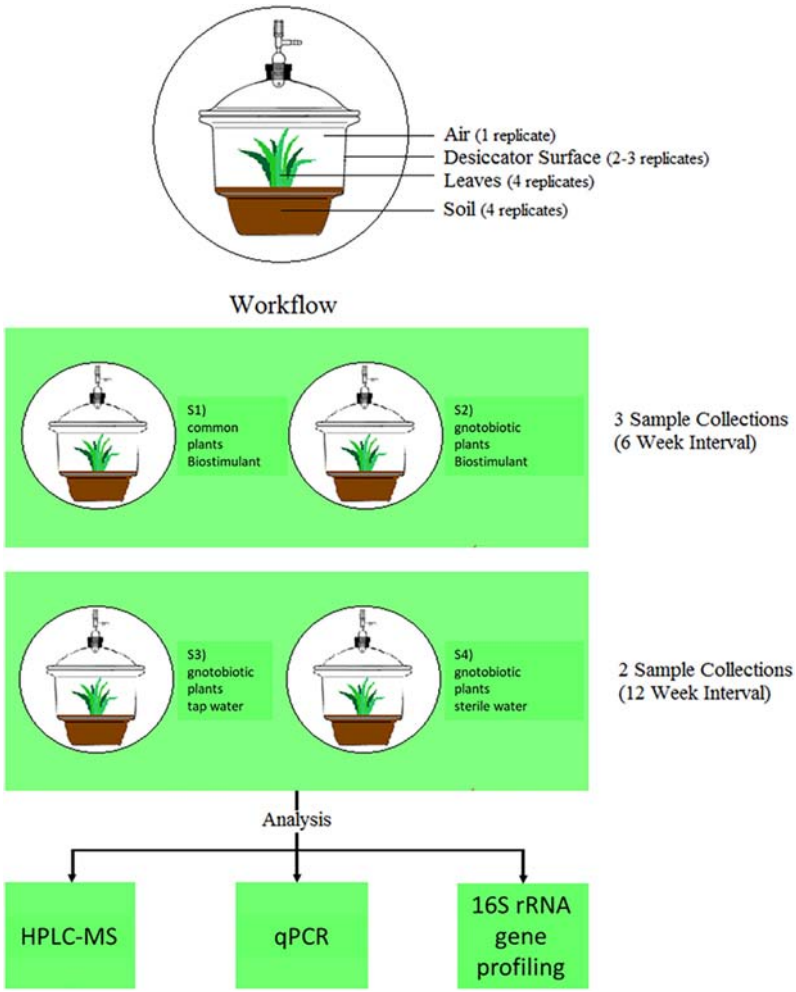


FIGURE 1 | Experimental setup and workflow of this study.

procedure, from pre-growth to the end of the experiment, the plants grew inside the greenhouse. Only for sample collection and aeration purposes, the plants were transferred to a clean bench for 1–3 h. The growth conditions inside the greenhouse (Binder KBWF 720, Tullingen, Germany) were set to 22°C with a day–night regime of 12:12 h (7 kLux).

Plant Treatments

The irrigation of the plants began after an air sampling in the frame of the first sample collection. In addition, the desiccators were opened once a week for an hour under laminar flow to further decrease the high moisture content, which promoted fungal growth. The plants were watered in an interval of 2 weeks with a volume of 50–100 ml. The biostimulant solution was prepared according to manual instructions[2]. The biostimulant contained vermicompost, malt sprouts, stone dust and organic herbs (stinging nettle, comfrey, field horsetail, valerian, marigold) and the following nutrients: 1.8 mg/l NH4-N, 22 mg/l NO3-N, 230 mg/l N Kjeldahl., 11.3 mg/l PO4-P, 290 mg/l K, 650 mg/l TOC. One tea bag (45 ml, 36.59 g $\pm$ 0.22 g) filled with the dried biostimulant was added to 2 L of autoclaved ultrapure water (BioScience-Grade, nuclease-free, autoclaved, DEPC-treated water, Carl Roth GmbH & Co. KG, Karlsruhe, Germany), under a laminar flow. The flask was closed, and the suspension was steeped for 24 h at room temperature before the tea bag was discarded. For each sampling event a new suspension of the biostimulant was prepared. The tap water was filled into sterile DNA-free glass ware and stored with the steeped biostimulant at 4°C in between the individual watering periods. The biostimulant (plant growth promoting agent "bio-guss universal compost-tea") was applied as a liquid once a week over a duration of 12 weeks (3 months) on gnotobiotic plants grown in sterile and common soil. Plants watered only with tap water or sterile water were used as a control. By the use of sterile tubes implemented through the faucet of the desiccator and by placing the opening of each tube directly (∼1 cm distance) above an uncovered region (no covering plant leaves) of plant soil in each system, spilling

[2]http://www.gartenleben.at/bioguss

of irrigation solutions on abiotic surfaces or plant leaves was prevented. Plants were transferred to incubation chambers to guarantee a constant microclimate with a mean temperature of 22.8°C, relative humidity of 79.3% and illumination of 5.6 kLux (**Supplementary Figure S1**) as determined with a data-logger (LOG 32 TH-PDF-data logger, DOSTMANN electronic GmBH, Germany and HD450: Datalogging Heavy Duty Light Meter, Extech, United States) over the whole course of the experiment. As high levels of relative humidity lead to mold formation, weekly aeration of the desiccators under a laminar flow were conducted. These aeration events are noticeable by respective repetitive drops in relative humidity (see **Supplementary Figure S1**).

Sample Collections

Samplings (see (Mahnert et al., 2015) for further details) of the biostimulant (500 µl), plant soil (100 mg near the plant stem), plant leaves and surrounding abiotic surfaces at three defined points in time together with control samples at two points in time of the air in the incubation chamber (13 l/min for 10 min), irrigation liquids (500 µl of tap water and sterile water), lab devices, sampling equipment and used reagents summed up to 158 samples for qPCR and 16S rRNA gene amplicon sequencing. In addition, samples of the biostimulant steeped for 1 and 24 h were investigated with HPLC-MS (Q Exactive™ Hybrid Quadrupole-Orbitrap™ Mass Spectrometer, Thermo Fisher Scientific, United States) in four replicates (see scheme of the workflow in **Figure 1**).

For samplings of the desiccator surfaces, sterilized desiccators (height: 38 cm, diameter: 33 cm) were placed inside the clean bench and the lid was opened without pressuring the faucet. A sterile 50 ml tube, containing a sterile and dampened alpha-wipe, was carefully taken out with a sterile tweezer. In the first sample collection the whole inner surface of the desiccator was sampled with constant pressure, turning the wipe over after sampling half of the whole inner-surface of the desiccator. Directly after sampling, the alpha-wipe was transferred to an Erlenmeyer flask, containing 40 mL 0.9% sterile DNA-free sodium chloride solution. Contaminating the sampling wipe with plant soil or the transfer of it was prevented at following sampling events. This procedure was repeated for all four desiccators. For negative controls (field blanks), one wipe which was directly transferred from the falcon tube to the flask without touching any surfaces was processed in parallel. After the first sampling, the *C. comosum* plants were transferred to their specific desiccators. The leaves in each desiccator were counted and measured with a sterilized ruler. Each plant leaf sample was represented in four replicates. Each replicate covered a quarter of the leaves' total surface area. Both sides of each leaf were sampled with constant pressure. Then, wipes were transferred into sterile Erlenmeyer flasks, which already contained 40 ml sterile, DNA-free 0.9% sodium chloride solution. Field blanks were processed as indicated above. With a sterile spatula, 100 mg of soil near the stem was transferred directly to a Lysing Matrix E Tube from the FastDNA Spin Kit for Soil in four replications. Subsequently the air from desiccators treated with the biostimulant was sampled with the SKC BioSampler (SKC Inc., PA, United States). All parts of the air sampler were autoclaved and dry-heated to achieve sterility and to degrade DNA. For the air sampling setup, the vacuum pump was connected to the desiccator over the faucet opening and to a beaker containing 10 ml PCR-grade water by sterile tubes. After the setup was installed, the vacuum pump was set to sample 13 l of air for 10 min. Sampling events were concluded with irrigating the plants with respective treatment types (biostimulant, tab-water or sterile water). An aliquot of 500 µl of each treatment was analyzed at each sample collection. Distances between the plant leaf surface and surrounding surfaces of the desiccator decreased from a maximum of 14 cm at the beginning to a minimum of 1 cm till the end of the experiment.

DNA Extraction

Samples from wipes and the air were concentrated by repeated centrifugation cycles at 3220 × *g* and 4°C for 5 min with filter tubes (Amicon Ultra-15 Centrifugal Filter Units, Merck, Germany) to 500 µl, after vortexing for 10 s and sonication at 40 kHz for 2 min in an ultrasonic bath (Transonic Digitals, Elma, United States). PMA treatment and light crosslinking was performed as described in (Moissl-Eichinger et al., 2015). Concentrated and treated cell suspensions were then homogenized with a FastPrep-24 Classic Instrument (MP Biomedicals, United States) 2× for 30 s at 6.5 m/s. Tubes were cooled on ice for 30 s between homogenization cycles. Then genomic DNA was extracted with the FastDNA® Spin Kit for Soil (MP Biomedicals, Solon, OH, United States) according to manufacturer instructions.

Quantitative PCR (qPCR)

Quantitative PCR was conducted with the primers 515f-(GTGC CAGCAGCCGC) and 927r-(CCCGTCAATTYMTTTGAGTT) (0.5 µl of 5 µM each) on a Rotorgene 6000 instrument (Celtic Diagnostics, South Africa). Beside primers, the 10 µl reaction mix contained 5 µl KAPA SYBR FAST qPCR Master Mix Universal, 3 µl PCR-grade water, and 1 µl template DNA. Amplifications were achieved through 40 cycles of denaturation at 95°C for 20 s, annealing at 54°C for 15 s and elongation at 72°C for 30 s. A melt curve from 72 to 95°C (5 s per 1°C increase) together with standards based on *Bacillus subtilis* B2G and no template controls served as quality controls for amplified products. qPCR runs with reaction efficiencies above 1.2 and R^2-values above 0.99 were considered to be of sufficient quality to determine microbial abundance.

16S rRNA Gene Amplicon Libraries

Amplicons were generated by two separate PCR reactions on a TPersonal thermocycler (Biometra, Germany). In the first step pads were added together with the primers [0.3 µl of 515f-(GTGYCAGCMGCCGCGGTAA)-pad and 926r-(CCGYCAATTYMTTTRAGTTT)-pad primers, 10 µM each] onto the target sequence in a 30 µl PCR reaction mix with 22.4 µl PCR-grade water, 6 µl Taq & Go PCR master mix and 1 µl template DNA. PCR products were amplified by 30 cycles of denaturation at 95°C for 45 s, annealing at 55°C for 45 s, and elongation at 72°C for 90 s. The PCR was repeated three times for each sample and 20 µL from each PCR reaction was pooled

and used as template for the second PCR. In the second step, individual barcodes were attached to the pads in a 50 µl PCR reaction mix (details see above) and 15 cycles of denaturation at 95°C, annealing at 53°C and elongation at 72°C for 30 s at each step. The second PCR step was repeated four times for each sample and checked for quality by gel electrophoresis. Pooled PCR products were purified with the Wizard SV Gel and PCR clean-up System kit (Promega, United States) before they were quantified with a NanoDrop UV-Vis instrument (Thermo Scientific, United States). 50 µg DNA of an equimolar concentrated amplicon pool was then sent to the GATC Biotech AG, (Konstanz, Germany) for Illumina HiSeq sequencing using an optimized protocol (Schwendner et al., 2017) to achieve 300 bp paired end reads in the rapid run mode after entry quality control and adapter ligation. Raw reads were deposited in the European Nucleotide Archive - ENA[3] under project ID PRJEB27998.

Bioinformatics and Statistics

Amplicon sequences were pre-processed in QIIME 1.9.1 (Caporaso et al., 2010) and analyzed with QIIME2 (versions 2017.10 – 2018.8) (Bolyen et al., 2018). Forward and reverse amplicon sequences were stitched with an overlap of 100 bp and redundant sequences were removed. Reads were imported into QIIME 2 and demultiplexed according to sample specific barcodes. Sequences were filtered and denoised into features with DADA2 (Callahan et al., 2016). Resulting feature tables were rarefied for a core diversity analysis including phylogenetic metrics (unifrac) of the alpha and beta diversity with default settings. Representative sequences were aligned and filtered with mafft (Katoh and Standley, 2013) before a phylogenetic tree was calculated and rooted with fasttree (Price et al., 2010). Kruskal–Wallis tests (Kruskal and Wallis, 1952) were calculated for alpha diversity metrics to define significance between categorical metadata columns. Likewise, PERMANOVA tests (Anderson, 2001) based on 999 permutations were executed to define significance for beta diversity metrics between categorical metadata. For numerical metadata columns, significant correlations were determined by Spearman rank correlations and mantel tests (Pearson, 1895; Spearman, 1904; Mantel, 1967). Community compositions were linked to environmental variables by bioenv tests (Clarke and Ainsworth, 1993). Taxonomic assignments of representative sequences were conducted through a naïve-bayes classifier (Pedregosa et al., 2012) trained on 16S rRNA gene OTUs clustered at 99% similarities within the Silva123 database release. Differential abundance of taxa was determined by ANCOM (Mandal et al., 2015) and gneiss (Morton et al., 2017). Longitudinal analysis (Bokulich et al., 2018a) were based on changes in Shannon diversity estimates and weighted unifrac distances using linear mixed effects modeling (Seabold and Perktold, 2010) and non-parametric microbial interdependence tests (Zhang et al., 2017). Sample metadata was predicted with supervised machine learning classification and regression methods (Bokulich et al., 2018b) and maturity index prediction (Subramanian et al., 2014). Predictions of potential functional capabilities and contributions of distinct ASVs to particular functions were executed in PICRUSt (Langille et al., 2013). BugBase (Ward et al., 2017) was used to predict potential microbial phenotypes and SourceTracker (Knights et al., 2011) was applied to estimate the potential origin of characteristic taxa. Two-way ANOVAs were calculated in R (R Core Team, 2014) to determine any groupings of the qPCR data by the factors treatment and time.

Metabolite Profiling

Metabolites of the biostimulant were analyzed by high-performance liquid chromatography (HPLC) coupled to mass spectrometry (MS). One bag of the biostimulant was transferred to 100 mL autoclaved water in four replicates. Two bags were steeped for 1 h and two were steeped for 24 h at room temperature. Afterwards respective bags of the biostimulant were carefully removed and 1 ml of the solutions were centrifuged at 13,500 × *g* and 4°C for 10 min. The resulting supernatants were analyzed with a HPLC-MS (Q Exactive™ Hybrid Quadrupole-Orbitrap™ Mass Spectrometer, Thermo Fisher Scientific, United States) on an Atlantis column at 0.3 mL/min including a gradient of 40% of Component B for 2 min – 100% of Component B for 15 min – and 40% of Component B for 5 min, and a run voltage of 3100 V and a capillary temperature of 330°C for 40 min. Component A contained 0.1% formic acid in double distilled H_2O, while Component B contained 0.1% formic acid solved in acetonitrile. The water, used for steeping, served as a blank. Positive and negative mode were separately executed with a resolution of 70.000, an AGC target of 10^6, Maximum IT set to 200 ms, a scan range of 100–1500 mass to charge ratios and a resolution of 17.500 for the MS2-Parameter. Metabolite analysis was performed with Compound Discoverer 2.1 (Thermo Fisher Scientific). Spectra were compared with database entries on mzCloud[4] and simulated spectra from CFM-ID (Allen et al., 2014).

RESULTS

Impact on Plant Growth

In the course of the experiment the leaf area increased in all systems (**Supplementary Figure S2**). The highest increase was evident for common plants irrigated with the biostimulant (8-fold), followed by gnotobiotic plants irrigated with the biostimulant (4-fold) and sterile water (1.5-fold). Only gnotobiotic plants irrigated with tap water showed a decline in plant growth from 0.17 to 0.16 m^2. The plants which were treated with the biostimulant (common and gnotobiotic plants) did not show a significantly higher growth compared to the other systems (two-way ANOVA: $P = 0.075$; **Supplementary Table S1**). However, the plants started with different leaf areas, which makes comparisons even less objective.

[3]https://www.ebi.ac.uk

[4]https://www.mzcloud.org/

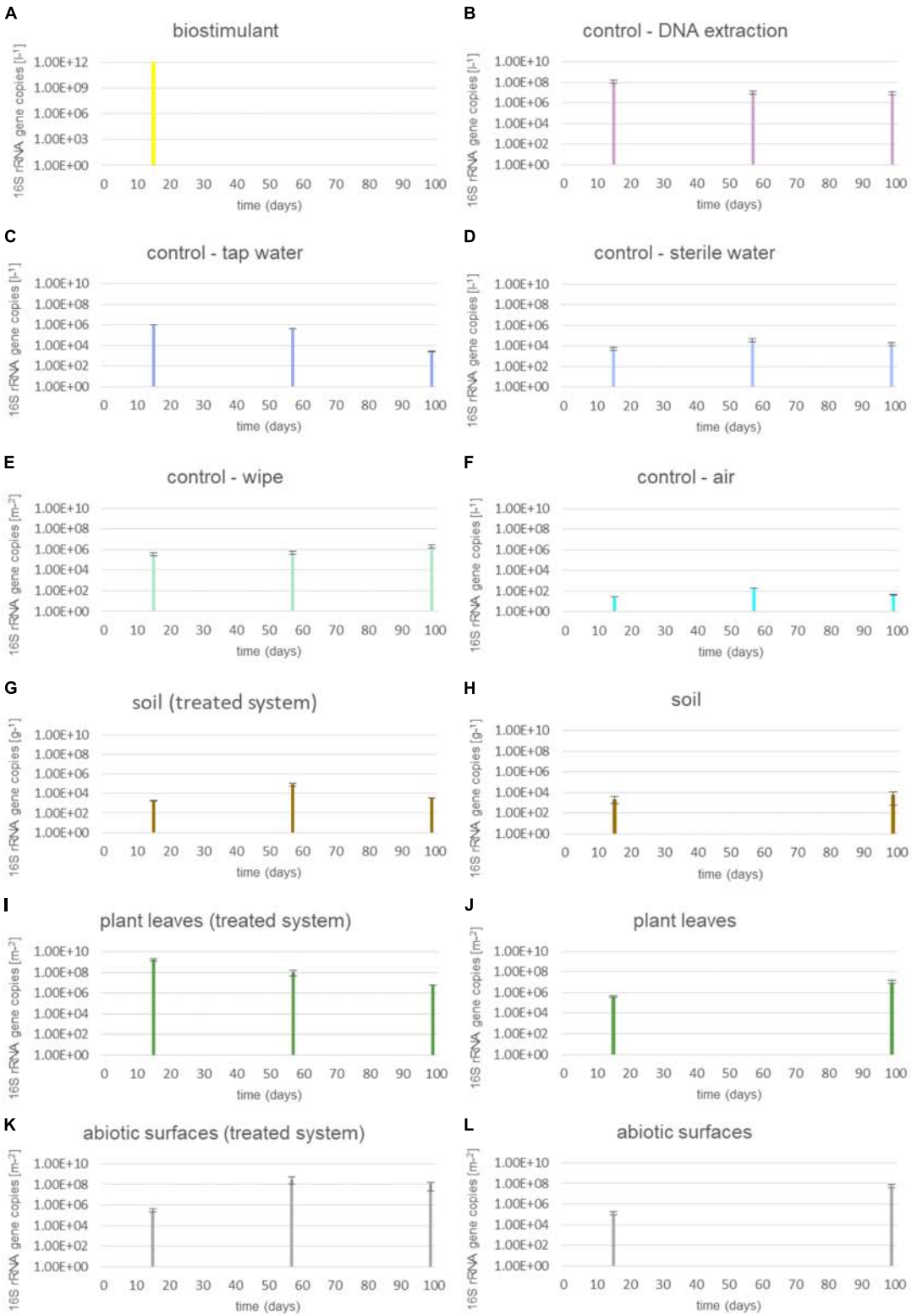


FIGURE 2 | Microbial abundance of samples treated with the biostimulant and untreated samples. **(A)** Steeped biostimulant, **(B)** control of DNA extraction reagents, **(C)** tap water control, **(D)** sterile water control, **(E)** wipe control, **(F)** air control, **(G)** soil of biostimulant treated systems, **(H)** soil of systems without contact to the biostimulant, **(I)** plant leaves of biostimulant treated systems, **(J)** plant leaves of systems without contact to the biostimulant, **(K)** desiccator surfaces of biostimulant treated systems, and **(L)** desiccator surfaces of systems without contact to the biostimulant. 16S rRNA gene copy numbers were extrapolated to 1l steeped biostimulant **(A)**, DNA extraction reagents **(B)**, irrigation waters **(C,D)**, or air **(F)**, 1 g soil **(G,H)**, 1 m^2 wipe **(E)**, plant leaf area **(I,J)** or desiccator surface **(K,L)**.

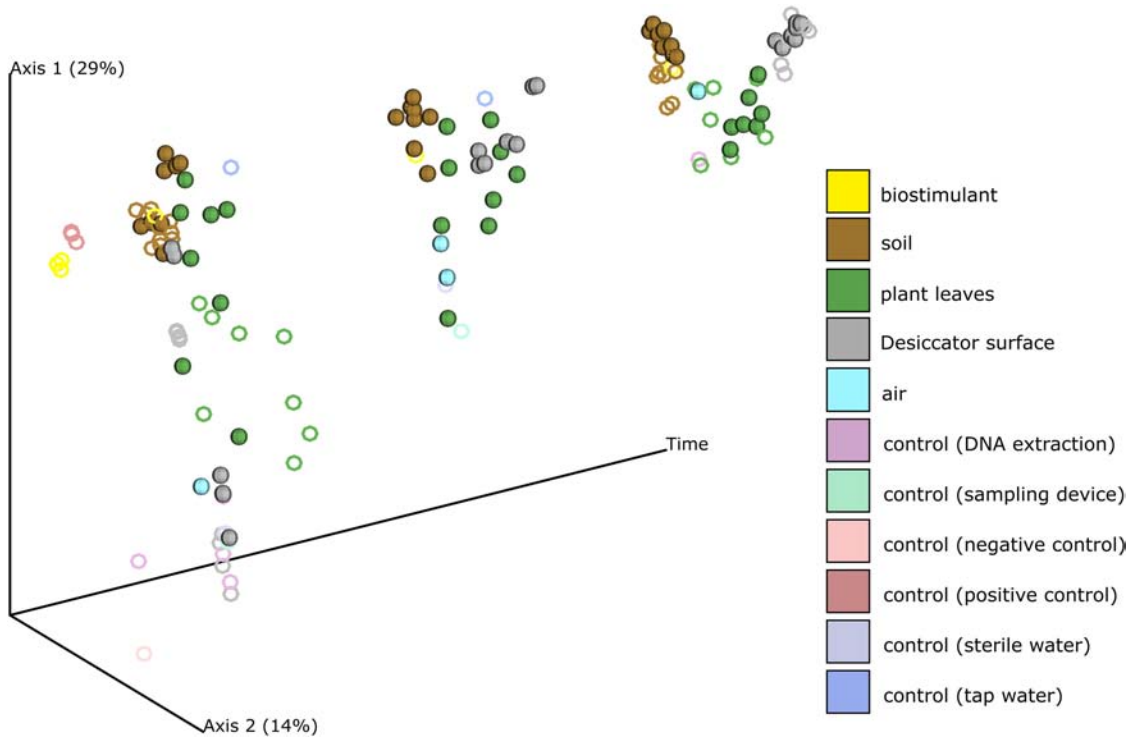


FIGURE 3 | PCoA of the changing microbial composition over time and after application of the biostimulant. Desiccator surfaces experienced the biggest shift in their microbial composition and became more leaf-like. Samples from biostimulant treated test systems are shown as spheres and samples from test systems treated with sterile and tap water are shown as rings.

Impact on Microbial Abundance

Microbial abundance of dry and dissolved biostimulant was very high ($\sim 10^{12}$ 16S rRNA gene copies per gram or liter). The microbial abundance increased in the soil of the plant ($\sim 10^{3}$ to $\sim 10^{9}$ 16S rRNA gene copies per g) and on surrounding abiotic surfaces ($\sim 10^{5}$ to $\sim 10^{7}$ 16S rRNA gene copies per m^2), while samples from plant leaves ($\sim 10^{8}$ to $\sim 10^{6}$ 16S rRNA gene copies per m^2) and controls showed a decrease in microbial abundance (**Figure 2**). A two-way ANOVA revealed a significant grouping of microbial abundances for plant leaves and surrounding abiotic surfaces for the factor time, but no significance for the type of treatment or an interaction of both factors (treatment and time; **Supplementary Table S2**).

The proportion of 16S rRNA gene copy numbers from intact microbial cells was determined through a treatment with the chemical PMA prior to DNA extraction procedures. This differentiated analysis showed that the dissolved biostimulant contained mainly intact microbial cells (up to 91%, 1.2×10^{11} 16S rRNA gene copies per liter; **Supplementary Figure S3**). On plant leaves the proportion of intact microbial cells was lower compared to the dissolved biostimulant. Interestingly, plant leaves treated with the biostimulant showed a higher proportion of intact cells (42%) compared to plants irrigated with tap or sterile water (20%). Nevertheless, any groupings by these factors were not significant (**Supplementary Table S3**).

Impact on Microbial Diversity

The 16S rRNA amplicon library resulted in a total of 54,330,655 sequences (forward and reverse, respectively) with a length of 301 bp. After filtering and denoising 158 samples contained on average 10,551 ASV (amplicon sequence variants; minimum 2, maximum 552,293) and 13,405,388 reads per sample (minimum 2, maximum 242,282).

Beside changes in microbial abundance we could also determine distinct changes of microbial composition on plant leaves and on surrounding abiotic surfaces of the plant (**Figure 3**, **Supplementary Figures S4**, **S5** and **Supplementary Tables S4**, **S5**). While samples from abiotic surfaces were similar to controls at the beginning of the experiment, their composition changed together with those from plant leaves along PCoA Axis 1 till the end of the experiment (**Supplementary Figure S6**). During the experiment not only plant leaves became more similar to each other, also samples from abiotic surfaces became more leaf-like. Clustering of samples according to their type of treatment was minor and not significant in pairwise comparisons (**Supplementary Figures S7**, **S8** and **Supplementary Table S5**).

This process was accompanied by a decrease of microbial diversity (Shannon diversity H' in the air: 6.1–5.3, controls: 5.0–3.6, surrounding abiotic surfaces: 6.6–5.0, plant leaves: 5.7–5.5, biostimulant: 7.8–4.2). Only soil samples showed an increase of microbial diversity during the course of the incubation period (H': 6.2–7.0). However, the overall decrease of microbial

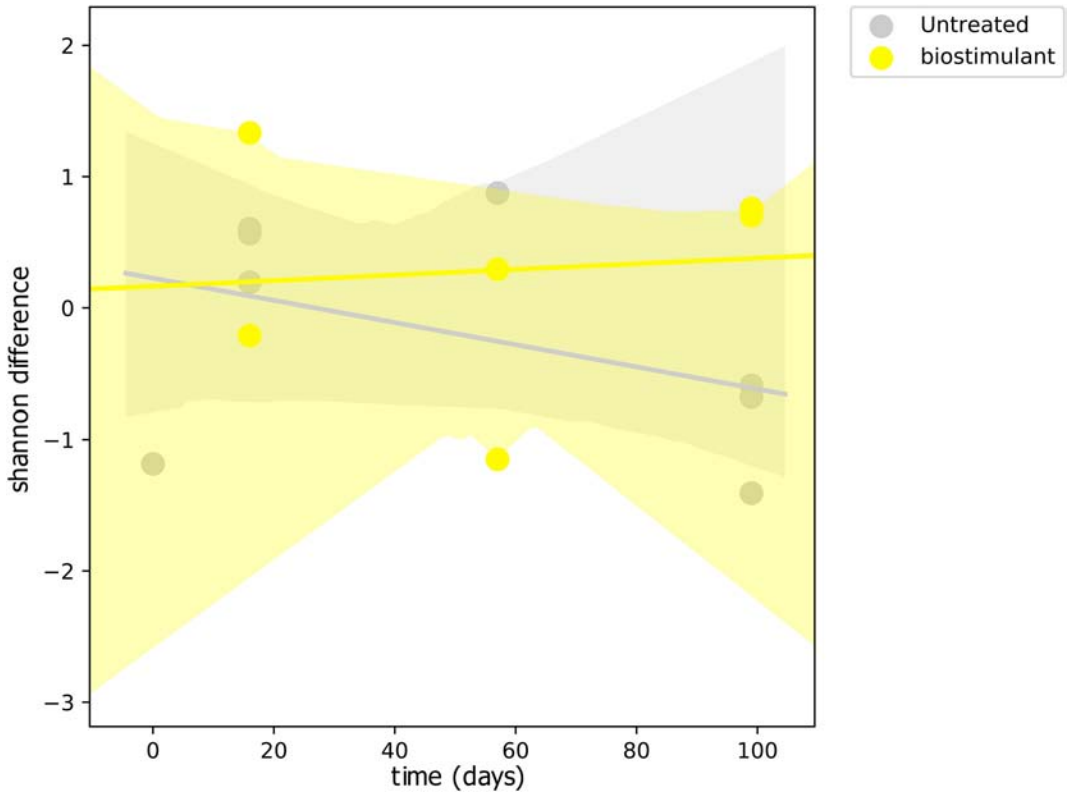


FIGURE 4 | Regression scatterplot to track the rate of change in microbial diversity (Shannon *H'*) from a baseline through the course of the experiment for samples treated with the biostimulant and untreated samples.

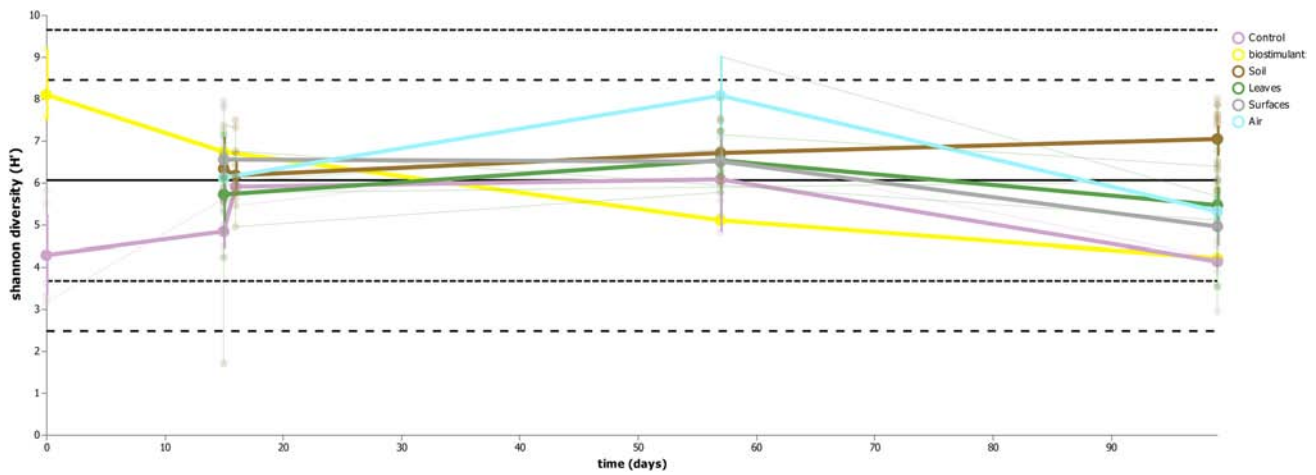


FIGURE 5 | Volatility plot indicating changes of microbial diversity (Shannon *H'*) for the main sample categories (controls, biostimulant, soil, plant leaves, desiccator surfaces and air).

diversity was much lower or even impeded for those samples which were treated with the biostimulant (in direct or indirect contact with the biostimulant: surrounding abiotic surfaces 6.8–5.3, plant leaves 5.5–5.4, soil 6.4–7.3; non-treated samples: surrounding abiotic surfaces 6.3–4.6, plant leaves 6.2–5.4, soil 5.8–6.8; see **Figures 4**, **5**). Nevertheless, pairwise difference tests based on a Wilcoxon signed-rank test were not significant (**Supplementary Table S6**). Changes of Shannon diversity were accompanied by a decrease of richness, phylogenetic diversity and evenness for almost all sample types. Only samples of the plant soil experienced an increase of these alpha diversity metrics (**Supplementary Figure S9**). The microbial composition

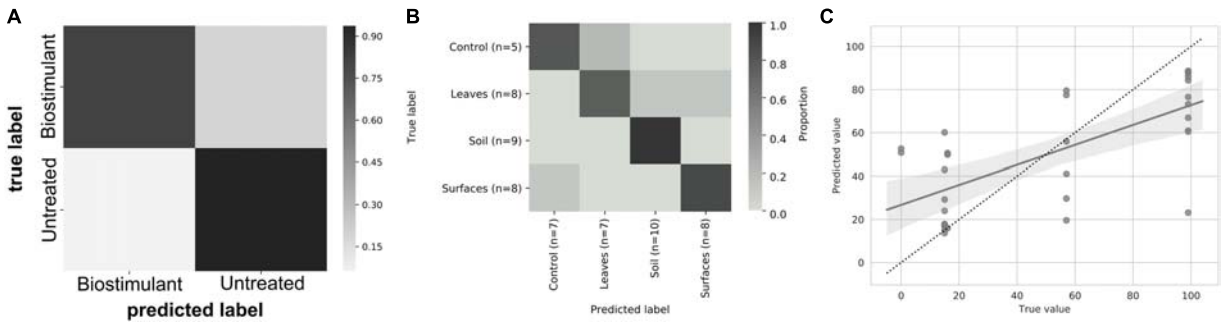


FIGURE 6 | Heatmap of the prediction ability of samples treated with the biostimulant **(A)**, the main sampling categories air, controls, plant leaves, soil, and desiccator surfaces **(B)**, and the day of sampling **(C)**. Machine learning tools based on random Forest classification and regression models were used to train the software to predict a certain metadata category from its ASVs (amplicon sequence variants) profile.

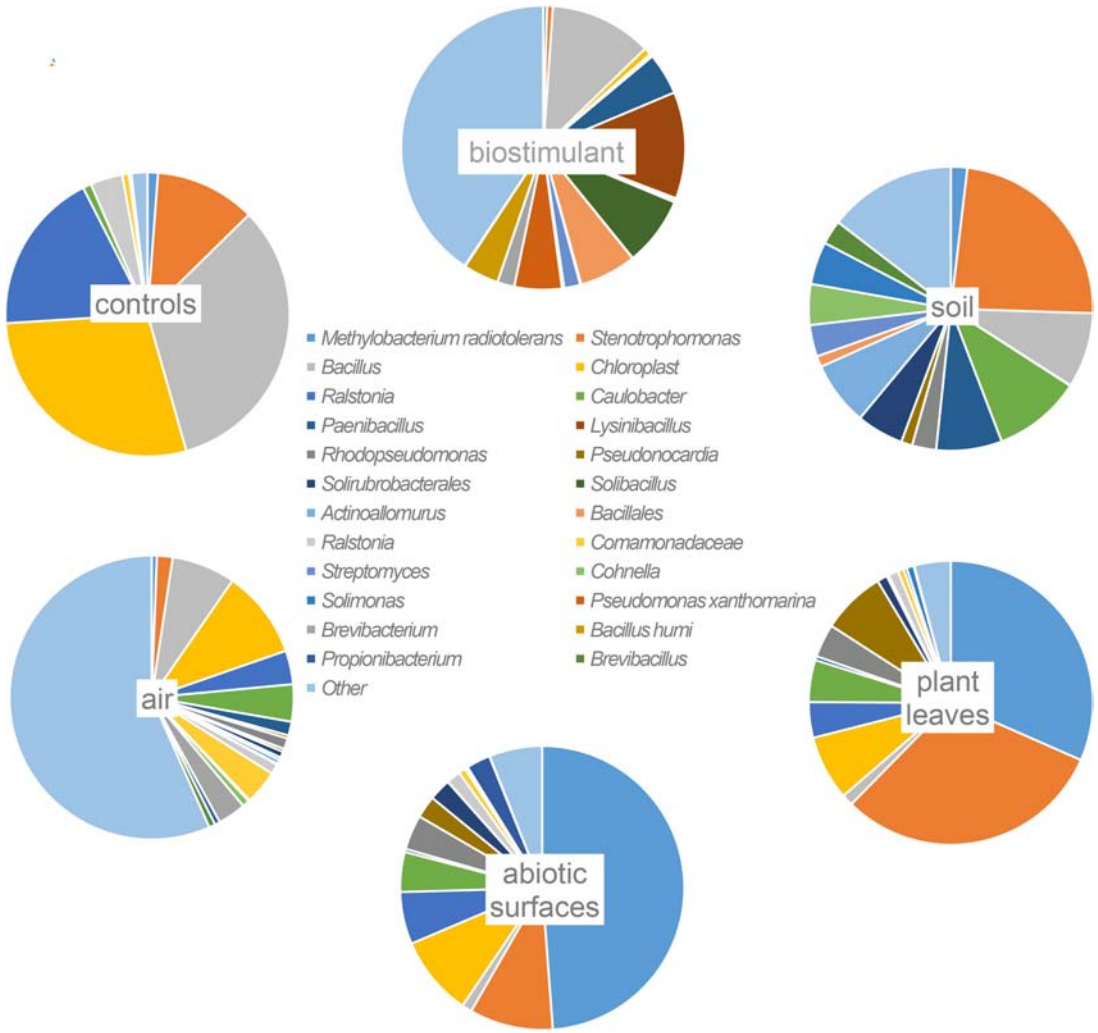


FIGURE 7 | Most abundant taxa (>10% relative abundance) on highest taxonomic levels per origin of samples (abiotic surfaces = desiccator surfaces).

of samples was distinct enough to predict a treatment with the biostimulant to an accuracy of 87.5% with random Forest classification and regression models (see **Figure 6A**). Likewise, the origin of samples could be easily predicted for soil samples (100%) and to a lesser extent for surrounding abiotic surfaces (87.5%) and controls (80%). Plant leaves were not

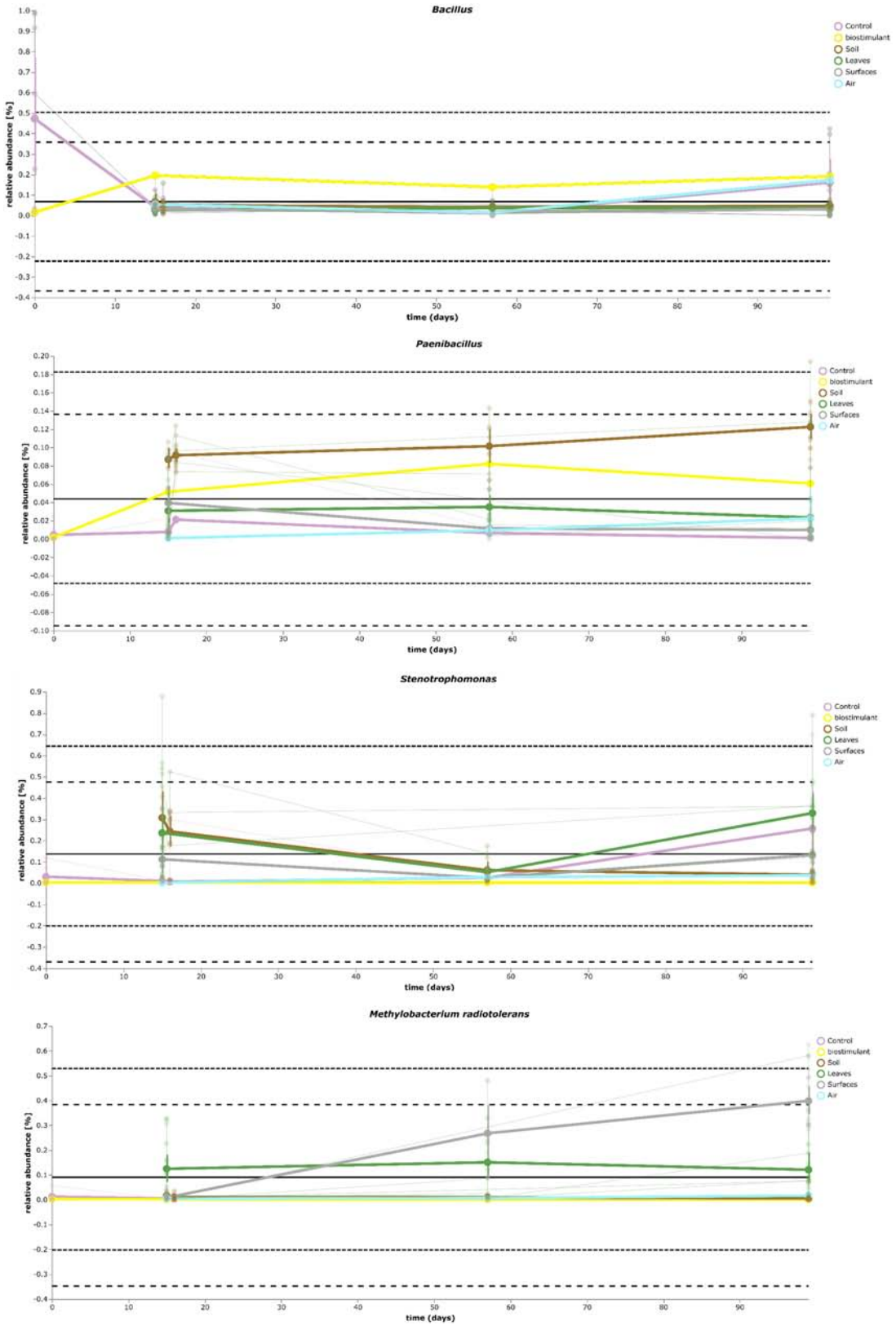


FIGURE 8 | Volatility plot of selected taxa on genus and species level showing distinct changes in relative abundance for different type of samples (biostimulant – *Bacillus*, soil – *Paenibacillus*, plant leaves – *Stenotrophomonas*, desiccator surfaces – *Methylobacterium radiotolerans*).

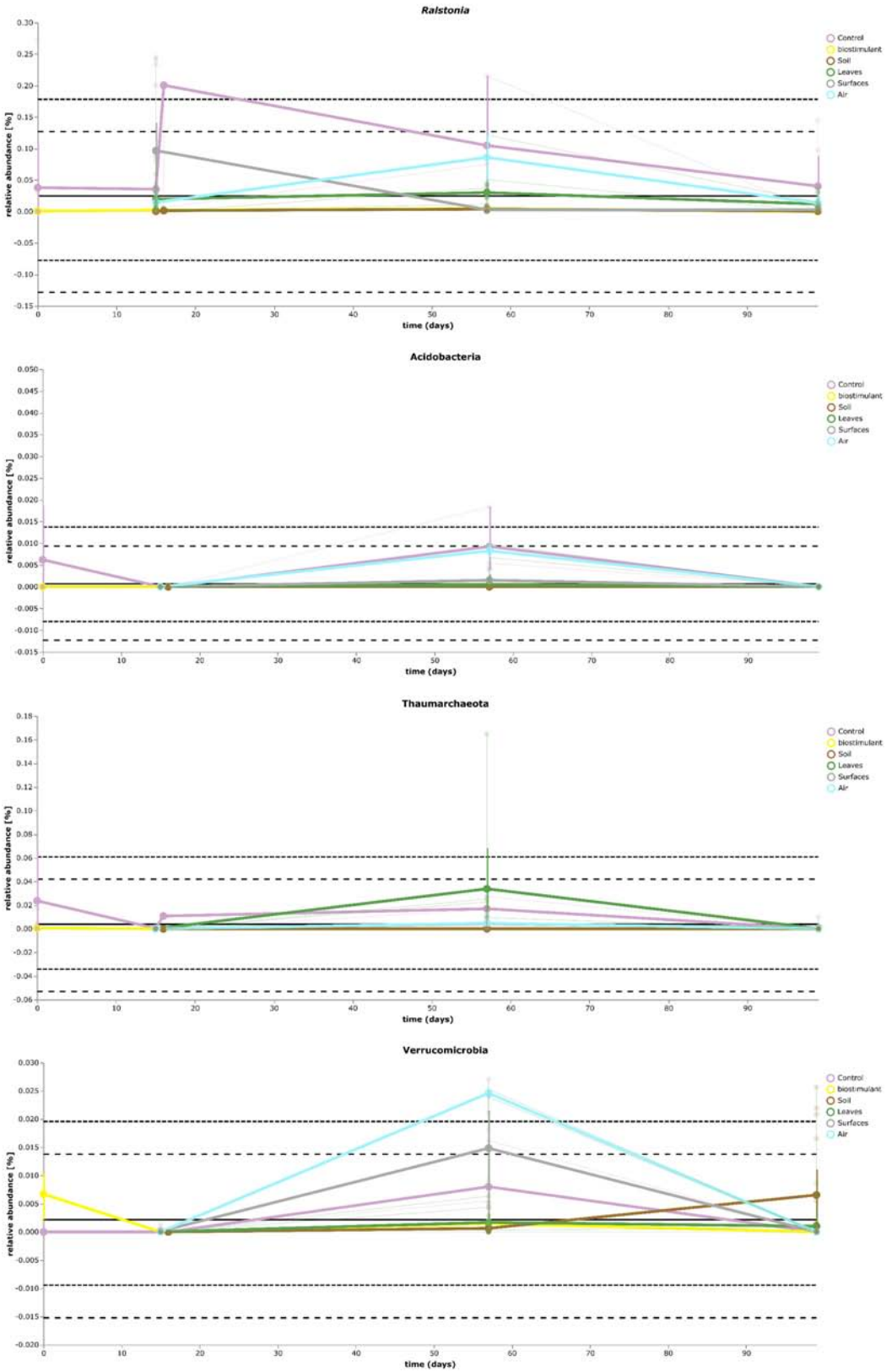


FIGURE 9 | Volatility plot of selected taxa on genus and species level showing distinct changes in relative abundance for different type of samples (controls – *Ralstonia*) and of the transient occurrence of new phyla (*Acidobacteria, Thaumarchaeota,* and *Verrucomicrobia*).

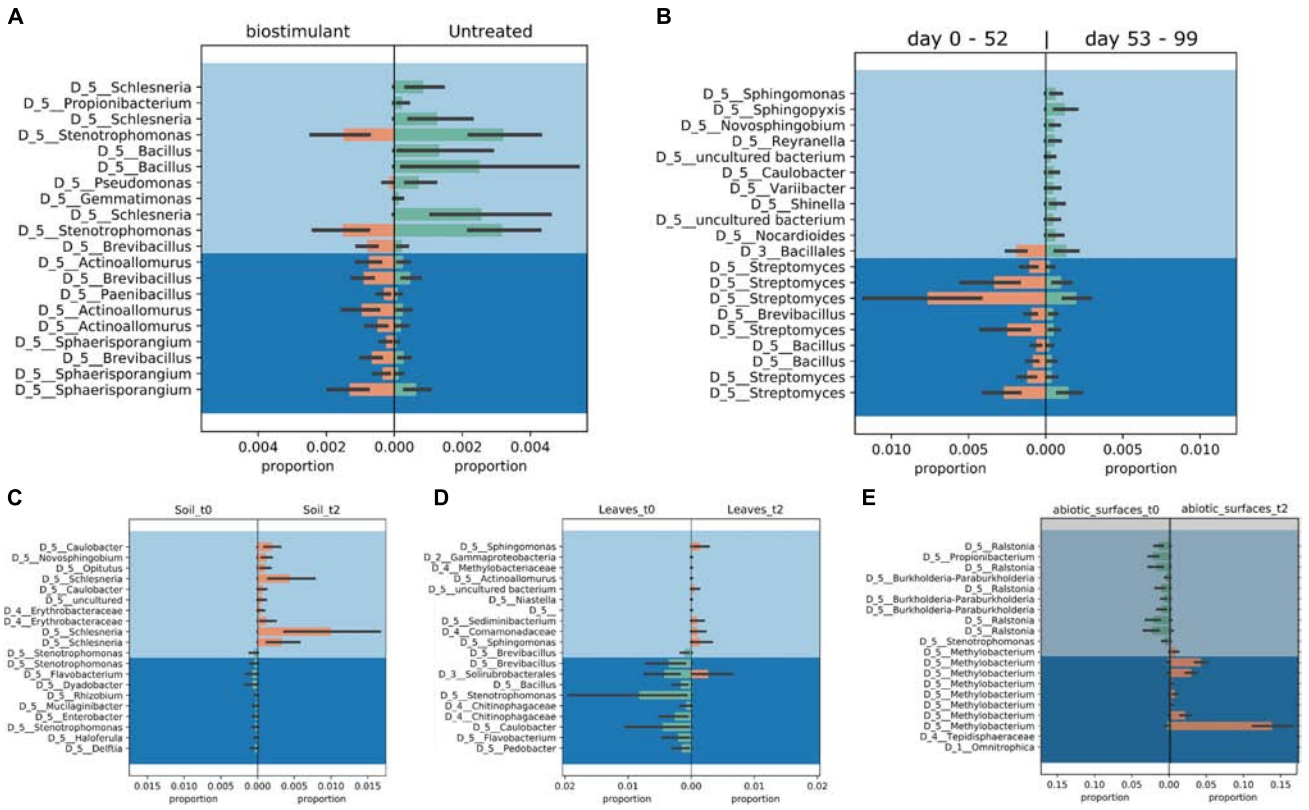


FIGURE 10 | Proportion plots of differential abundance analysis using balances in gneiss according to type of treatment **(A)**, time of sampling **(B)**, soil **(C)**, plant leaves **(D)**, and samples of the desiccator surfaces **(E)**.

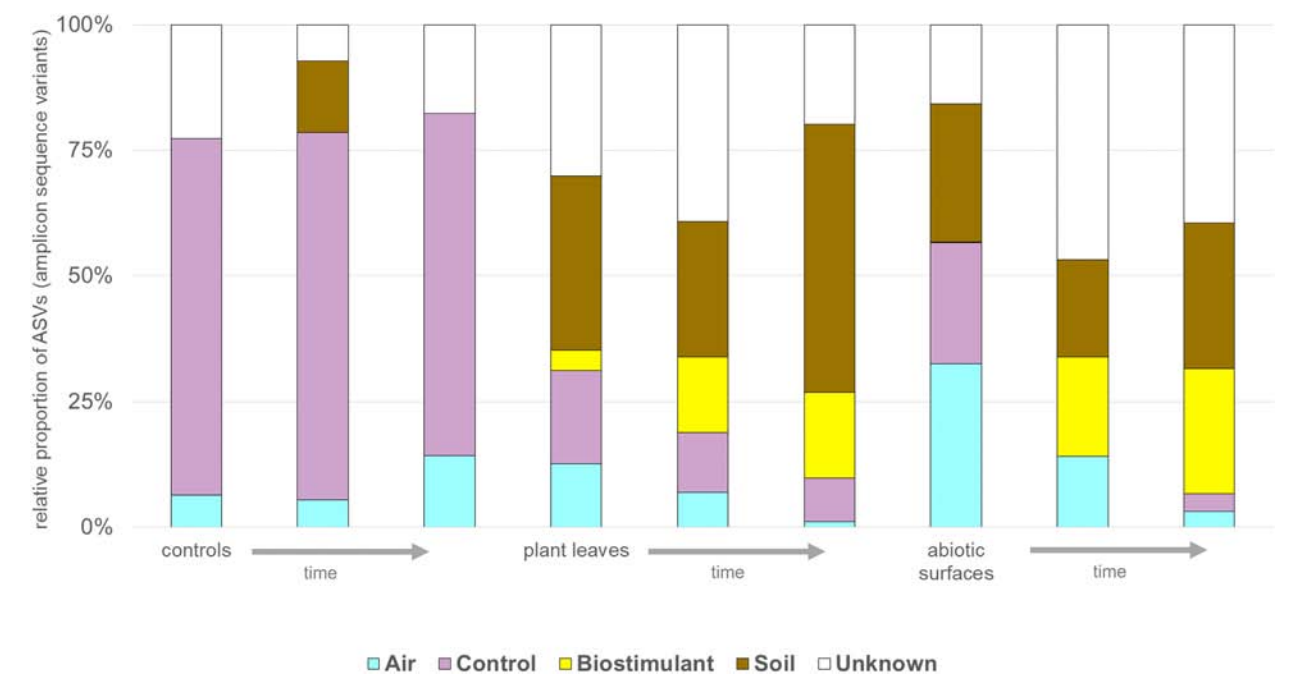


FIGURE 11 | The potential sources of microbes determined with SourceTracker. Change in proportions [%] of microbes from air, controls, the biostimulant, soil and unknown sources for control, plant leaf and desiccator surfaces (abiotic surfaces) samples over time (indicated by an arrow).

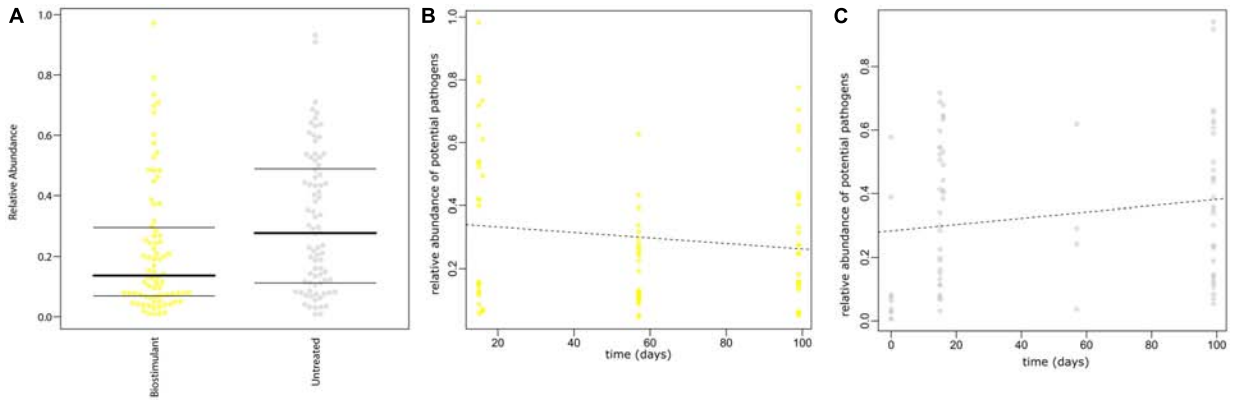


FIGURE 12 | (A) Prediction of potential pathogenic phenotypes for treated samples with the biostimulant and untreated samples. **(B)** Prediction of potential pathogenic phenotypes over time for samples treated with the biostimulant and **(C)** for untreated samples. Predictions are based on the Greengenes database reference set clustered at 97% similarity and precalculated files created with the help of PICRUSt, IMG, KEGG and PATRIC. The plot shows the relative proportion [%] of potential pathogenic traits inferred from the 16S rRNA gene profile.

that characteristic to predict microbial compositions (75%) (**Figure 6B**) as well as the day of sampling (R = 0.68, P = 1.7 * 10^{-5}) (**Figure 6C**).

Impact on Microbial Composition

Most ASVs were assigned to the phyla *Proteobacteria*, *Firmicutes*, and *Actinobacteria*. Compared to gnotobiotic plants, leaves and soil of common plants showed higher relative abundances of *Bacteroidetes*. Further differences on phylum level were apparent between the dry and dissolved biostimulant. In contrast to its dry counterpart the steeped biostimulant showed a higher relative abundance of *Firmicutes* (dissolved biostimulant 76%, dry biostimulant 17%), while all other assigned phyla showed a lower relative abundance in comparison to the dry biostimulant. At the end of the experiment, *Proteobacteria* was the most abundant microbial phylum with an average of 80.1 ± 12.1% on plant leaves and on surrounding abiotic surfaces. In contrast, soil samples showed a balanced distribution of *Firmicutes, Proteobacteria,* and *Actinobacteria*. On genus and species level (see **Figure 7**) most sequences could be assigned to *Methylobacterium radiotolerans* (up to 75%), *Stenotrophomonas* sp. (up to 63%), *Lysinibacillus* sp. (up to 20%), *Bacillus* sp. (up to 19%), *Caulobacter* sp. (15%) and *Paenibacillus* sp. (11%). While *Caulobacter* and *Paenibacillus* were typically observed in samples of the plant soil, the biostimulant was rich in signatures of *Lysinibacillus* and *Bacillus*. During the experiment a predominance of *Stenotrophomonas* formed on plant leaves and *Methylobacterium radiotolerans* dominated on surrounding abiotic surfaces (**Figure 8**). Noteworthy, new phyla, e.g., *Verrucomicrobia* (air and surfaces), *Acidobacteria* (air and controls) and *Thaumarchaeota* (plant leaves) manifest after the treatment with the biostimulant (**Figure 9**). Compared to real biological samples, controls and technical samples showed other lineages like *Ralstonia* (**Figures 7**, **9**) for tap and sterile water or sequences of *Propionibacterium* on alpha wipes. Differential abundance analysis using balances in gneiss revealed that proportions of *Paenibacillus*, *Brevibacillus*, *Actinoallomurus,* and *Sphaerisporangium* were higher in samples and test systems treated with the biostimulant (**Figure 10A**). During the whole incubation period the proportion of sequences assigned to *Caulobacter, Novosphingobium*, or *Schlesneria* increased in particular in the plant soil (**Figures 10B,C**), *Sphingomonas* on plant leaves (**Figure 10D**) and *Methylobacterium* on the surrounding surfaces of the desiccator (**Figure 10E**). Most of these bacterial genera are typical representatives of plant-associated microorganisms, living in the phyllosphere and rhizosphere of plants (*Methylobacterium radiotolerans, Rhodopseudomonas*) or on abiotic surfaces (*Stenotrophomonas*) and water-bearing environments (*Caulobacter*). A core microbiome analysis showed seven ASVs, which were shared to proportions of 80 and 90% with all samples. For samples of the biostimulant and from soil, shared ASVs (80%) were assigned to *Bacillus humi* and *Janibacter*. *Stenotrophomonas,* and *Methylobacterium radiotolerans* were shared (80%) between samples from the biostimulant, plant leaves and surrounding surfaces of the desiccator. Likewise samples from soil, plant leaves and desiccator surfaces as well as samples from plant leaves and desiccator surfaces, respectively, shared (90%) the same ASV assigned to *Methylobacterium radiotolerans*.

SourceTracker was used to identify potential sources of observed microbes (see **Figure 11**). At the beginning of the experiment plant leaf samples and samples from surrounding abiotic surfaces were almost void of typical microbial signatures of the biostimulant. However, during the incubation period, their proportion increased on plant leaves (4–17%) and even highly significant in the case of samples from surrounding abiotic surfaces (0–25%; two-way ANOVA $P = 1.1 * 10^{-7}$). Nevertheless, beside the factor time no significant increase could be determined for the type of treatment or an interaction of both factors (treatment and time; **Supplementary Table S7**). In addition, according to phenotype predictions with BugBase, significantly lower proportions of potential pathogens (Mann–Whitney–Wilcoxon Test, FDR-corrected P = 0.006, **Supplementary**

Table S8) were detected in samples treated with the biostimulant (**Figure 12A**). Correlations of this decline in potential pathogens over time were not significant (Spearman's rank correlation, R: −0.06, P = 0.6) for samples treated with the biostimulant, but the increase in potential pathogens over time was significant for untreated samples (Spearman's rank correlation, R: 0.3, P = 0.003) (**Figures 12B,C** and **Supplementary Tables S9**, **S10**).

Impact on the Metabolic Profile

HPLC-MS measurements of the steeped biostimulant after 1 and 24 h of incubation revealed several plant growth promoting substances (see **Table 1**). Each of the five most common compounds (except 4-amino-3-hydroxybenzoic acid) are important metabolites in biological degradation processes. The longer the incubation time, the more of the compounds dissolved in the suspension (e.g., 4-amino-3-hydroxybenzoic acid increased 13-fold after 24 h). However, no new compounds were found after 24 h compared to 1 h of steeping the biostimulant.

We used PICRUSt to predict functional capabilities from detected microbial compositions (**Supplementary Table S11**) and determine the contributions of certain taxa to detected metabolites of the HPLC-MS analysis. This co-observation of metabolites, predicted functions and potential microbial contributors revealed that species of *Pseudomonas* could have contributed to the detected metabolites in the biostimulant to proportions of up to 0.2%.

DISCUSSION

In this study we showed that biostimulants for plants applied to soil have a potential to shift the microbiota on the above-ground parts of the plant as well as in the surrounding. They supported especially ASVs with beneficial representatives and counteract the loss of microbial biodiversity. The obtained results support the idea that biostimulants can have a positive impact on plant growth and performance by shifting the microbiome and metabolome as well. This can explain the often-reported plant growth promoting effect for biostimulants. However, our experimental setup was not sufficient to significantly distinguish between effects from incubating conditions over time on the microbiome and actual effects of the biostimulant. In addition, the underlying mechanism of microbial transfer is still unknown. Since simple spilling of the irrigation solution was prevented, we suspect other vectors like microscopic aerosols, or actual microbial locomotion from the rhizosphere through the endosphere to the phyllosphere and microbial deposition from plant leaves to surrounding abiotic surfaces as most promising explanations of the observed phenomena. Our ideas are based on the reported transfer of microorganisms from other holobionts to their surroundings (Qian et al., 2012) and the obvious connection between different sampled microenvironments and microbiomes (Badri et al., 2013). Nevertheless, extensive analysis of the changing air quality as a main target for microbial transfer was not possible due to the detection limit of the applied methods and therefore this analysis was beyond the scope of this study.

At phylum level, the treatment resulted in comparison to the untreated control in an increase of *Bacteroidetes* and a surprising peak of new phyla at the second sampling event, e.g., *Verrucomicrobia*, *Acidobacteria,* and *Thaumarchaeota. Verrucomicrobia* comprise mainly as-yet uncultivated species; several have been already identified in association with plant hosts (Bragina et al., 2015). Species of the phylum *Acidobacteria* (e.g., *Granulicella paludicola*, *G. pectinivorans*, *G. aggregans, G. rosea, Acidicapsa borealis, A. ligni,* and *Terriglobus tenax*) were shown to actively interact with plants and act as plant growth-promoting bacteria (Kielak et al., 2016). Interestingly, plant hosts were shown to select particular groups of *Acidobacteria* and *Verrucomicrobia* (Da Rocha et al., 2013). Recently,

TABLE 1 | High-performance liquid chromatography (HPLC)-MS results for the steeped biostimulant.

Name	Sample/ control ratio	KEGG pathways	Mean area (1 h)	Mean area (24 h)	Difference 1 vs. 24 h [%]
Indole-3-acetic acid	4.8	Metabolic pathways, Biosynthesis of plant hormones and alkaloids derived from terpenoid and polyketide, Plant hormone signal transduction, Tryptophan metabolism	607016.2	2666757.8	439.3
Syringic acid	2.3	Microbial metabolism in diverse environments, Aminobenzoate degradation	731223.7	1649582.1	225.6
4-Amino-3- Hydroxybenzoic acid	13.4	–*	42164.6	589748.7	1398.7
2-Naphthalene sulfonic acid	15.1	Naphthalene degradation	49270.5	319698.5	648.9
Acetophenone	3.4	Microbial metabolism in diverse environments, Ethylbenzene-, DDT-, Bisphenol- and aromatic compounds degradation	99621.9	212796.9	213.6

**No KEGG pathway entry found.*

Sterile H_2O was used for steeping the biostimulant and served as a control. The five most abundant compounds are listed, which showed a sample to control ratio over 2. Detected metabolites were set into the context of KEGG pathways (http://www.genome.jp/kegg/pathway.html) to identify their potential role in biological systems.

representatives from *Thaumarchaeota* were identified to be also plant-associated with potential beneficial functions such as the production of phytohormones, which were identified by metagenomic mining (Taffner et al., 2018). Unfortunately isolates of all three phyla are difficult or even impossible to obtain. Therefore, the observation that these groups can be enriched by biostimulants is an important finding for targeted microbiome engineering. The microbiome shift was also visible at genus level; here the proportion of potentially beneficial microorganisms like *Methylobacterium, Stenotrophomonas,* and *Caulobacter* increased relatively and significantly for *Brevibacillus, Actinoallomurus, Paenibacillus,* and *Sphaerisporangium*. Species of *Brevibacillus* have potential to act as biological control agents as they were shown to produce chitinases to degrade fungal cell walls (Hassi et al., 2012) or can act pesticidal against insects, nematodes and mollusks (Ruiu, 2013) and plant-associated endophytic Actinobacteria like *Actinoallomurus* and *Sphaerisporangium* were already suggested as plant-growth promoting agents in the past (Qin et al., 2011; Hamedi and Mohammadipanah, 2014). Furthermore, the overall loss of microbial diversity was reduced, which took place under our experimental conditions, and the built environment became more plant-like.

Our model biostimulant is a mixture containing dried vermicompost, compost and plant residues. These mixtures are typical for biostimulants and therefore it is often difficult to identify the active ingredient. Results of our study showed that the biostimulant contain a high number of intact microbial cells, which definitely contribute to the microbiome shift and effect. Obviously, the positive effect of the biostimulant was not derived from its microbial content alone. The metabolites, which were determined in the biostimulant, contained properties, which can be useful for both the plants and the human's well-being. For instance, syringic acid acts antifungal (Chong et al., 2012) and indole-3-acetic acid has plant growth promoting effects (Estelle, 2001). Moreover, acetophenone and syringic acid even have reported health benefits, with improvement against hypoglycemia and diabetes (Muthukumaran et al., 2013; Jiang et al., 2018). However, it was not investigated in this study, if these properties could be passed on to the benefit of human health. Hence, follow-up studies should extend our findings to human well-being, other plant species and biostimulants as well as experimental settings that better represent normal room conditions. Unfortunately the metabolome was not investigated at all sampling events in parallel with the microbiome. Moreover late stage effects of the microbiome and metabolome were not monitored. In addition, actual functional metagenomics of the treated microbiomes would be helpful to identify changed metabolic capabilities beyond simple correlations of predicted pathways based on static databases and measured metabolites.

We used an indoor plant as a model; all house plants have amazing capacities beyond simple embellishments, e.g., to improve indoor air quality (Sriprapat et al., 2014), and human performance in built environments (Bringslimark et al., 2009). As we could show before, plants can also shape the microbiome in an indoor environment (Mahnert et al., 2015). Therefore, indoor plants can act as vectors to transfer beneficial microbiota and increase biodiversity of microbial wastelands in built environments (Gibbons, 2016). Even more goal-driven is the application of a defined microbial consortia as a biostimulant on an indoor plant to manipulate the microbiome of an indoor environment. As we could show, beneficial properties of the biostimulant (high diversity of intact and beneficial microbiota) may be transferrable beyond the plant itself and extendable to the close vicinity of it. However, it is important to note that necessary compromises of the experimental set-up could limit general validity of our results. Most indoor environments are characterized by a few indoor plants in vast open spaces. Here, the proportion of plant-leaf to surrounding abiotic surfaces, the number of occupants their actions, the microclimate and longitudinal parameters can be completely different. In addition, effects of the substrate were pretty much ignored. The rhizosphere of common soil is a hotspot for diverse microbial interactions, which are absent in the sterilized soil we applied. Furthermore the impact of fertilizers and nutrients was not investigated. Therefore future studies should also include additional control experiments with synthetic nutrient mixtures, common soil with an active rhizosphere as well as non-soil based substrates. This would help to differentiate between actual drivers (nutrients, metabolites, or microbes) of observed microbial changes. Nevertheless, our experimental set-up tried to limit unknown influences by environmental parameters and establish sterile replicable test systems with defined settings by the use and comparison of sterile, DNA-free sampling equipment, plant soil, gnotobiotic plants, a constant microclimate and many controls of the environment. Apart from these limitations, our results indicate that core and shared microbial signatures of the biostimulant (e.g., *Methylobacterium*) were transferred from the irrigated soil not only to the plant surface itself, but also to surrounding abiotic surfaces. The detected microbiota may already support a healthy environment for the treated plant. For instance, *Lysinibacillus* and *Bacillus* function as bio-insecticides (Berry, 2012), and *Bacillus a*nd *Paenibacillus* augment plant growth (Bloemberg and Lugtenberg, 2001).

Hence, we envision combined effects of biostimulants not only for plant, but also human health in the future. Both plants as well as human beings rely on beneficial microbiota and overall diversity (Berg et al., 2017). However, common human activities in the environment reduce microbial diversity and therefore destabilize important microbial networks (Blaser, 2016; van der Heijden and Hartmann, 2016). This processes could facilitate the entry and establishment of pathogens in a system with serious consequences for the plant or human holobiont (Kennedy et al., 2002). As a proof of principle this study should be a first step to design biostimulants not only for plants, but also other holobionts in respective target environments in the future.

DATA AVAILABILITY

16S rRNA gene amplicon raw data is available at the European Nucleotide Archive - ENA (https://www.ebi.ac.uk) under project ID PRJEB27998.

AUTHOR CONTRIBUTIONS

AM and GB study design. MS plant propagations. MH conducting experiments and measurements. AM and MH data analysis. AM, MH, and GB wrote the manuscript. All authors approved the final manuscript.

FUNDING

This study was supported by an "Innovation check plus" (858649) from the Austrian Research Promotion Agency (FFG) and the company GARTENleben GmbH, Zwettl, and a grant of the Austrian Science Fund (FWF) and the federal state government of Styria to GB (P29285-BBL). The authors also gratefully acknowledge support from the central lab of NAWI Graz.

ACKNOWLEDGMENTS

We like to thank Elisabeth Koppensteiner and Daniela Tüchler for proposing "bio-guss universal compost tea" as a biostimulant in this study. We thank Tomislav Cernava, Daria Rybakova, Henry Müller, Barbara Fetz, and Angelika Schäfer (all Graz) for their helpful support in experimental preparations and during conducted measurements.

SUPPLEMENTARY MATERIAL

The Supplementary Material for this article can be found online at: https://www.frontiersin.org/articles/10.3389/fmicb.2018.02985/full#supplementary-material

REFERENCES

Allen, F., Pon, A., Wilson, M., Greiner, R., and Wishart, D. (2014). CFM-ID: a web server for annotation, spectrum prediction and metabolite identification from tandem mass spectra. *Nucleic Acids Res.* 42, 94–99. doi: 10.1093/nar/gku436

Anderson, M. J. (2001). A new method for non-parametric multivariate analysis of variance. *Austral Ecol.* 26, 32–46.

Badri, D. V., Zolla, G., Bakker, M. G., Manter, D. K., and Vivanco, J. M. (2013). Potential impact of soil microbiomes on the leaf metabolome and on herbivore feeding behavior. *New Phytol.* 198, 264–273. doi: 10.1111/nph.12124

Berg, G. (2009). Plant-microbe interactions promoting plant growth and health: perspectives for controlled use of microorganisms in agriculture. *Appl. Microbiol. Biotechnol.* 84, 11–18. doi: 10.1007/s00253-009-2092-7

Berg, G., Köberl, M., Rybakova, D., Müller, H., Grosch, R., and Smalla, K. (2017). Plant microbial diversity is suggested as the key to future biocontrol and health trends. *FEMS Microbiol. Ecol.* 93:fix050. doi: 10.1093/femsec/fix050

Berg, G., Krechel, A., Ditz, M., Sikora, R. A., Ulrich, A., and Hallmann, J. (2005). Endophytic and ectophytic potato-associated bacterial communities differ in structure and antagonistic function against plant pathogenic fungi. *FEMS Microbiol. Ecol.* 51, 215–229. doi: 10.1016/j.femsec.2004.08.006

Berg, G., Mahnert, A., and Moissl-Eichinger, C. (2014). Beneficial effects of plant-associated microbes on indoor microbiomes and human health? *Front. Microbiol.* 5:15. doi: 10.3389/fmicb.2014.00015

Berry, C. (2012). The bacterium, *Lysinibacillus sphaericus*, as an insect pathogen. *J. Invertebr. Pathol.* 109, 1–10. doi: 10.1016/j.jip.2011.11.008

Blaser, M. J. (2016). Antibiotic use and its consequences for the normal microbiome. *Science* 352, 544–545. doi: 10.1126/science.aad9358

Bloemberg, G. V., and Lugtenberg, B. J. J. (2001). Molecular basis of plant growth promotion and biocontrol by rhizobacteria. *Curr. Opin. Plant Biol.* 4, 343–350. doi: 10.1016/S1369-5266(00)00183-7

Bokulich, N. A., Dillon, M. R., Zhang, Y., Rideout, J. R., Bolyen, E., Li, H., et al. (2018a). q2-longitudinal: longitudinal and paired-sample analyses of microbiome data. *mSystems* 3, 1–9. doi: 10.1128/mSystems.00219-18

Bokulich, N. A., Dillon, M., Bolyen, E., Kaehler, B. D., Huttley, G. A., and Caporaso, J. G. (2018b). q2-sample-classifier: machine-learning tools for microbiome classification and regression. *bioRxiv* [Preprint]. doi: 10.1101/306167

Bolyen, E., Rideout, J. R., Dillon, M. R., Bokulich, N. A., Abnet, C., Al-Ghalith, G. A., et al. (2018). QIIME 2: reproducible, interactive, scalable, and extensible microbiome data science. *PeerJ Prepr.* 6:e27295v1. 1–22. [Preprints]. doi: 10.7287/peerj.preprints.27295v1

Bragina, A., Berg, C., and Berg, G. (2015). The core microbiome bonds the Alpine bog vegetation to a transkingdom metacommunity. *Mol. Ecol.* 24, 4795–4807. doi: 10.1111/mec.13342

Brilli, F., Fares, S., Ghirardo, A., de Visser, P., Calatayud, V., Muñoz, A., et al. (2018). Plants for sustainable improvement of indoor air quality. *Trends Plant Sci.* 23, 507–512. doi: 10.1016/j.tplants.2018.03.004

Bringslimark, T., Hartig, T., and Patil, G. G. (2009). The psychological benefits of indoor plants: a critical review of the experimental literature. *J. Environ. Psychol.* 29, 422–433. doi: 10.1016/j.jenvp.2009.05.001

Bulgarelli, D., Schlaeppi, K., Spaepen, S., Ver Loren van Themaat, E., and Schulze-Lefert, P. (2013). Structure and functions of the bacterial microbiota of plants. *Annu. Rev. Plant Biol.* 64, 807–838. doi: 10.1146/annurev-arplant-050312-120106

Callahan, B. J., Mcmurdie, P. J., Rosen, M. J., Han, A. W., Johnson, A. J., and Holmes, S. P. (2016). DADA2: high resolution sample inference from amplicon data. *Nat. Methods* 13, 581–583. doi: 10.1038/nmeth.3869

Caporaso, J. G., Kuczynski, J., Stombaugh, J., Bittinger, K., Bushman, F. D., Costello, E. K., et al. (2010). QIIME allows analysis of high- throughput community sequencing data. *Nat. Methods* 7, 335–336. doi: 10.1038/NMETH.F.303

Chong, K. P., Atong, M., and Rossall, S. (2012). The role of syringic acid in the interaction between oil palm and *Ganoderma boninense*, the causal agent of basal stem rot. *Plant Pathol.* 61, 953–963. doi: 10.1111/j.1365-3059.2011.02577.x

Clarke, K. R., and Ainsworth, M. (1993). A method of linking multivariate community structure to environmental variables. *Mar. Ecol. Prog. Ser.* 92, 205–219. doi: 10.3354/meps092205

Da Rocha, U. N., Plugge, C. M., George, I., Van Elsas, J. D., and Van Overbeek, L. S. (2013). The rhizosphere selects for particular groups of *Acidobacteria* and *Verrucomicrobia*. *PLoS One* 8:e82443. doi: 10.1371/journal.pone.0082443

du Jardin, P. (2015). Plant biostimulants: definition, concept, main categories and regulation. *Sci. Hortic.* 196, 3–14. doi: 10.1016/j.scienta.2015.09.021

Erlacher, A., Cardinale, M., Grosch, R., Grube, M., and Berg, G. (2014). The impact of the pathogen *Rhizoctonia solani* and its beneficial counterpart *Bacillus amyloliquefaciens* on the indigenous lettuce microbiome. *Front. Microbiol.* 5:175. doi: 10.3389/fmicb.2014.00175

Estelle, M. (2001). Plant hormones: transporters on the move. *Nature* 413, 374–375. doi: 10.1038/35096672

Gibbons, S. M. (2016). The built environment is a microbial wasteland. *mSystems* 1:e00033. doi: 10.1128/mSystems.00033-16.Copyright

Hamedi, J., and Mohammadipanah, F. (2014). Biotechnological application and taxonomical distribution of plant growth promoting actinobacteria. *J. Ind. Microbiol. Biotechnol.* 42, 157–171. doi: 10.1007/s10295-014-1537-x

Hardoim, P. R., van Overbeek, L. S., Berg, G., Pirttilä, A. M., Compant, S., Campisano, A., et al. (2015). The hidden world within plants: ecological and evolutionary considerations for defining functioning of microbial endophytes. *Microbiol. Mol. Biol. Rev.* 79, 293–320. doi: 10.1128/MMBR.00050-14

Hassi, M., El Guendouzi, S., Haggoud, A., David, S., Ibnsouda, S., Houari, A., et al. (2012). Antimycobacterial activity of a *Brevibacillus laterosporus* strain isolated from a moroccan soil. *Braz. J. Microbiol.* 43, 1516–1522. doi: 10.1590/S1517-83822012000400036

Jiang, H. W., Gu, S. S., Cao, L., Jiang, S. S., Lin, J., Chen, Y., et al. (2018). Potential hypoglycemic effect of acetophenones from the root bark of *Cynanchum wilfordii*. *Nat. Prod. Res.* doi: 10.1080/14786419.2018.1443100 [Epub ahead of print].

Katoh, K., and Standley, D. M. (2013). MAFFT multiple sequence alignment software version 7: improvements in performance and usability. *Mol. Biol. Evol.* 30, 772–780. doi: 10.1093/molbev/mst010

Kennedy, T. A., Naeem, S., Howe, K. M., Knops, J. M. H., Tilman, D., and Reich, P. (2002). Biodiversity as a barrier to ecological invasion. *Nature* 417, 636–638. doi: 10.1038/nature00776

Kielak, A. M., Cipriano, M. A. P., and Kuramae, E. E. (2016). Acidobacteria strains from subdivision 1 act as plant growth-promoting bacteria. *Arch. Microbiol.* 198, 987–993. doi: 10.1007/s00203-016-1260-2

Knights, D., Kuczynski, J., Charlson, E. S., Zaneveld, J., Mozer, M. C., Collman, R. G., et al. (2011). Bayesian community-wide culture-independent microbial source tracking. *Nat. Methods* 8, 761–763. doi: 10.1038/nmeth.1650

Kröber, M., Wibberg, D., Grosch, R., Eikmeyer, F., Verwaaijen, B., Chowdhury, P., et al. (2014). Effect of the strain *Bacillus amyloliquefaciens* FZB42 on the microbial community in the rhizosphere of lettuce under field conditions analyzed by whole metagenome sequencing. *Front. Microbiol.* 5:252. doi: 10.3389/fmicb.2014.00252

Kruskal, W. H., and Wallis, W. A. (1952). Use of ranks in one-criterion variance analysis. *J. Am. Stat. Assoc.* 47, 583–621. doi: 10.1080/01621459.1952.10483441

Langille, M. G. I., Zaneveld, J., Caporaso, J. G., McDonald, D., Knights, D., Reyes, J. A., et al. (2013). Predictive functional profiling of microbial communities using 16S rRNA marker gene sequences. *Nat. Biotechnol.* 31, 814–821. doi: 10.1038/nbt.2676

Lim, S. L., Wu, T. Y., Lim, P. N., and Shak, K. P. Y. (2015). The use of vermicompost in organic farming: overview, effects on soil and economics. *J. Sci. Food Agric.* 95, 1143–1156. doi: 10.1002/jsfa.6849

Mahnert, A., Moissl-Eichinger, C., and Berg, G. (2015). Microbiome interplay: plants alter microbial abundance and diversity within the built environment. *Front. Microbiol.* 6:887. doi: 10.3389/fmicb.2015.00887

Mandal, S., Van Treuren, W., White, R. A., Eggesbø, M., Knight, R., and Peddada, S. D. (2015). Analysis of Composition of Microbiomes (ANCOM): a novel method for studying microbial composition. *Microb. Ecol. Health Dis.* 26:27663. doi: 10.3402/mehd.v26.27663

Mantel, N. (1967). The detection of disease clustering and a generalized regression approach. *Cancer Res.* 27, 209–220. doi: 10.1038/214637b0

Moissl-Eichinger, C., Auerbach, A. K., Probst, A. J., Mahnert, A., Tom, L., Piceno, Y., et al. (2015). Quo vadis? Microbial profiling revealed strong effects of cleanroom maintenance and routes of contamination in indoor environments. *Sci. Rep.* 5:9156. doi: 10.1038/srep09156

Morton, J. T., Sanders, J., Quinn, R. A., Mcdonald, D., Gonzalez, A., Vázquez-Baeza, Y., et al. (2017). Balance trees reveal microbial niche differentiation. *mSystems* 2:e00162-16. doi: 10.1128/mSystems.00162-16

Muthukumaran, J., Srinivasan, S., Venkatesan, R. S., Ramachandran, V., and Muruganathan, U. (2013). Syringic acid, a novel natural phenolic acid, normalizes hyperglycemia with special reference to glycoprotein components in experimental diabetic rats. *J. Acute Dis.* 2, 304–309. doi: 10.1016/S2221-6189(13)60149-3

Pearson, K. (1895). Note on regression and inheritance in the case of two parents. *Proc. R. Soc. Lond.* 58, 240–242. doi: 10.1098/rspl.1895.0041

Pedregosa, F., Varoquaux, G., Gramfort, A., Michel, V., Thirion, B., Grisel, O., et al. (2012). Scikit-learn: machine learning in python. *J. Mach. Learn. Res.* 12, 2825–2830. doi: 10.1007/s13398-014-0173-7.2

Philippot, L., Raaijmakers, J. M., Lemanceau, P., and van der Putten, W. H. (2013). Going back to the roots: the microbial ecology of the rhizosphere. *Nat. Rev. Microbiol.* 11, 789–799. doi: 10.1038/nrmicro3109

Price, M. N., Dehal, P. S., and Arkin, A. P. (2010). FastTree 2 - Approximately maximum-likelihood trees for large alignments. *PLoS One* 5:e9490. doi: 10.1371/journal.pone.0009490

Qian, J., Hospodsky, D., Yamamoto, N., Nazaroff, W. W., and Peccia, J. (2012). Size-resolved emission rates of airborne bacteria and fungi in an occupied classroom. *Indoor Air* 22, 339–351. doi: 10.1111/j.1600-0668.2012.00769.x

Qin, S., Xing, K., Jiang, J. H., Xu, L. H., and Li, W. J. (2011). Biodiversity, bioactive natural products and biotechnological potential of plant-associated endophytic actinobacteria. *Appl. Microbiol. Biotechnol.* 89, 457–473. doi: 10.1007/s00253-010-2923-6

R Core Team (2014). *R: A Language and Environment for Statistical Computing*. Vienna: R Foundation for Statistical Computing.

Ruiu, L. (2013). *Brevibacillus laterosporus*, a pathogen of invertebrates and a broad-spectrum antimicrobial species. *Insects* 4, 476–492. doi: 10.3390/insects4030476

Schwendner, P., Mahnert, A., Koskinen, K., Moissl-Eichinger, C., Wirth, R., Berg, G., et al. (2017). Preparing for the manned Mars journey: microbiome dynamics in the confined Mars500 habitat during simulated Mars flight and landing. *Microbiome* 5:129. doi: 10.1186/s40168-017-0345-8

Seabold, S., and Perktold, J. (2010). "Statsmodels: econometric and statistical modeling with python," in *Proceedings of the 9th Python in Science Conference*, Austin, TX, 57–61.

Spearman, C. (1904). The proof and measurement of association between two things. *Am. J. Psychol.* 15, 72–101. doi: 10.2307/1412159

Sriprapat, W., Suksabye, P., Areephak, S., Klantup, P., Waraha, A., Sawattan, A., et al. (2014). Uptake of toluene and ethylbenzene by plants: removal of volatile indoor air contaminants. *Ecotoxicol. Environ. Saf.* 102, 147–151. doi: 10.1016/j.ecoenv.2014.01.032

Subramanian, S., Huq, S., Yatsunenko, T., Haque, R., Mahfuz, M., Alam, M. A., et al. (2014). Persistent gut microbiota immaturity in malnourished Bangladeshi children. *Nature* 510, 417–421. doi: 10.1038/nature13421

Taffner, J., Erlacher, A., Bragina, A., Berg, C., and Moissl-eichinger, C. (2018). What is the role of Archaea in plants? new insights from the vegetation of alpine bogs. *mSphere* 3:e00122-18. doi: 10.1128/mSphere.00122-18

Turner, T. R., James, E. K., and Poole, P. S. (2013). The plant microbiome. *Genome Biol.* 14:209. doi: 10.1186/gb-2013-14-6-209

van der Heijden, M. G. A., and Hartmann, M. (2016). Networking in the plant microbiome. *PLoS Biol.* 14:e1002378. doi: 10.1371/journal.pbio.1002378

Vandenkoornhuyse, P., Quaiser, A., Duhamel, M., Le Van, A., and Dufresne, A. (2015). The importance of the microbiome of the plant holobiont. *New Phytol.* 206, 1196–1206. doi: 10.1111/nph.13312

Ward, T., Larson, J., Meulemans, J., Hillmann, B., Lynch, J., Sidiropoulos, D., et al. (2017). BugBase predicts organism level microbiome phenotypes. *bioRxiv* [Preprint]. doi: 10.1101/133462

Yakhin, O. I., Lubyanov, A. A., Yakhin, I. A., and Brown, P. H. (2017). Biostimulants in plant science: a global perspective. *Front. Plant Sci.* 7:2049. doi: 10.3389/fpls.2016.02049

Zhang, Y., Han, S. W., Cox, L. M., and Li, H. (2017). A multivariate distance-based analytic framework for microbial interdependence association test in longitudinal study. *Genet. Epidemiol.* 41, 769–778. doi: 10.1002/gepi.22065

Conflict of Interest Statement: The product "bio-guss universal compost tea" was provided by the GARTENleben GmbH. The company suggested the application of this product as a biostimulant in this study.

frontiers in Microbiology

ORIGINAL RESEARCH
published: 06 December 2018
doi: 10.3389/fmicb.2018.02986

Effect of Tillage Treatment on the Diversity of Soil Arbuscular Mycorrhizal Fungal and Soil Aggregate-Associated Carbon Content

*Xingli Lu[1], Xingneng Lu[2] and Yuncheng Liao[3]**

[1] College of Agronomy, Ningxia University, Yinchuan, China, [2] Yinchuan Provincial Sub-branch, The People's Bank of China, Yinchuan, China, [3] College of Agronomy, Northwest A&F University, Yangling, China

No-tillage agriculture can sustain productivity and protect the environment. A comprehensive understanding of soil arbuscular mycorrhizal (AM) fungal diversity and soil carbon distribution within aggregate fractions is essential to the evaluation of no-tillage agriculture. The long-term field experiment included two tillage treatments (1) no tillage with straw returned to the soil (NTS), and (2) conventional mouldboard-plowing tillage without straw (CT), and was conducted on the Loess Plateau, north-western China, from October 2009. The soil samples were collected from the surface layer (0–20 cm depth) at the maturation stage of the summer maize (*Zea mays* L.) for analyzing aggregates separated by the dry-sieving method. The organic carbon content in the bulk soil and different particle size aggregates were measured using the dichromate oxidization method. The species compositions of soil AM fungi were compared by applying high-throughput sequencing of 18S rRNA. The results showed that the NTS had 9.1–12.2% higher percentage of soil macro-aggregates, resulting in 9.8% increase in mean weight diameter and 10.0% increase in bulk soil organic carbon content as compared with CT treatment. In addition, the NTS treatment had significantly higher percentages of *Septoglomus* and *Glomus* than the CT treatment. We also found some significant differences in the fungal communities of the soils of the two treatments. There was a strong positive relationship between bulk soil organic carbon and the percentages of *Septoglomus* and *Glomus*. Our results suggested that the NTS treatment had a protective effect on AM fungal community structures, which might play a key role in the development of agricultural sustainability in the Loess Plateau of China.

Keywords: AM fungi, no-tillage, soil carbon, maize field, Loess Plateau

OPEN ACCESS

Edited by:
Giuseppe Colla, Università degli Studi della Tuscia, Italy

Reviewed by:
Sabine Dagmar Zimmermann, Centre National de la Recherche Scientifique (CNRS), France
Marcela Claudia Pagano, Universidade Federal de Minas Gerais, Brazil

****Correspondence:***
Yuncheng Liao
liaoyuncheng1969@163.com

Specialty section:
This article was submitted to Plant Microbe Interactions, a section of the journal Frontiers in Microbiology

Received: *28 June 2018*
Accepted: *19 November 2018*
Published: *06 December 2018*

Citation:
Lu X, Lu X and Liao Y (2018) Effect of Tillage Treatment on the Diversity of Soil Arbuscular Mycorrhizal Fungal and Soil Aggregate-Associated Carbon Content. Front. Microbiol. 9:2986. doi: 10.3389/fmicb.2018.02986

INTRODUCTION

With increasing global interest in climate change, there has been increasing interest in the potential for carbon (C) sequestration in agricultural soil. Soil is not only the basis of crop production but is also the key facilitator of C sequestration in terrestrial ecosystems. As the largest C pool in the terrestrial ecosystem, soil has high ecological value. Carbon dioxide (CO_2) emissions from soil

play a key role in C balance at the continental scale. At the global scale, the top 1 m layer of soil contains about 1,500 Pg of soil organic carbon (SOC; Song et al., 2016). The accumulation of SOC is considered to be the best choice for long-term C sequestration in the terrestrial ecosystem. Moreover, SOC and CO_2 can be mutually converted. Once a soil ecosystem is destroyed, the rate of decomposition of organic carbon in the soil will be accelerated, greenhouse gas emissions will increase, and the greenhouse effect will be intensified, resulting in global warming (Zhu et al., 2016; Forte et al., 2017; Krauss et al., 2017).

In addition, soil aggregate stability and SOC are important indicators of soil quality and environmental sustainability in agro-ecosystems. Firstly, the decomposition and transformation of SOC are affected by aggregation construction (Zhang et al., 2008; Zhao et al., 2015, 2018). It has been reported that stable aggregates can physically prevent SOC against rapid decomposition (Sun et al., 2018). Secondly, SOC is considered to be the main binding agent contributing to aggregate stability (Chenu et al., 2000; Bhattacharyya et al., 2011). Moreover, the SOC content in macro-aggregates is an important index of soil aggregate stability and C loss, as influenced by various management methods (Sheehy et al., 2015). For example, the quantity and stabilization mechanisms of SOC, as related to soil aggregates, are influenced by tillage practices (Crittenden et al., 2015). Previous studies have shown that unreasonable tillage will destroy the stability of a soil ecosystem over the long term, including its soil aggregate stability, soil porosity and soil nutrients, causing a series of changes in the soil's physical and chemical characteristics (Bartz et al., 2014; Crittenden et al., 2015; Buchi et al., 2017). Extensive experiments have reported that conservation tillage, such as the adoption of a no-tillage regime, can increase soil macro-aggregates formation and stability, and offer a good protective effect for SOC (Mikha et al., 2013; Kumar et al., 2014; Dai et al., 2015), as compared with conventional tillage systems.

The total area of the Loess Plateau, China, is 640,000 ha and soil erosion affects 60.9% of it. Thus, soil erosion is one of the main environmental problems on the Loess Plateau (Huang et al., 2002). Wang et al. (2015) reported that one of the main causes of soil erosion on the Loess Plateau is the adoption of irrational management measures. Traditional tillage not only increases soil erosion but has also led to a continuous reduction in soil fertility by removing large amounts of crop straw, which is associated with great mechanical disturbance (Zhang et al., 2016). Arbuscular mycorrhizal (AM) fungi have an important role in biogeochemical cycles and contribute to many terrestrial ecosystem functions (Harley and Smith, 1983; Piotrowski et al., 2004; Wang et al., 2018). For example, AM fungi have a vital role in improving crop growth and enhancing crop resistance to plant disease (Rillig, 2004). Furthermore, the formation and conservation of soil aggregates are always influenced by the extra-radical hyphae of AM fungi (Rillig et al., 2010; Dai et al., 2015). The community composition of AM fungi is also easily influenced by variations in land-use types and agricultural management methods (Martinez and Johnson, 2010; Xiang et al., 2014; Zhao et al., 2015). Thus, there is concern about the responsible conservation of AM fungi in cultivated fields for sustainable crop management. However, there is limited systematic information pertinent to soil aggregates, their associated C content and AM fungal diversity under different tillage regimes on the Loess Plateau.

Thus, to estimate the impacts of no-tillage with straw return (NTS) practices on AM fungal diversity, bulk SOC, and soil aggregates and their associated C contents, and reveal the main factors that affect SOC in bulk soil in relation to conventional mouldboard plowing tillage without straw (CT), a long-term experiment applying high-throughput sequencing of 18S rRNA was conducted in the Loess Plateau, China. We hypothesized that tillage regimes influence AM fungal community composition via alteration of soil physical and chemical characteristics, which ultimately influence bulk SOC. The objectives of the present study were to: (i) explore the changes in AM fungal community composition after 7 years of no-tillage, and (ii) study the relationships between these changes and soil physical and chemical characteristics, such as soil aggregate composition, associated C contents, and bulk SOC, to determine the key factors influencing bulk SOC.

MATERIALS AND METHODS

Site

The experiment was conducted at the Northwest A&F University farm (latitude 34°21′ N, longitude 108°10′ E). The experiment included two tillage treatments: (1) no tillage with crop straw return (NTS) and (2) conventional tillage without crop straw (CT). The same tillage treatments were used over 7 years (2009–2015) in plots measuring 18.3 m × 15 m. The two tillage treatments were designed in a randomized block and included three replications. The crop system was a winter wheat (*Triticum aestivum* L.)-summer maize (*Zea mays* L.) rotation system.

The fields were cultivated twice: once after harvesting winter wheat in June and once after the summer maize harvest in October. The field was plowed to 20–25 cm depth in the CT treatment, and then a rotavator was applied to plow the soil to 15 cm depth. No-tillage machinery disturbed the soils in the NTS plots either before or after the establishment of the trial, except during sowing when a no-tillage planter was used.

Soil DNA Extraction

At the maize harvesting stage (October, 2015) three replicates of soil samples from the NTS and CT treatments were selected. The soil specimens were taken from 20 points at surface depth (0–20 cm) for each plot. Samples were mixed and sieved through a 2 mm square aperture mesh to remove stones and plant material (including above-ground materials and roots) and kept at −80°C until analysis. Microbial DNA was taken from 0.25 g of fresh soil by applying a TIANamp soil DNA kit according to the manufacturer's instructions. The A260/280 ratio and agarose gel electrophoresis were applied to control the DNA quality and integrity. The genomic DNA was kept at

−20°C until PCR amplification and metagenomic sequencing were conducted.

PCR Amplification and Preparation of the Amplicon Libraries

PCR amplification was performed in a GeneAmp PCR System 9700 (Life Technologies, Carlsbad, CA, United States). The hypervariable regions (V3–V4) of 18S rDNA were used to distinguish the species of fungi. We synthesized the primers based on the changeable region of V3–V4 (F: 5′- GCCTCCCTCGCGCCATCAG -3′, R:5′-GCCTTGCCAGCCCGCTCAG -3′) in the hypervariable region of 18S rDNA for PCR (Lin et al., 2012). PCRs were performed in a 25 μl reaction, which contained 12.5 μl 2 × KAPA HiFi HotStart ReadyMix, 0.25 μmol L^{-1} of each primer and 10 ng of DNA template. Thermocycling conditions included starting denaturation at 95°C for 3 min, 25 cycles at 95°C for 30 s, Tm for 30 s, 72°C for 30 s, and 72°C for 5 min. In process of the amplification reaction, the indexes allowing sample multiplexing during sequencing were integrated between the Illumina Miseq adaptor and the reverse primer. The PCR products for each specimen were combined to prepare the PCR amplicon libraries. The PCR products were quantified by applying the Agilent 2100 Bioanalyzer System (Santa Clara, CA, United States) after purification and they were then combined at equal concentrations. Amplicon sequencing was conducted based on the Illumina Miseq platform at Beijing Ori-Gene Science and Technology Co., Ltd. (Beijing, China). The PCR product was purified using Ampure XP beads. In addition, the PCR product was recovered using a QIAquick Gel Extraction kit.

Processing of Sequencing Data

The 18S data were purified as follows: (1) We removed sequences with sequencing quality scores less than 20, removed sequences containing N, and removed sequences of >10 bp; (2) We removed sequences with primer mismatch (>4 bp) and; (3) removed primer sequences except for short (less than 200 bp) and overly long (>500 bp) sequences; (4) We used UCHIME software as a reference to remove chimeras from the height abundance sequence (Schloss et al., 2009). The sequence was loaded into OTUs (operational taxonomic units) to which the 97% identity was applied. Then, representative sequences were selected according to the most abundant sequence in each OTU. We deposited the raw sequence data into the NCBI Sequence Read Archive database with accession number SRP150029.

The Chao 1 and Shannon indexes were determined, and principal component analysis (PCA) was performed using the UniFrac distance matrix (Lozupone and Knight, 2005). The rarefaction curves of the Chao 1 and Shannon indexes were applied to compare the fungal diversity and richness in the different tillage systems. PCA was performed based on the sequences and OTUs obtained using R 3.12 software. LEfSe software was used to analyze significant differences between the microorganisms in different treatments.

Analysis of Soil Properties

All three replicates were used in the analysis of soil chemistry characteristics. The SOCs in bulk soil and aggregations were determined using the dichromate oxidation method (Bao, 2000). Soil aggregates were divided using a dry-sieving method based on Huang et al. (2007). Soil aggregates with diameters >2 mm (large macro-aggregates), 2–0.25 mm (small macro-aggregates), 0.053–0.25 mm (micro-aggregates) and <0.053 mm (silt and clay fractions) were separated by shaking the sieves mechanically with an amplitude 1.5 mm for 2 min. The mean weight diameter (MWD) was applied to represent soil aggregate stability and was calculated according to He et al. (2018):

$$MWD = \sum_{1}^{n+1} \frac{r_{i-1} + r_i}{2} \times m_i \quad (1)$$

where MWD is the mean weight diameter (mm), r_i is the diameter of each part class (mm), and m_i is the weight proportion of soil aggregates compared to the total weight in each class.

The differences in the percentage of soil aggregate contents, their associated C contents, and MWD between tillage treatments, were tested using SPSS 17.0.

RESULTS

Impact of Tillage Regimes on Soil Aggregates Distribution

The results showed that different tillage treatments significantly ($P < 0.05$) changed the distribution of soil aggregates. Soil aggregates were mainly composed of large aggregates. However, the percentage of large macro-aggregates was significantly ($P < 0.05$) greater in NTS, being 9.1% (>2 mm) and 12.2% (0.25–2 mm) higher than in the CT treatment, respectively (**Figure 1** and **Supplementary Table S1**). Meanwhile, NTS

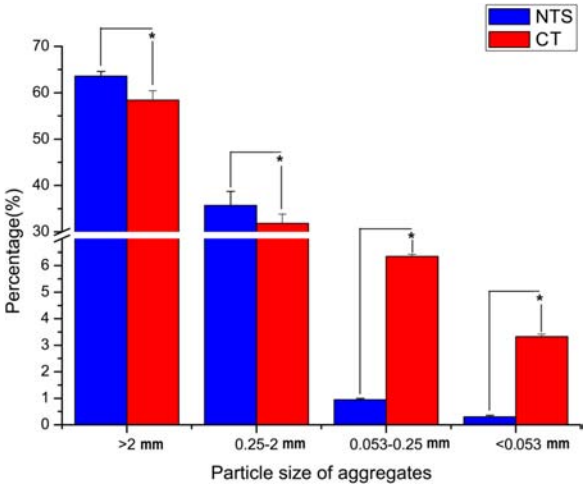


FIGURE 1 | Effect of tillage treatments on soil aggregates size distributions. *$p < 0.05$.

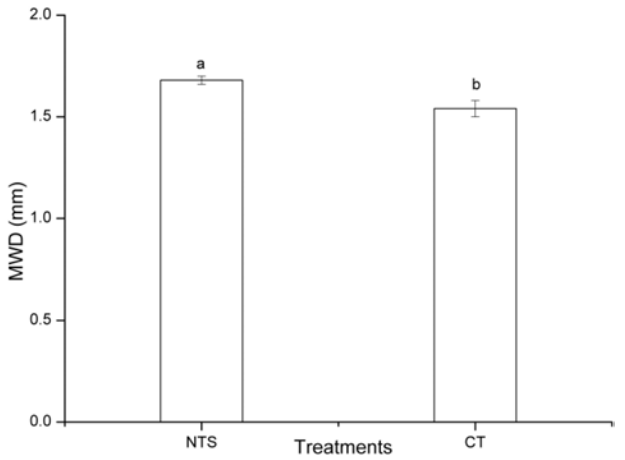


FIGURE 2 | The MWD (mean weight diameter) under no tillage with crop straw returning (NTS) and conventional tillage without the crop straw (CT) treatments. Different letters above the standard error bars indicate significant differences between treatments at the $p < 0.05$ level.

treatment significantly ($P < 0.05$) increased the MWD by 9.8% as compared with CT treatment (**Figure 2** and **Supplementary Table S2**). However, NTS significantly ($P < 0.05$) decreased the proportions of micro-aggregates (0.053–0.25 mm), and silt and clay fractions (<0.053 mm) by 88.1 and 92.6%, respectively (**Figure 1** and **Supplementary Table S1**).

Effect of Tillage on SOC in Aggregates

As shown in **Figure 3**, NTS soil had higher SOC in all aggregates and bulk soil than those of CT treatment. Compared to the CT treatment, SOC in the large macro-aggregates, small macro-aggregates, micro-aggregates, and silt and clay parts was 25.2, 35.1, 91.1, and 9.2% greater, respectively. Moreover, NTS treatment significantly increased SOC in bulk soil by 10.0% as compared with CT (**Figure 3** and **Supplementary Table S3**). All the above results show that NTS treatment can significantly improve the physical and chemical characteristics of soil.

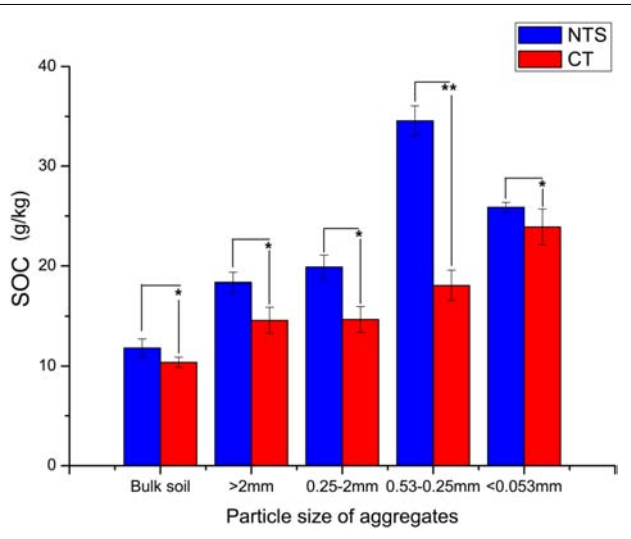


FIGURE 3 | The SOC (soil organic carbon content) in bulk soil and its aggregations under no tillage with crop straw returning (NTS) and conventional tillage without the crop straw (CT) treatments. *$p < 0.05$ and **$p < 0.01$.

Analysis of Rarefaction Curves

The Chao1 and Shannon indexes were used to reflect species diversity. The greater their values, the higher the species diversity of the sample. The rarefaction curves of the Chao 1 and Shannon indexes in different tillage systems are shown in **Figure 4**. When the number of sequences exceeded 1000, all amplified dilution curves reached a plateau, which meant that the sequence-derived diversity and abundance assessed in the present study could sufficiently characterize the fungal species in each sample (**Figure 4**). Alpha diversity analysis suggested that there was a significant difference in the richness index (Chao1) between the NTS and CT treatments. The Chao 1 value of the NTS treatment was significantly higher than that of the CT treatment. The results indicate that NTS treatment is beneficial to fungi.

Overall Taxonomic Information

A total of 507,263 quality sequences were obtained by quality control analyses of the raw data. According to the 97% species similarity level, a total of 688 OTUs were derived in this study. The results show that the OTU number of the NTS treatment was significantly higher than that of the CT treatment (**Figure 5**). Moreover, the Venn diagram intuitively indicates the common and special OTUs. Venn analysis showed that only 58% of OTUs (399) were shared by the different treatments. There were 209 and 80 particular OTUs in the NTS and CT treatments, respectively (**Figure 5**).

To further analyze the differences between species, LEfSe software was used to determine significant differences in the fungal species present in the different treatments. The results showed that the relative abundances of *Oligohymenophorea*, *Conthreep*, *Hypotrichia*, *Spirotrichea*, *Intramacronucleata*, *Lobulomycetaceae*, *Lobulomycetales*, *Chrysophyceae,* and *Vischeria* were significantly increased in the NTS treatment, while the relative abundances of *Ascomycota*, *Chaetothyriales*, *Onygenales*, *Eurotiomycetes*, *Strophariaceae*, *Incertae Sedis Rhodosporidium*, *Sporidiobolales*, *Incertae Sedis Chytridiomycetes*, *Glomerales*, *Incertae Sedis Glomeromycetes,* and *Incertae Sedis Endogonales* were significantly decreased in the NTS treatment (**Figure 6**). These results indicate that there is a close correlation between the soil tillage method and the composition of microorganisms.

Composition of AM Fungi Under Different Tillage Treatments

Arbuscular mycorrhizal fungi are common fungal species in soil and play a key role in plant growth and plant resistance to plant disease. We also analyzed the species composition of AM fungi. For different tillage treatments, the *Glomeromycotina* sequences varied in number from 4551 to 47,055, becoming 4 to 29 of the OTUs, respectively. The sequence proportions of

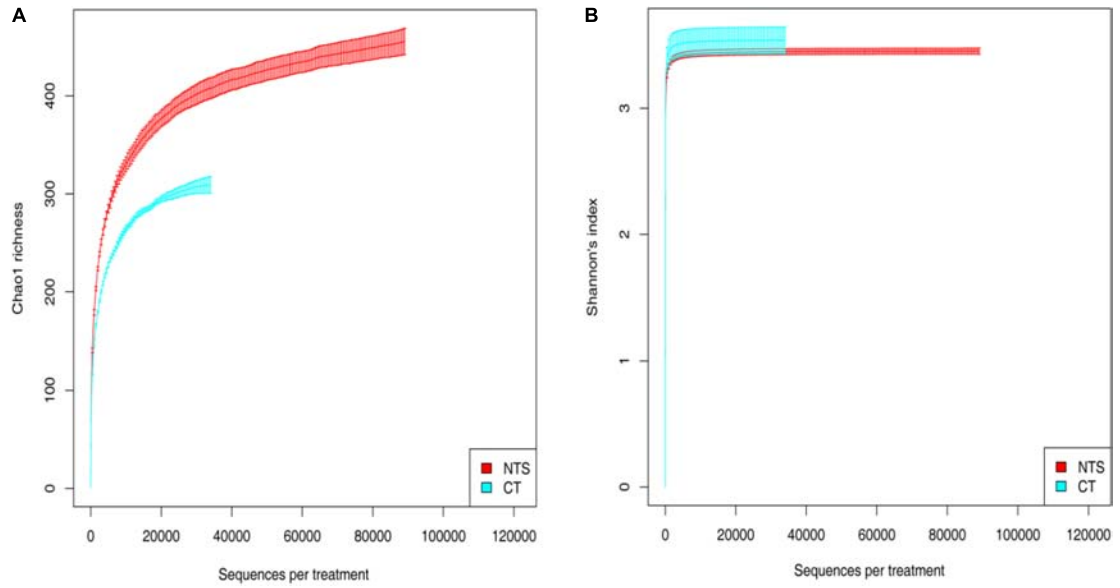


FIGURE 4 | Rarefaction curves of Chao 1 **(A)**, and Shannon index **(B)** for no tillage with crop straw returning (NTS) and conventional tillage without the crop straw (CT) treatments.

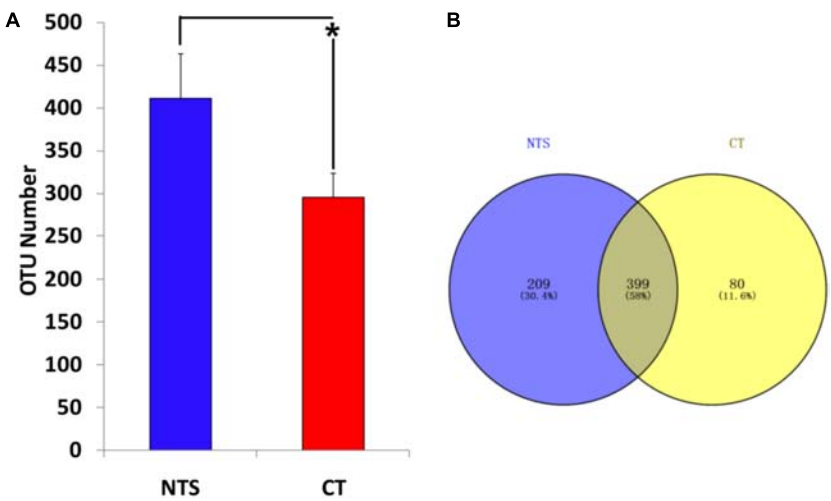


FIGURE 5 | The OTU number **(A)** and venn diagram **(B)** of soil Arbuscular mycorrhizal (AM) fungi under no tillage with crop straw return (NTS) and conventional tillage without the crop straw (CT) treatments. *$p < 0.05$.

the different *Glomeromycotina* species are shown in **Figure 7**. The NTS treatment had higher proportions of *Septoglomus* and *Glomus* than CT. The proportions of *Glomeromycetes* varied in the two treatments (**Supplementary Table S4**).

Relationship Between Soil AM Fungal Communities and Aggregate Contents

Pearson correlation coefficients and PCA were applied to evaluate the relationship between soil AM fungal communities (i.e., the percentages of *Septoglomus*, *Glomus*, and *Glomerales* unclassified) and soil physical and chemical properties (i.e., SOC in bulk soil, soil aggregates and their associated C contents). The PCA (**Figure 8**) showed that that the first component explained 85.6% of the total variance. Moreover, it was negatively associated with the percentage of large and small macro-aggregates and the SOCs in macro- and micro-aggregates and the silt and clay parts, the proportions of *Glomus*, and *Septoglomus*. The second component of the PCA explained 8.0% of the overall variance and was positively correlated with the percentage of large macro-aggregates, the SOCs in macro-aggregates and the silt and clay fraction, the percentage of *Glomus*.

Moreover, the PCA and Pearson correlation coefficient results showed that the proportion of *Septoglomus* was positively

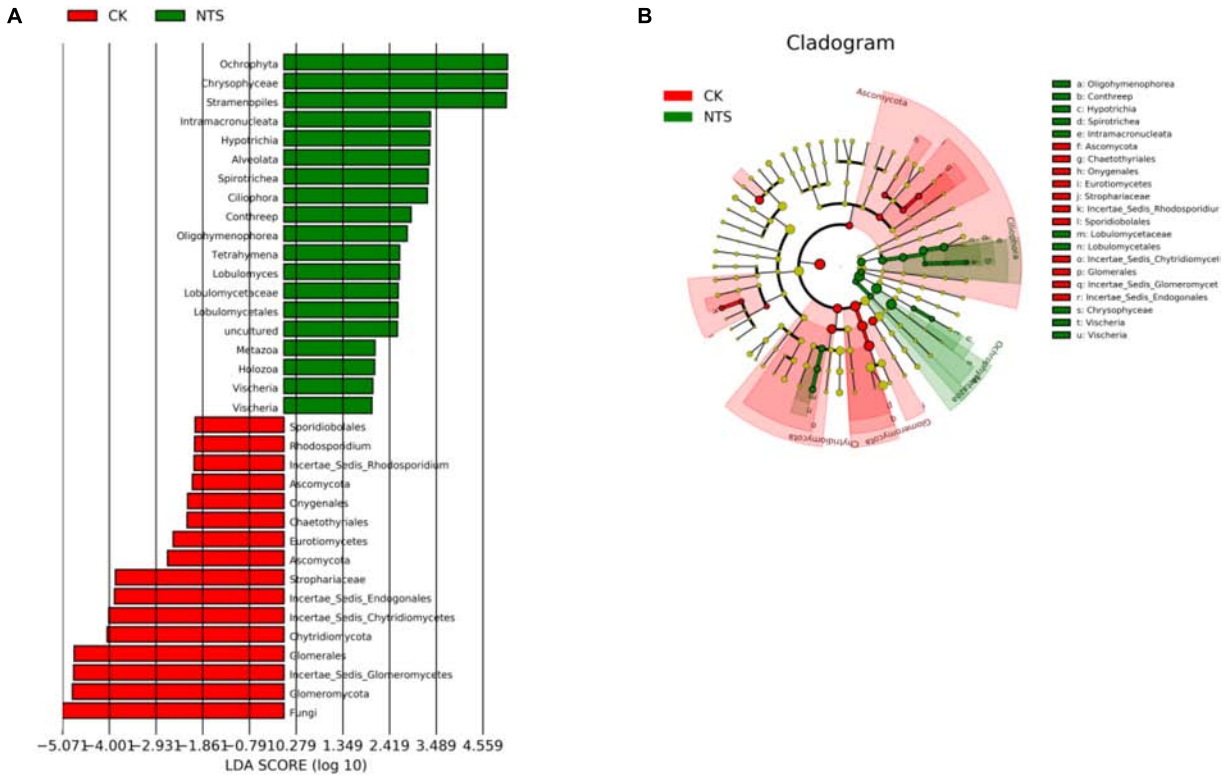


FIGURE 6 | The differences in the LDA (Linear Discriminant Analysis) distribution histogram **(A)** and evolutionary branch diagram **(B)** of special microorganisms under two tillage treatments: no-tillage with crop straw return (NTS) and conventional tillage without crop straw (CT).

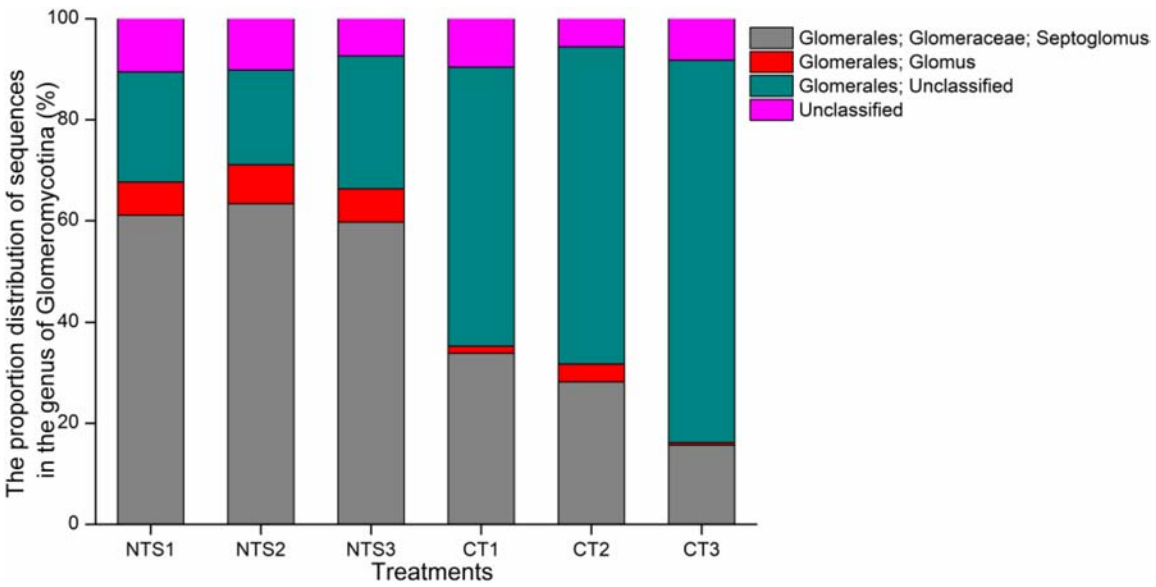


FIGURE 7 | The proportion of sequences in *Glomeromycotina* under no tillage with crop straw return (NTS) and conventional tillage without the crop straw (CT) treatments.

associated with that of *Glomus*, the percentages of soil macro-aggregates and their associated C content, the SOC in micro-aggregates, and the SOC in bulk soil. The proportion of *Septoglomus* was negatively correlated with that of *Glomerales* unclassified, the percentages of micro-aggregates, and the silt and clay fraction. Similarly, the percentage of large macro-aggregates,

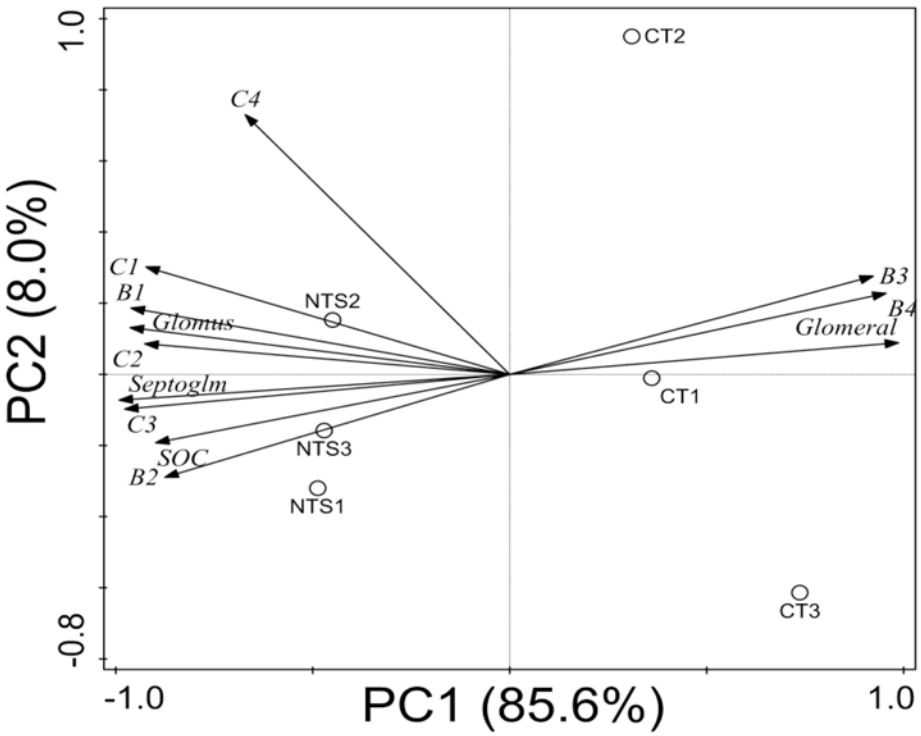


FIGURE 8 | Biplot of principal components PC1 and PC2 from all soil samples and variables: percentage of soil large macro-aggregates (B1); percentage of small macro-aggregates (B2); percentage of micro-aggregates (B3); percentage of the silt and clay fraction (B4); SOC in large macro-aggregates (C1); SOC in small macro-aggregates (C2); SOC in micro-aggregates (C3); SOC in the silt and clay parts (C4); mean weight diameter (MWD); percentage of *Septoglomus* (*Septoglm*); percentage of *Glomus* (*Glomus*); percentage of *Glomerales* unclassified (*Glomeral*) sequences for the following cases: no-tillage with crop straw (NTS) and conventional tillage without crop straw (CT), based on correlations of the 12 variables specified in **Table 1**.

and the SOC in bulk soil and macro- and micro-aggregates, also are important contributors to the proportion of *Glomus*. In addition to these relationships, the percentage of large macro-aggregates was positively correlated with bulk soil and macro- and micro-aggregates SOC.

In addition, a highly significant multiple linear regression equation was obtained for the purposes of predicting the main influences on bulk SOC ($P < 0.01$, $R^2 = 0.85$, standard error of the estimate = 0.078; factors considered redundant or irrelevant were excluded from the model according to the step-wise method; **Table 2**). The results indicate that the percentage of large aggregates was the determining factor of bulk SOC in this region.

DISCUSSION

Soil Aggregates and Their Associated C Contents

This study indicates that, after 7 years of winter wheat-summer maize rotation, NTS treatment improved the MWD, the percentages of large and small macro-aggregates compared to CT treatment. Similarly, previous studies have also reported that tillage treatment decreased soil macro-aggregates and soil stability, which might be due to the mechanical disruption of macro-aggregates caused by frequent tillage treatments (Bottinelli et al., 2017; Somasundaram et al., 2018). The mean aggregates size is known to decrease as tillage is intensified (Sheehy et al., 2015). When compared to conventionally tillage treatment, no tillage with crop straw return could enhance soil structural stability, this might be due to the increased bulk SOC concentration in the semi-arid and semi-humid area of North China (Du et al., 2017).

Moreover, our results showed that NTS had the higher SOC in all aggregates than those of CT treatment. Tillage management has been found to cause measurable changes in the SOC contents of organic-mineral fractions (Sun et al., 2013). Similarly, zero tillage could reduce the damage to soil aggregates and enhance the concentration and stability of related SOC, which resulted in higher SOC under NTS in comparison with CT treatment (Song et al., 2016). Our results show that NTS treatment could effectively increase SOC in bulk soil mainly by enhancing the percentage of large macro-aggregates as compared with CT, which might be useful to alleviate the greenhouse effect to some extent.

Composition of AM Fungi

Soil fungal diversity analysis showed that NTS treatment could significantly improve soil microbial composition and diversity. No difference in maize yield was observed between NTS and CT treatments in the present study (**Supplementary Table**

TABLE 1 | Pearson correlation coefficients between the soil properties and percentages of sequences in *Glomeromycotina*.

	Septoglomus	*Glomus*	*Glomerales*	B1	B2	B3	B4	C1	C2	C3	C4	SOC
Septoglomus	1.000	0.927**	−0.999**	0.942**	0.921**	−0.914*	−0.950**	0.907*	0.900*	0.973**	0.614ns	0.903*
Glomus		1.000	−0.923**	0.978**	0.750ns	−0.885*	−0.910*	0.896*	0.872*	0.926**	0.743ns	0.867*
Glomerales			1.000	−0.943**	−0.921**	0.914*	0.950**	−0.902*	−0.879*	−0.971**	−0.596ns	−0.899*
B1				1.000	0.795ns	−0.825*	−0.867*	0.928**	0.836*	0.906*	0.786ns	0.864*
B2					1.000	−0.786ns	−0.832*	0.716ns	0.762ns	0.834*	0.410ns	0.923**
B3						1.000	0.995**	−0.779ns	−0.860*	−0.968**	−0.394ns	−0.820*
B4							1.000	−0.824*	−0.887*	−0.985**	−0.457ns	−0.853*
C1								1.000	0.893*	0.906*	0.818*	0.691ns
C2									1.000	0.920**	0.683ns	0.773ns
C3										1.000	0.562ns	0.826*
C4											1.000	0.510ns
SOC												1.000

*ns, there was no significant difference. *$p < 0.05$, **$p < 0.01$. B1, percentage of soil large macro-aggregates; B2, percentage of small macro-aggregates; B3, percentage of micro-aggregates; B4, percentage of the silt and clay parts; C1, SOC in large macro-aggregates; C2, SOC in small macro-aggregates; C3, SOC in micro-aggregates; C4, SOC in the silt and clay fraction; SOC, SOC in bulk soil; Septoglomus, percentage of Septoglomus; Glomus, percentage of Glomus; Glomerales, percentage of Glomerales unclassified.*

TABLE 2 | Multiple linear regression models for SOC under different tillage regimes.

Variables	Estimated parameters	SE	*P*-value	R^2
Intercept	−1.420	2.637		
B1	0.374	0.078	0.009	0.851

B1, intercept parameters; SE, Standard error.

S5). Similarly, other previous studies also showed that tillage methods could influence soil microbial activity and structure by altering the habitat of soil microbes, such as the soil's gas permeability, soil texture and microbial substrates (Wang et al., 2017), thus affecting SOC. In addition, tillage could change the soil physical structure, strongly undermine the underground mycelium of soil mycorrhizal fungi, and reduce their extension range, infection rate, and community diversity (Anderson et al., 1987; Kabir, 2005). Similarly, other previous studies have also reported that no-till treatment could improve the abundance and the diversity of soil AM fungi as compared to plowed plots, which ultimately improved the plant growth (Boddington and Dodd, 2000; Wetzel et al., 2014; Hu et al., 2015). Moreover, our results showed that the percentage of macro-aggregates, its associated C content, SOCs in small macro-aggregates and micro-aggregates, and SOC in bulk soil were related with the percentage of *Septoglomus*. Similarly, Qin et al. (2017) also showed that the percentage of macro-aggregates (0.25–2 mm) was positively related with soil AM fungi biomass.

However, other studies showed that long-term no-till treatment could decrease the soil AM fungal propagules because of the higher soil bulk density, and the lower C utilization efficiency of soil organisms as compared with the plowed plots (Fu et al., 2000; Curaqueo et al., 2011; Schluter et al., 2018). These inconsistent conclusions may be due to the differences in soil properties, climatic conditions and the duration of no-till treatment studied. In addition, we also found some significant differences in the soil fungal communities of the two treatments that might have a close relationship with plant growth and the physical and chemical characteristics of soil. Our results suggested that the effect of tillage treatment on AM fungi community might be more important at a long-time scale. Thus, it is a need for a long-term study to focus on the effect of various tillage treatments on AM fungi community and its relationship with crop growth and soil properties (i.e., soil physical and chemical characteristics) during crop growth on Loess Plateau in China.

CONCLUSION

In this study, tillage treatment changed soil aggregate distributions and their associated C contents, with the NTS treatment having more macro-aggregates and associated C contents than the CT treatment. Meanwhile, NTS treatment significantly increased the percentages of *Septoglomus* and *Glomus* compared with CT treatment. We also found some

significant differences in soil fungal communities between the two treatments. In addition, Pearson correlation coefficients and PCA identified a close relationship between SOC levels and the proportions of *Septoglomus* and *Glomus* in the soil community. Step-wise regression analysis indicated that NTS promoted SOC at the surface soil layer (0–20 cm), probably by enhancing the percentage of large macro-aggregates therein. Above all, our results indicate that NTS conditions favor the maintenance of AM fungi, soil structure and SOC and might play a key role in the development of agricultural sustainability in the Loess Plateau of China. This long-term study was based on a 7-year field trial and provides insights into the consequences of agricultural practices on soil properties and microorganisms, thereby playing a role in agricultural sustainability. However, further study is needed to investigate the relationships between soil AM communities, soil physical and chemical characteristics, and crop production in the Loess Plateau, China.

AUTHOR CONTRIBUTIONS

XlL and YL designed the experiments. XlL and XnL carried out the experiments and performed the analyses. XlL contributed to writing the paper. YL checked the paper.

FUNDING

This study was financially supported by the National Natural Science Foundation of China (31860361 and 3107 1375).

ACKNOWLEDGMENTS

The authors appreciate the editors and the two reviewers for their great comments for the improvement of the manuscript.

SUPPLEMENTARY MATERIAL

The Supplementary Material for this article can be found online at: https://www.frontiersin.org/articles/10.3389/fmicb.2018.02986/full#supplementary-material

REFERENCES

Anderson, E. L., Millner, P. D., and Kunishi, H. M. (1987). Maize root length, density and mycorrhizal infection as influenced by tillage and soil phosphorus. *J. Plant Nutr.* 10, 1349–1356. doi: 10.1080/01904168709363667

Bao, S. (2000). *Soil Agro-Chemistrical Analysis*. Beijing: China Agriculture Press.

Bartz, M. L. C., Brown, G. G., Rosac, M. G., Filhoc, O. K., James, S. W., Decaëns, T., et al. (2014). Earthworm richness in land-use systems in Santa Catarina. *Brazil. Appl. Soil Ecol.* 83, 59–70. doi: 10.1016/j.apsoil.2014.03.003

Bhattacharyya, R., Kundu, S., Srivastva, A. K., Gupta, H. S., Chaudhary, V. P., and Bhatt, J. C. (2011). Long term fertilization effects on soil organic carbon pools in a sandy loam soil of the Indian Himalayas. *Plant Soil.* 341, 109–124. doi: 10.1007/s11104-010-0627-4

Boddington, C. L., and Dodd, J. C. (2000). The effect of agricultural practices on the development of indigenous arbuscular mycorrhizal fungi. I. Field studies in an Indonesian ultisol. *Plant Soil.* 218, 137–144. doi: 10.1023/A:1014966801446

Bottinelli, N., Angers, D. A., Hallaire, V., Michot, D., Le Guillou, C., Cluzeau, D., et al. (2017). Tillage and fertilization practices affect soil aggregate stability in a Humic Cambisol of Northwest France. *Soil Till. Res.* 170, 14–17. doi: 10.1016/j.still.2017.02.008

Buchi, L., Wendling, M., Amosse, C., Jeangros, B., Sinaj, S., and Charles, R. (2017). Long and short term changes in crop yield and soil properties induced by the reduction of soil tillage in a long term experiment in Switzerland. *Soil Till. Res.* 174, 120–129. doi: 10.1016/j.still.2017.07.002

Chenu, C., Le Bissonnais, Y., and Arrouays, D. (2000). Organic matter influence on clay wettability and soil aggregate stability. *Soil Sci. Soc. Am. J.* 64, 1479–1486. doi: 10.2136/sssaj2000.6441479x

Crittenden, S. J., Poot, N., Heinen, M., van Balen, D. J. M., and Pulleman, M. M. (2015). Soil physical quality in contrasting tillage systems in organic and conventional farming. *Soil Till. Res.* 154, 136–144. doi: 10.1016/j.still.2015.06.018

Curaqueo, G., Barea, J. M., Acevedo, E., Rubio, R., Cornejo, P., and Borie, F. (2011). Effects of different tillage system on arbuscular mycorrhizal fungal propagules and physical properties in a Mediterranean agroecosystem in central Chile. *Soil Till. Res.* 113, 11–18. doi: 10.1016/j.still.2011.02.004

Dai, J., Hu, J. L., Zhu, A. N., Wang, R., Zhang, J. B., and Wong, M. H. (2015). No tillage enhances arbuscular mycorrhizal fungal population, glomalin-related soil protein content, and organic carbon accumulation in soil macroaggregates. *J. Soil. Sediment.* 15, 1055–1062. doi: 10.1007/s11368-015-1091-9

Du, Z. L., Han, X., Wang, Y. Q., Gu, R. S., Li, Y. C., Wang, D. Y., et al. (2017). Changes in soil organic carbon concentration, chemical composition, and aggregate stability as influenced by tillage systems in the semi-arid and semi-humid area of North China. *Can. J. Sci.* 98, 91–102. doi: 10.1139/CJSS-2017-0045

Forte, A., Fiorentino, N., Fagnano, M., and Fierro, A. (2017). Mitigation impact of minimum tillage on CO_2 and N_2O emissions from a Mediterranean maize cropped soil under low-water input management. *Soil Till. Res.* 166, 167–178. doi: 10.1016/j.still.2016.09.014

Fu, S. L., Coleman, D. C., Schartz, R., Potter, R., Hendrix, P. F., and Crossley, D. A. (2000). C-14 distribution in soil organisms and respiration after the decomposition of crop residue in conventional tillage and no-till agroecosystems at Georgia Piedimont. *Soil Till. Res.* 57, 31–41. doi: 10.1016/S0167-1987(00)00133-1

Harley, J. L., and Smith, S. E. (1983). *Mycorrhizal Symbiosis*. London: Academic Press.

He, Y. B., Xu, C., Gu, F., Wang, Y., and Chen, J. Z. (2018). Soil aggregate stability improves greatly in response to soil water dynamics under natural rains in long-term organic fertilization. *Soil Till. Res.* 184, 281–290. doi: 10.1016/j.still.2018.08.008

Hu, J. L., Yang, A., Zhu, A., Wang, J. H., Dai, J., Wong, M. H., et al. (2015). Arbuscular mycorrhizal fungal diversity, root colonization, and soil alkaline phosphatase activity in response to maize-wheat rotation and no-tillage in North China. *J. Microbiol.* 53, 454–461. doi: 10.1007/s12275-015-5108-2

Huang, Q. R., Hu, F., Yuan, Y. H., Liu, M. Q., and Li, H. X. (2007). Effects of long-term fertilization on aggregates characteristic of red paddy soil. *Soils* 39, 608–613.

Huang, Z. L., Fu, B. J., and Li, D. C. (2002). Restructure and restoration of ecosystem in Loess Plateau based on restoration ecology. *Journal. of Soil and Water Conservation.* 16, 122–125.

Kabir, Z. (2005). Tillage or no-tillage: impact on mycorrhizae. *Can. J. Plant Sci.* 85, 23–29. doi: 10.4141/P03-160

Krauss, M., Ruser, R., Müller, T., Hansen, S., Mäder, P., and Gattinger, A. (2017). Impact of reduced tillage on greenhouse gas emissions and soil carbon stocks in an organic grass-clover ley - winter wheat cropping sequence. *Agric. Ecosyst. Environ.* 239, 324–333. doi: 10.1016/j.agee.2017.01.029

Kumar, S., Nakajima, T., Mbonimpa, E. G., Gautam, S., Somireddy, U. R., Kadono, A., et al. (2014). Long-term tillage and drainage influences on soil

organic carbon dynamics, aggregate stability and corn yield. *Soil Sci. Plant Nutr.* 60, 108–118. doi: 10.1080/00380768.2013.878643

Lin, X. G., Feng, Y. Z., Zhang, H. Y., Chen, R. R., Wang, J. H., Zhang, J. B., et al. (2012). Long-term balanced fertilization decreases arbuscular mycorrhizal fungal diversity in an arable soil in North China revealed by 454 pyrosequencing. *Environ. Sci. Technol.* 46, 5764–5771. doi: 10.1021/es3001695

Lozupone, C., and Knight, R. (2005). UniFrac: a new phylogenetic method for comparing microbial communities. *Appl. Environ. Microb.* 71, 8228–8235. doi: 10.1128/AEM.71.12.8228-8235.2005

Martinez, T. N., and Johnson, N. C. (2010). Agricultural management influences propagule densities and functioning of arbuscular mycorrhizas in low-and high-input agroecosystems in arid environments. *Appl. Soil Ecol.* 46, 300–306. doi: 10.1016/j.apsoil.2010.07.001

Mikha, M. M., Vigil, M. F., and Benjamin, J. G. (2013). Long-term tillage impacts on soil aggregation and carbon dynamics under wheat-fallow in the central Great Plains. *Soil Sci. Soc. Am. J.* 77, 594–605. doi: 10.2136/sssaj2012.0125

Piotrowski, J. S., Denich, T., Klironomos, J. N., Graham, J. M., and Rillig, M. C. (2004). The effects of arbuscular mycorrhizas on soil aggregation depend on the interaction between plant and fungal species. *New Phytol.* 164, 365–373. doi: 10.1111/j.1469-8137.2004.01181.x

Qin, H., Niu, L. M., Wu, Q. F., Chen, J. H., Li, Y. C., Liang, C. F., et al. (2017). Bamboo forest expansion increases soil organic carbon through its effect on soil arbuscular mycorrhizal fungal community and abundance. *Plant Soil.* 420, 407–421. doi: 10.1007/s11104-017-3415-6

Rillig, M. C. (2004). Arbuscular mycorrhizae and terrestrial ecosystem processes. *Ecol. Lett.* 7, 740–754. doi: 10.1111/j.1461-0248.2004.00620.x

Rillig, M. C., Mardatin, N. F., Leifheit, E. F., and Antunes, P. M. (2010). Mycelium of arbuscular mycorrhizal fungi increases soil water repellency and is sufficient to maintain water-stable soil aggregates. *Soil Biol. Biochem.* 42, 1189–1191. doi: 10.1016/j.soilbio.2010.03.027

Schloss, P. D., Westcott, S. L., Ryabin, T., Hall, J. R., Hartmann, M., Hollister, E. B., et al. (2009). Introducing mothur: open-source, platform-independent, community-supported software for describing and comparing microbial communities. *Appl. Environ. Microb.* 75, 7537–7541. doi: 10.1128/AEM.01541-09

Schluter, S., Grossmann, C., Diel, J., Wu, G. M., Tischer, S., Deubel, A., et al. (2018). Long-term effects of conventional and reduced tillage on soil structure, soil ecological and soil hydraulic properties. *Geoderma.* 332, 10–19. doi: 10.1016/j.geoderma.2018.07.001

Sheehy, J., Regina, K., Alakukku, L., and Six, J. (2015). Impact of no-till and reduced tillage on aggregation and aggregate-associated carbon in Northern European agroecosystems. *Soil Till. Res.* 150, 107–113. doi: 10.1016/j.still.2015.01.015

Somasundaram, J., Chaudhary, R. S., Awanish Kumar, D., Biswas, A. K., Sinha, N. K., Mohanty, M., et al. (2018). Effect of contrasting tillage and cropping systems on soil aggregation, carbon pools and aggregate-associated carbon in rainfed Vertisols. *Eur. J. Soil Sci.* 69, 879–891. doi: 10.1111/ejss.12692

Song, K., Yang, J. J., Xue, Y., Lv, W. G., Zheng, X. Q., and Pan, J. J. (2016). Influence of tillage practices and straw incorporation on soil aggregates, organic carbon, and crop yields in a rice-wheat rotation system. *Sci. Rep-UK.* 6, :36602. doi: 10.1038/srep36602

Sun, H., Wang, C., Wang, X., and Rees, R. (2013). Changes in soil organic carbon and its chemical fractions under different tillage practices on loess soils of the Guanzhong Plain in north-west China. *Soil Use Manage.* 29, 344–353. doi: 10.1111/sum.12046

Sun, L. P., Jing, H., Wang, G. L., and Liu, G. B. (2018). Nitrogen addition increases the contents of glomalin-related soil protein and soil organic carbon but retains aggregate stability in a *Pinus tabulaeformis* forest. *Peer J.* 6, e5039. doi: 10.7717/peerj.5039

Wang, F., Mu, X. M., Li, R., Fleskens, L., Stringer, L. C., and Ritsema, C. J. (2015). Co-evolution of soil and water conservation policy and human–environment linkages in the Yellow River Basin since 1949. *Sci. Total Environ.* 508, 166–177. doi: 10.1016/j.scitotenv.2014.11.055

Wang, X. X., Wang, X. J., Sun, Y., Cheng, Y., Liu, S. T., Chen, X. P., et al. (2018). Arbuscular mycorrhizal fungi negatively affect nitrogen acquisition and grain yield of maize in a N deficient soil. *Front. Microbiol.* 9:418. doi: 10.3389/fmicb.2018.00418

Wang, Z. T., Li, T., Wen, X. X., Liu, Y., Han, J., Liao, Y. C., et al. (2017). Fungal communities in rhizosphere soil under conservation tillage shift in response to plant growth. *Front. Microbiol.* 8:1301. doi: 10.3389/fmicb.2017.01301

Wetzel, K., Silva, G., Matczinski, U., Oehl, F., and Fester, T. (2014). Superior differentiation of arbuscular mycorrhizal fungal communities from till and no-till plots by morphological spore identification when compared to T-RFLP. *Soil Biol. Biochem.* 72, 88–96. doi: 10.1016/j.soilbio.2014.01.033

Xiang, D., Verbruggen, E., Hu, Y. J., Veresoglou, S. D., Rilling, M. C., Zhou, W. P., et al. (2014). Land use influences arbuscular mycorrhizal fungal communities in the farming-pastoral ecotone of northern China. *New Phytol.* 204, 968–978. doi: 10.1111/nph.12961

Zhang, H., Zhang, Y., Yan, C., Liu, E., and Chen, B. (2016). Soil nitrogen and its fractions between long-term conventional and no-tillage systems with straw retention in dryland farming in northern China. *Geoderma.* 269, 138–144. doi: 10.1016/j.geoderma.2016.02.001

Zhang, Z., Wei, C., Xie, D., Gao, M., and Zeng, X. (2008). Effects of land use patterns on soil aggregate stability in Sichuan Basin. China. *Particuology.* 6, 157–166. doi: 10.1016/j.partic.2008.03.001

Zhao, C. C., Fu, S. L., Mathew, R. P., Lawrence, K. S., and Feng, Y. C. (2015). Soil microbial community structure and activity in a 100-year-old fertilization and crop rotation experiment. *J. Plant Ecol-UK.* 8, 623–632. doi: 10.1093/jpe/rtv007

Zhao, F. Z., Fan, X. D., Ren, C. J., Zhang, L., Han, X. H., Yang, G. H., et al. (2018). Changes of the organic carbon content and stability of soil aggregates affected by soil bacterial community after afforestation. *Catena.* 171, 622–631. doi: 10.1016/j.catena.2018.08.006

Zhu, X. Y., Chang, L., Liu, J., Zhou, M. H., Li, J. J., Gao, B., et al. (2016). Exploring the relationships between soil fauna, different tillage regimes and CO_2 and N_2O emissions from black soil in China. *Soil Biol. Biochem.* 103, 106–116. doi: 10.1016/j.soilbio.2016.08.019

Conflict of Interest Statement: The authors declare that the research was conducted in the absence of any commercial or financial relationships that could be construed as a potential conflict of interest.

frontiers in Plant Science

MINI REVIEW
published: 07 December 2018
doi: 10.3389/fpls.2018.01782

Renewable Sources of Plant Biostimulation: Microalgae as a Sustainable Means to Improve Crop Performance

Pasquale Chiaiese[1], Giandomenico Corrado[1], Giuseppe Colla[2], Marios C. Kyriacou[3] and Youssef Rouphael[1]*

[1] Department of Agricultural Sciences, University of Naples Federico II, Portici, Italy, [2] Department of Agriculture and Forest Sciences, University of Tuscia, Viterbo, Italy, [3] Department of Vegetable Crops, Agricultural Research Institute, Nicosia, Cyprus

Plant biostimulants (PBs) attract interest in modern agriculture as a tool to enhance crop performance, resilience to environmental stress, and nutrient use efficiency. PBs encompass diverse organic and inorganic substances (humic acids and protein hydrolysates) as well as prokaryotes (e.g., plant growth promoting bacteria) and eukaryotes such as mycorrhiza and macroalgae (seaweed). Microalgae, which comprise eukaryotic and prokaryotic cyanobacteria (blue-green algae), are attracting growing interest from scientists, extension specialists, private industry and plant growers because of their versatile nature: simple unicellular structure, high photosynthetic efficiency, ability for heterotrophic growth, adaptability to domestic and industrial wastewater, amenability to metabolic engineering, and possibility to yield valuable co-products. On the other hand, large-scale biomass production and harvesting still represent a bottleneck for some applications. Although it is long known that microalgae produce several complex macromolecules that are active on higher plants, their targeted applications in crop science is still in its infancy. This paper presents an overview of the main extraction methods from microalgae, their bioactive compounds, and application methods in agriculture. Mechanisms of biostimulation that influence plant performance, physiology, resilience to abiotic stress as well as the plant microbiome are also outlined. Considering current state-of-the-art, perspectives for future research on microalgae-based biostimulants are discussed, ranging from the development of crop-tailored, highly effective products to their application for increasing sustainability in agriculture.

Keywords: active molecules, plant-microbiome, modern agriculture, plant response, algae extracts, synergetic properties

OPEN ACCESS

Edited by:
Antonio Ferrante,
Università degli Studi di Milano, Italy

Reviewed by:
Spyridon Alexandros Petropoulos,
University of Thessaly, Greece
Andrea Ertani,
Università degli Studi di Padova, Italy

****Correspondence:***
Pasquale Chiaiese
chiaiese@unina.it

Specialty section:
This article was submitted to Crop and Product Physiology, a section of the journal Frontiers in Plant Science

Received: *12 October 2018*
Accepted: *16 November 2018*
Published: *07 December 2018*

Citation:
Chiaiese P, Corrado G, Colla G, Kyriacou MC and Rouphael Y (2018) Renewable Sources of Plant Biostimulation: Microalgae as a Sustainable Means to Improve Crop Performance. Front. Plant Sci. 9:1782. doi: 10.3389/fpls.2018.01782

MICROALGAE AS A RENEWABLE SOURCE OF PLANT BIOSTIMULANTS

Global demographic pressure on agricultural production calls for novel and sustainable approaches toward satisfying the ever-growing demand for plant biomass destined for human food, animal feed, and energy production. Conventional agricultural practice has relied overwhelmingly on non-renewable inputs of fertilizers and pesticides. Although their introduction has allowed

substantial progress for humankind (Cooper and Dobson, 2007), agro-chemicals also pose a serious, unresolved threat to human health and the environment (Fenner et al., 2013). Moreover, supply and application of these inputs is becoming increasingly costly because of resource depletion and the increasing global demand for mineral fertilizers. Finally, the use of chemical inputs in agriculture is restricted by a tightening legal framework because of mounting public concern. Plant biostimulants (PBs) can play a pivotal role in addressing sustainability challenges because they can reduce dependency on fertilizers, especially on off-farm chemical inputs. Moreover, PBs are also useful to improve yield and its stability under environmental stress (Bulgari et al., 2014; Calvo et al., 2014; Colla and Rouphael, 2015; Yakhin et al., 2017).

According to recent EU Regulation, PBs are defined mainly through their claimed action, in other words '*more by the plant response they elicit than by their makeup*.' Therefore, PBs embrace a wide range of organic non-microbial substances (humic acids, protein hydrolysates, and seaweed extracts) and microbial organisms (mycorrhizal fungi and N-fixing bacteria) (du Jardin, 2015; European Commission, 2016; Rouphael and Colla, 2018; Rouphael et al., 2018). Extracts of brown, green and red macroalgae (seaweed), which are mainly collected from sea water, represent an important category of organic biostimulants (Battacharyya et al., 2015; Rouphael et al., 2017). A wide array of chemicals has been identified in seaweed extracts, including polysaccharides, phenolics, fatty acids, vitamins, osmolytes, phytohormones, and hormone-like compounds involved in signaling plant response to abiotic stress (Khan et al., 2009; Battacharyya et al., 2015). Currently, brown macroalgae are the most common seaweed used in agriculture with dozens of commercial products (Craigie, 2011). The beneficial effects of seaweed extract applications, attributed to multiple mechanisms, include improvement of seedling establishment, flowering and fruit setting, as well as tolerance to a wide range of abiotic stress (Khan et al., 2009; Craigie, 2011; Battacharyya et al., 2015; De Pascale et al., 2018; Ertani et al., 2018). However, macroalgae are typically harvested from sea, which hampers the standardization of the raw material quality. The chemical composition of seaweed varies according to the tissue age, environmental conditions, nutrient availability, and time of harvesting (Marinho-Soriano et al., 2006; Marsham et al., 2007). Moreover, the spread of water pollution limits the use of seaweed biomass for biostimulant production in many parts of the globe. For this reason, the scientific community and the private industry are giving more emphasis to the more costly controlled production of '*in-house algae*.' Ideally, a successful biostimulant should be not only sustainable and effective, but also based on organic by-products and able to favor the closure of the nutrient loops in agriculture.

A promising alternative toward the standardization of raw material and cost reduction in algal biomass production would be the use of microalgae (Barone et al., 2018a,b). They embrace a wide range of evolutionarily diverse phototrophic and unicellular organisms. It has been estimated that the number of microalgae specie ranges from 50,000 to 1 million. Their long and complex evolutionary history is reflected in the vast range of metabolic compounds that they are able to produce. In any aquatic eco-system, microalgae are among the most important primary trophic producers (Sharma and Rai, 2011). They have successfully colonized also terrestrial niches, where they can be found in population densities ranging between 3 and 100 million cells per gram of topsoil (Kutílek and Nielsen, 2015). The very wide genetic variability in these microorganisms has yet to be fully explored and microalgae-based commercial products are currently used mainly as nutritional supplements. Presently, only few microalgal species (*Chlorella* spp., *Dunaliella* spp., *Haematoccocus* spp., *Isochrysis* spp., *Nannochloropsis* spp., *Porphyridium* spp., and *Spirulina* spp.) are industrially exploited (Walker et al., 2005; Wijffels et al., 2013). For instance, *Spirulina* spp. and *Chlorella* spp. have a large economic value for the production of functional food and dietary supplements (Schiavon et al., 2017); while *Dunaliella salina* and *Haematococcus pluvialis* are used to obtain two popular antioxidants, β-carotene and astaxanthin (Bajpai et al., 2013). Microalgae have also gained increasing interest as a source of renewable energy (e.g., biofuel production) and for industrial and domestic wastewater bioremediation (Spolaore et al., 2006; Chiaiese et al., 2011; Renuka et al., 2018).

Microalgae can be autotrophic or heterotrophic. As solar conversion in some microalgae species is very efficient, the most common procedure for cultivation of this microorganism is presently the autotrophic growth (Renuka et al., 2018). The basic cultivation system consists of open-ponds used for food supplement and antioxidant production, with highly variable productivity depending on species and environmental conditions. Open system cultivation of microalgae is thus limited to certain robust species, such as *Spirulina* spp., *Dunaliella* spp., and *Chlorella* spp., that are able to grow under extreme conditions. Reduction of growing area and protection against potential contamination can be obtained in closed-ponds, referred to as photobioreactors. This type of cultivation method is often used for the production of high added-value molecules, such as pharmaceutical compounds. However, the main disadvantage of photobioreactors is the high capital cost for designing and operating. A viable alternative for growing microalgae is in heterotrophic conditions exploiting existing industrial bacterial-bioreactors (Kim et al., 2013; Kovar et al., 2014; Venkata Mohan et al., 2015; Hu et al., 2018). The main advantages of this cultivation system is the high cell concentration, which can reach up to 100 g L^{-1}; in photobioreactors, the maximum density is around 40 g L^{-1}, even lower in open-ponds (*c.* 10 g L^{-1}). A recent sustainable energy-based strategy for growing microalgae relies on using wastewater of industrial, domestic and agricultural origin in bioreactors that allow for the removal of contaminants during the production of microalgal biomass. Therefore, microalgae have the potential to reduce the negative discharges to the environment by, for instance, re-using nutrients and products and valorizing waste from different sources, including those related to agriculture.

This mini-review presents the various methods of production of microalgal extracts, their biologically active compounds and application methods in agriculture. The biostimulant action by which they affect plant performance, physiological status, resilience to environmental stressors as well as their effects

on plant microbiome are also covered. Several perspectives for future research relevant to microalgae-based biostimulants are proposed, encompassing the development of specific and effective products as well as their applications for advancing sustainability in modern agriculture.

EXTRACTION TECHNIQUES, CHEMICAL CHARACTERIZATION AND APPLICATION METHODS OF MICROALGAE-DERIVED BIOSTIMULANTS

Numerous studies have been carried out on techniques to obtain microalgal extracts (Samarasinghe et al., 2012; Michalak and Chojnacka, 2014, 2015 and refrences cited therein). The extraction techniques require as a first step the removal of the cell wall for releasing the bioactive compounds (Samarasinghe et al., 2012). This can be achieved by: (i) mechanical/physical means (e.g., autoclaving, homogenization, microwaving, pulsed electric field technology, sonication, liquid nitrogen), (ii) chemical means (e.g., sodium hydroxide, hydrochloric or sulfuric acids, osmotic shock, nitrous acid), and (iii) enzymatic means (e.g., cellulase, protease). Mechanical/physical pretreatment methods are more energy-demanding, while enzymatic methods are gaining popularity within the industry, although cell lysing enzymes represent an additional cost (Michalak and Chojnacka, 2014). The significant advantages of the enzymatic pretreatment methods over the chemical ones have been attributed mainly to the more gentle cell-wall disruption, not involving chemical and/or physical treatment. It is therefore expected that the resulting algal extracts will retain higher levels of bioactive compounds (Michalak and Chojnacka, 2014, 2015).

The choice of extraction method used to obtain biologically active compounds from the microalgal biomass is mainly dictated by the type of raw material and by the target molecule(s) (Michalak and Chojnacka, 2014). Conventional techniques include the use of organic solvents. The main drawbacks of traditional solvent extraction techniques are the high volumes of solvents used, the lack of high throughput and the length of the process. Novel extraction methods such as microwave assisted extraction, pressured liquid extraction, supercritical fluid extraction and ultrasound assisted extraction have been adopted, enabling the delivery of extracts in a solvent free-environment, safer for both plants and humans (Michalak and Chojnacka, 2014, 2015).

Microalgae produce a remarkable diversity of biologically active metabolites. The quantity and quality of bioactive metabolites in microalgal extracts largely depend on the extraction technique and the microalgal species used (Puglisi et al., 2018). The content of primary metabolites, carbohydrates and lipids in microalgae is very high (55–70% fresh weight) when they are cultivated under optimal conditions. Carbohydrates are usually one of the major components of microalgal extracts. In *Chlorella* spp., *Chlamydomonas* spp., *Dunaliella* spp. and *Spirulina* spp., carbohydrates may account for up to 46% of the dry weight (DW) extract (Spolaore et al., 2006; Pinzon et al., 2014; Tibbetts et al., 2015). In addition to common carbohydrates, microalgae can contain floridean, myxophycean, and chrysolaminarin starch (Lee, 2008). Protein usually accounts for 18–46% (DW) of microalgal extract (Becker, 2013). The presence of some amino acids such as tryptophan and arginine in microalgal extracts is expected to increase significantly the growth and yield of cultivated crops because these two amino acids are the metabolic precursors of key phytohormones (Colla et al., 2013, 2014, 2016). Tryptophan is pivotal for plant metabolism as it serves as building block for proteins, precursors of plant hormones such as auxin and salicylic acid, and for aromatic secondary compounds with multiple biological functions (Colla et al., 2016). In addition to tryptophan, arginine serves as precursor to polyamines, which partake in many important biological processes such as embryogenesis, organogenesis (particularly flower initiation and development, fruit setting, ripening, and leaf senescence), as well as in protection against osmotic stress (Kalamaki et al., 2009).

To deliver microalgae to agronomic and horticultural crops, a number of application methods have been adopted, depending on the microalgal product and formulation. The modes of application include: (i) soil amendment with algal formulations using suitable carriers, (ii) soil amendment with algal dry biomass (e.g., pellets, granules or powder) or suspended liquid culture, and (iii) foliar spray (using culture supernatant, leachate, and algal compost tea) or substrate/soil drench with algal culture (Coppens et al., 2015; Bushong et al., 2016; Renuka et al., 2018). The foliar application method appeared to be the most effective if applied under high relative humidity conditions and when leaf stomata are open, in order to increase the permeability and uptake of the product.

BIOSTIMULATORY ACTION OF MICROALGAL EXTRACTS

Microalgae-derived products have multi-functional properties in agriculture, facilitating nutrient uptake, improving crop performance, physiological status and tolerance to abiotic stress (Renuka et al., 2018). Despite the fact that microalgae produce compounds that are active on higher plants (de Morais et al., 2015; Borowitzka, 2016), the practical applications of microalgae in crop science are limited.

In recent years, experimental studies testing the action of microalgal extracts, under open-field and greenhouse conditions, have demonstrated that they stimulate germination, seedling growth, shoot, and root biomass in several crops such as lettuce, red amaranth, pack choi, tomato, and pepper (Faheed and Abd-El Fattah, 2008; Garcia-Gonzalez and Sommerfeld, 2016; Barone et al., 2018a,b; El Arroussi et al., 2018). Promotion of growth (on both fresh and dry weight basis) at the early stages of development was reported for lettuce germinated in a *C. vulgaris* containing medium (at 2 and 3 g dry microalgae extract kg^{-1} of soil) (Faheed and Abd-El Fattah, 2008). In the same study, improvement of plant growth (i.e., shoot,

root dry weight, and length) was associated to the stimulation of carotenoid and chlorophyll pigment biosynthesis, which may have improved the photosynthetic activity. Similarly, the application of *Spirulina platensis* enhanced plant growth in different leafy vegetables such as rocket, bayam red, and pak choi (Wuang et al., 2016). Garcia-Gonzalez and Sommerfeld (2016) and El Arroussi et al. (2018) indicated that also fruit vegetables, such as tomato and pepper, are positively affected by the application of microalgal extracts. For instance, seed priming and foliar application at different concentrations (0, 0.75, 1.875, 3.75, 5.625, and 7.5 g mL^{-1}) of aqueous cell extracts or dry biomass of green alga *Acutodesmus dimorphus* promoted seed germination, plant growth, and floral production in a dose-dependent manner (Garcia-Gonzalez and Sommerfeld, 2016). Interestingly, the authors demonstrated the presence of a bell-shaped concentration–response curve, with maximum benefit at 3.75 g mL^{-1} extract. Spraying at 3-day intervals with the polysaccharide extract of blue-green alga *S. platensis* increased plant size, root dry weight, the size and number of nodes up to 100%, for tomato and up to 50% for pepper (El Arroussi et al., 2018).

Characterization of the biostimulant action conferred by the extracts of microalgae *C. vulgaris* or *Scenedesmus quadricauda* applied at two concentrations (2 and 4 ml L^{-1}) on sugar beet grown in a hydroponic Hoagland solution was carried out by Barone et al. (2018a). Treated seedlings incurred changes in root architecture (higher total root length, surface, and number of root tips). In addition, differences between the two application rates were not observed. The different changes induced by microalgal extracts on root morphology have been reflected also at the molecular level with an upregulation of several genes involved in various biological pathways of primary and secondary metabolism. Zhang et al. (2017) and Barone et al. (2018b) suggested that the hydroponic co-cultivation of tomato plants with *C. vulgaris*, *S. quadricauda* or *C. infusionum* had a positive effect on crop performance in terms of fresh and dry plant weight. A putative biostimulatory mechanism involved may be associated to the continuous algal photosynthesis constantly delivering oxygen to the hydroponic nutrient solution (Barone et al., 2018a). Another possible mechanism involved in the biostimulatory action of microalgae belonging to *Chlorophyta* spp. and *Cyanophyta* spp. is the production and excretion of hormones (auxins and cytokynins) into the growing substrate/soil and surrounding environment (Jäger et al., 2010; Renuka et al., 2018). Microalgal extract applications can also mitigate the detrimental effects imposed on crops by the two major agents of abiotic stress, salinity, and drought (Abd El-Baky et al., 2010; El Arroussi et al., 2018). For instance, El Arroussi et al. (2018) reported that *D. salina* exopolysaccharides mitigate the effect of different salinity levels in tomato by increasing the antioxidant enzymatic activity, phenolic compounds and key metabolites such as neophytadiene, tocopherol, stigmasterol, and 2,4-ditert-butylphenol, which are considered components of the main mechanisms against oxidative stress. The application of aqueous extracts of *Chlorella ellipsoida* and *Spirulina maxima* on wheat (Abd El-Baky et al., 2010) and *Nannochloris* on tomato (Oancea et al., 2013) also mitigated salt stress impact.

Microalgae-based plant biostimulation could be attributed also to the modulation of microbial communities residing in both the phyllosphere and the rhizosphere (Ranjan et al., 2016; Renuka et al., 2018). For instance, inoculation with blue-green algae such as *Calothrix elenkinii* stimulated the phyllosphere and rhizosphere microbiomes (Priya et al., 2015; Manjunath et al., 2016). One of the main mechanisms responsible for the improvement of soil microbial communities in response to inoculation with blue-green algae relates to the production of exopolysaccharides. Exopolysaccharides secreted by many microalgal species provide organic carbon for the growth and development of beneficial microbes, leading to the formation of useful biofilms in the rhizosphere (Xiao and Zheng, 2016). Their association with soil elements helps in the solubilization, mineralization, and bioavailability of macro and micronutrients, thus improving crop performance (Drever and Stillings, 1997).

CONCLUDING REMARKS AND CHALLENGES AHEAD

The main advantage of microalgae-based biostimulants is that their production requires limited non-renewable resources and bears an overall reduced environmental impact. Compared to other photosynthetic organisms, microalgae are potentially more suitable for biotechnological improvement, especially for metabolic engineering (Fu et al., 2016; Guiheneuf et al., 2016; Jagadevan et al., 2018). However, the advancement of their applications in agriculture is hampered by various factors. While there is a general consensus on the potential benefits of the interaction between microalgae and crops, there is limited scientific evidence underpinning this interaction, compared to other organic/inorganic and microbial PBs. The vast diversity of microscopic algae still remains largely unexplored and little work has been done for the selection and genetic improvement of microalgae accession for agriculture.

In the coming years, research efforts should focus on: (1) elucidating the microalgae × plant species × environment interaction, in order to select optimal combinations; (2) optimizing application parameters (e.g., timing, mode, rate of application, and plant developmental stage); (3) quantitative and qualitative characterization of microbial communities as modulated by microalgal PBs; (4) determining the persistence of effects subsequent to microalgal PBs foliar application; (5) the impact of climatic (e.g., radiation, and relative humidity) and plant morphological factors (e.g., cuticle thickness and leaf permeability) on the effectiveness of microalgal PBs; (6) developing tailored microalgal strains with compositions and formulations adapted to specific environments and (7) identifying potentially synergistic green/blue-green microalgal combinations providing complementary traits (e.g., production of phytohormones, and siderophores, N fixation). Finally, the synergistic

effects among green and blue-green algae should be at the centre of future research aiming to design and develop efficient microalgae-based products with specific biostimulation action.

AUTHOR CONTRIBUTIONS

All authors listed have made a substantial, direct and intellectual contribution to the work, and approved it for publication.

REFERENCES

Abd El-Baky, H. H., Rl-Baz, F. K., and El Baroty, G. S. (2010). Enhancing antioxidant availability in wheat grains from plants grown under seawater stress in response to microalgae extract treatments. *J. Sci. Food Agric.* 90, 299–303. doi: 10.1002/jsfa.3815

Bajpai, R., Prokop, A., and Zappi, M. (2013). *Algal Biorefineries: Volume 1: Cultivation of Cells and Products*. Berlin: Springer, doi: 10.1007/978-94-007-7494-0

Barone, V., Baglieri, A., Stevanato, P., Broccanello, C., Bertoldo, G., Bertaggia, M., et al. (2018a). Root morphological and molecular responses induced by microalgae extracts in sugar beet (*Beta vulgaris* L.). *J. Appl. Phycol.* 30, 1061–1072. doi: 10.1007/s10811-017-1283-3

Barone, V., Puglisi, I., Fragalà, F., Lo Piero, A. R., Giuffrida, F., and Baglieri, A. (2018b). Novel bioprocess for the cultivation of microalgae in hydroponic growing system of tomato plants. *J. Appl. Phycol.* doi: 10.1007/s10811-018-1518-y

Battacharyya, D., Babgohari, M. Z., Rathor, P., and Prithiviraj, B. (2015). Seaweed extracts as biostimulants in horticulture. *Sci. Hortic.* 196, 39–48. doi: 10.1016/j.scienta.2015.09.012

Becker, E. W. (2013). "Microalgae in human and animal nutrition," in *Handbook of Microalgal Culture*, eds A. Richmond and Q. Hu (Hoboken, NJ: Wiley), 461–503. doi: 10.1002/9781118567166.ch25

Borowitzka, M. A. (2016). "Chemically-mediated interactions in microalgae," in *The Physiology of Microalgae*, eds M. A. Borowitzka, J. Beardall, and J. A. Raven (Cham: Springer International Publishing), 321–357.

Bulgari, R., Cocetta, G., Trivellini, A., Vernieri, P., and Ferrante, A. (2014). Biostimulants and crop responses: a review. *Biol. Agric. Hortic.* 31, 1–17. doi: 10.1080/01448765.2014.964649

Bushong, J. T., Miller, E. C., Mullock, J. L., Arnall, D. B., and Raun, W. R. (2016). Irrigated and rain-fed maize response to different nitrogen fertilizer application methods. *J. Plant Nutr.* 39, 1874–1890. doi: 10.1080/01904167.2016.1187747

Calvo, P., Nelson, L., and Kloepper, J. W. (2014). Agricultural uses of plant biostimulants. *Plant Soil* 383, 3–41. doi: 10.1007/s11104-014-2131-8

Chiaiese, P., Palomba, F., Tatino, F., Lanzillo, C., Pinto, G., Pollio, A., et al. (2011). Engineered tobacco and microalgae secreting the fungal laccase POXA1b reduce phenol content in olive oil mill wastewater. *Enzyme Microb. Technol.* 49, 540–546. doi: 10.1016/j.enzmictec.2011.06.002

Colla, G., and Rouphael, Y. (2015). Biostimulants in horticulture. *Sci. Hortic.* 196, 1–2. doi: 10.1016/j.scienta.2015.10.044

Colla, G., Rouphael, Y., Canaguier, R., Svecova, E., and Cardarelli, M. (2014). Biostimulant action of a plant-derived protein hydrolysate produced through enzymatic hydrolysis. *Front. Plant Sci.* 5:448. doi: 10.3389/fpls.2014.00448

Colla, G., Rouphael, Y., Lucini, L., Canaguier, R., Stefanoni, W., Fiorillo, A., et al. (2016). Protein hydrolysate-based biostimulants: origin, biological activity and application methods. *Acta Hortic.* 1148, 27–34. doi: 10.17660/ActaHortic.2016.1148.3

Colla, G., Svecova, E., Rouphael, Y., Cardarelli, M., Reynaud, H., Canaguier, R., et al. (2013). Effectiveness of a plant-derived protein hydrolysate to improve crop performances under different growing conditions. *Acta Hortic.* 1009, 175–179. doi: 10.17660/actahortic.2013.1009.21

Cooper, J., and Dobson, H. (2007). The benefits of pesticides to mankind and the environment. *Crop Pro.* 26, 1337–1348. doi: 10.1016/j.cropro.2007.03.022

Coppens, J., Grunert, O., Van Den Hende, S., Vanhoutte, I., Boon, N., Haesaert, G., et al. (2015). The use of microalgae as a high-value organic slow-release fertilizer results in tomatoes with increased carotenoid and sugar levels. *J. Appl. Phycol.* 28, 2367–2377. doi: 10.1007/s10811-015-0775-2

Craigie, J. S. (2011). Seaweed extract stimuli in plant science and agricolture. *J. Appl. Phycol.* 23, 371–393. doi: 10.1007/s10811-010-9560-4

de Morais, M. G., da Silva Vaz, B., de Morais, E. G., and Costa, J. A. V. (2015). Biologically active metabolites synthesized by microalgae. *Bio. Med. Res. Inter.* 2015:835761. doi: 10.1155/2015/835761

De Pascale, S., Rouphael, Y., and Colla, G. (2018). Plant biostimulants: innovative tool for enhancing plant nutrition in organic farming. *Eur. J. Hortic. Sci.* 82, 277–285. doi: 10.17660/eJHS.2017/82.6.2

Drever, J. I., and Stillings, L. L. (1997). The role of organic acids in mineral weathering. *Colloids Surf. Physicochem. Eng. Asp.* 120, 167–181. doi: 10.1016/S0927-7757(96)03720-X

du Jardin, P. (2015). Plant biostimulants: definition, concept, main categories and regulation. *Sci. Hortic.* 196, 3–14. doi: 10.1016/j.scienta.2015.09.021

El Arroussi, H., Benhima, R., Elbaouchi, A., Sijilmassi, B., El Mernissi, N., Aafsar, A., et al. (2018). *Dunaliella salina* exopolysaccharides: a promising biostimulant for salt stress tolerance in tomato (*Solanum lycopersicum*). *J. Appl. Phycol.* 30, 2929–2941. doi: 10.1007/s10811-017-1382-1

Ertani, A., Francioso, O., Tinti, A., Schiavon, M., Pizzeghello, D., and Nardi, S. (2018). Evaluation of seaweed extracts from *Laminaria* and *Ascophyllum nodosum* spp. as biostimulants in *Zea mays* L. Using a combination of chemical, biochemical and morphological approaches. *Front. Plant Sci.* 9:428. doi: 10.3389/fpls.2018.00428

European Commission (2016). *Proposal for a Regulation Laying Down Rules on the Making Available on the Market of Ce Marked Fertilising Products and Amending Regulations* Technical Report EC 1069/2009 and EC1107/2009. Brussels: European Commission

Faheed, F. A., and Abd-El Fattah, Z. (2008). Effect of *Chlorella vulgaris* as bio-fertilizer on growth parameters and metabolomic aspects of lettuce plant. *J. Agric. Soc. Sci.* 4, 165–169.

Fenner, K., Canonica, S., Wackett, L. P., and Elsner, M. (2013). Evaluating pesticide degradation in the environment: blind spots and emerging opportunities. *Science* 341, 752–758. doi: 10.1126/science.1236281

Fu, W., Chaiboonchoe, A., Khraiwesh, B., Nelson, D. R., Al-Khairy, D., Mystikou, A., et al. (2016). Algal cell factories: approaches, applications, and potentials. *Mar. Drugs* 14:225. doi: 10.3390/md14120225

Garcia-Gonzalez, J., and Sommerfeld, M. (2016). Biofertilizer and biostimulant properties of the microalgae *Acutodesmus dimorphus*. *J. Appl. Phycol.* 28, 1051–1061. doi: 10.1007/s10811-015-0625-2

Guiheneuf, F., Khan, A., and Tran, L. S. (2016). Genetic engineering: a promising tool to engender physiological, biochemical, and molecular stress resilience in green microalgae. *Front. Plant Sci.* 31:400. doi: 10.3389/fpls.2016.00400

Hu, J., Nagarajan, D., Zhang, Q., Chang, J. S., and Lee, D. J. (2018). Heterotrophic cultivation of microalgae for pigment production: a review. *Biotechnol. Adv.* 36, 54–67. doi: 10.1016/j.biotechadv.2017.09.009

Jagadevan, S., Banerjee, A., Banerjee, C., Guria, C., Tiwari, R., Baweja, M., et al. (2018). Recent developments in synthetic biology and metabolic engineering in microalgae towards biofuel production. *Biotechnol. Biofuels* 11:185. doi: 10.1186/s13068-018-1181-1

Jäger, K., Bartók, T., Ördög, V., and Barnabás, B. (2010). Improvement of maize (*Zea mays* L.) anther culture responses by algae-derived natural substances. *S. Afr. J. Bot.* 76, 511–516. doi: 10.1016/j.sajb.2010.03.009

Kalamaki, M. S., Merkouropoulos, G., and Kanellis, A. K. (2009). Can ornithine accumulation modulate abiotic stress tolerance in Arabidopsis? *Plant Signal. Behav.* 4, 1099–1101. doi: 10.4161/psb.4.11.9873

Khan, W., Rayirath, U. P., Subramanian, S., Jithesh, M. N., Rayorath, P., Hodges, D. M., et al. (2009). Seaweed extracts as biostimulants of plant growth and development. *J. Plant Growth Regul.* 28, 386–399. doi: 10.1007/s00344-009-9103-x

Kim, S., Park, J.-E., Cho, Y.-B., and Hwang, S.-J. (2013). Growth rate, organic carbon and nutrient removal rates of *Chlorella sorokiniana* in autotrophic, heterotrophic and mixotrophic conditions. *Biores. Technol.* 144, 8–13. doi: 10.1016/j.biortech.2013.06.068

Kovar, K., Přibyl, P., and Wyss, M. (2014). "Microalgae grown under heterotrophic and mixotrophic conditions," in *Industrial Scale Suspension Culture of Living Cells*, eds H. P. Meyer and D. R. Schmidhalter (Hoboken, NJ: Wiley), 164–185. doi: 10.1002/9783527683321.ch04

Kutílek, M., and Nielsen, D. R. (2015). *Soil: The Skin of the Planet Earth*. Berlin: Springer, 13–19. doi: 10.1007/978-94-017-9789-4_3

Lee, R. E. (2008). "Basic characteristics of the algae," in *Phycology*, 4 Edn, ed. R. E. Lee (Cambridge: Cambridge University Press), 3–30. doi: 10.1017/cbo9780511812897.002

Manjunath, M., Kanchan, A., Ranjan, K., Venkatachalam, S., Prasanna, R., Ramakrishnan, B., et al. (2016). Beneficial cyanobacteria and eubacteria synergistically enhance bioavailability of soil nutrients and yield of okra. *Heliyon* 2:e00066. doi: 10.1016/j.heliyon.2016.e00066

Marinho-Soriano, E., Fonseca, P. C., Carneiro, M. A. A., and Moreira, W. S. C. (2006). Seasonal variation in the chemical composition of two tropical seaweeds. *Biores. Technol.* 97, 2402–2406. doi: 10.1016/j.biortech.2005.10.014

Marsham, S., Scott, G. W., and Tobin, M. L. (2007). Comparison of nutritive chemistry of a range of temperate seaweeds. *Food Chem.* 100, 1331–1336. doi: 10.1016/j.foodchem.2005.11.029

Michalak, I., and Chojnacka, K. (2014). Algal extracts: Technology and advances. *Eng. Life Sci.* 14, 581–591. doi: 10.1002/elsc.201400139

Michalak, I., and Chojnacka, K. (2015). Algae as production systems of bioactive compounds. *Eng. Life Sci.* 15, 160–176. doi: 10.1002/elsc.201400191

Oancea, L., Velea, S., Fătu, V., Mincea, C., and Ilie, L. (2013). Micro-algae based plant biostimulant and its effect on water stressed tomato plants. *J. Plant Prot.* 6, 104–114.

Pinzon, A. Y., Gonzalez-Delgado, A. D., and Kafarov, V. (2014). Optimization of microalgae composition for development of a typology of biorefinery based on profitability analysis. *Chem. Eng. Trans.* 37, 457–462.

Priya, H., Prasanna, R., Ramakrishnan, B., Bidyarani, N., Babu, S., Thapa, S., et al. (2015). Influence of cyanobacterial inoculation on the culturable microbiome and growth of rice. *Microbiol. Res.* 171, 78–89. doi: 10.1016/j.micres.2014.12.011

Puglisi, I., Barone, V., Sidella, S., Coppa, M., Broccanello, C., Gennari, M., et al. (2018). Biostimulant activity of humic-like substances from agro-industrial waste of *Chlorella vulgaris* and *Scenedesmus quadricauda*. *Eur. J. Phycol.* 53, 433–442. doi: 10.1080/0.9670262.2018.1458997

Ranjan, K., Priya, H., Ramakrishnan, B., Prasanna, R., Venkatachalam, S., Thapa, S., et al. (2016). Cyanobacterial inoculation modifies the rhizosphere microbiome of rice planted to a tropical alluvial soil. *Appl. Soil Ecol.* 108, 195–203. doi: 10.1016/j.apsoil.2016.08.010

Renuka, N., Guldhe, A., Prasanna, R., Singh, P., and Bux, F. (2018). Microalgae as multi-functional options in modern agriculture: current trends, prospects and challenges. *Biotechnol. Adv.* 36, 1255–1273. doi: 10.1016/j.biotechadv.2018.04.004

Rouphael, Y., and Colla, G. (2018). Synergistic biostimulatory action: Designing the next generation of plant biostimulants for sustainable agriculture. *Front. Plant Sci.* 9:1655. doi: 10.3389/fpls.2018.01655

Rouphael, Y., De Micco, V., Arena, C., Raimondi, G., Colla, G., and De Pascale, S. (2017). Effect of *Ecklonia maxima* seaweed extract on yield, mineral composition, gas exchange, and leaf anatomy of zucchini squash grown under saline conditions. *J. Appl. Phycol.* 29, 459–470. doi: 10.1007/s10811-016-0937-x

Rouphael, Y., Spíchal, L., Panzarová, K., Casa, R., and Colla, G. (2018). High-throughput plant phenotyping for developing novel biostimulants: from lab to field or from field to lab? *Front. Plant Sci.* 9:1197. doi: 10.3389/fpls.2018.01197

Samarasinghe, N., Fernando, S., and Faulkner, B. (2012). Effect of high pressure homogenization on aqueous phase solvent extraction of lipids from nannochloris *Oculata microalgae*. *J. Ener. Nat. Res.* 1, 1–7. doi: 10.11648/j.jenr.20120101.11

Schiavon, M., Ertani, A., Parrasia, S., and Vecchia, F. D. (2017). Selenium accumulation and metabolism in algae. *Aquat. Toxicol.* 189, 1–8. doi: 10.1016/j.aquatox.2017.05.011

Sharma, N. K., and Rai, A. K. (2011). Biodiversity and biogeography of microalgae: progress and pitfalls. *Environ. Rev.* 19, 1–15. doi: 10.1139/a10-020

Spolaore, P., Joannis-Cassan, C., Duran, E., and Ismbert, A. (2006). Commercial applications of microalgae. *J. Biosc. Bioneng.* 101, 87–96. doi: 10.1263/jbb.101.87

Tibbetts, S. M., Milley, J. E., and Lall, S. P. (2015). Chemical composition and nutritional properties of fresh water and marine microalgal biomass cultured in photobioreactors. *J. Appl. Phycol.* 27, 1109–1119. doi: 10.1007/s10811-014-0428-x

Venkata Mohan, S., Rohit, M. V., Chiranjeevi, P., Chandra, R., and Navaneeth, B. (2015). Heterotrophic microalgae cultivation to synergize biodiesel production with waste remediation: progress and perspectives. *Biores. Technol.* 184, 169–178. doi: 10.1016/j.biortech.2014.10.056

Walker, T. L., Purton, S., Becker, D. K., and Collet, C. (2005). Microalgae as bioreactors. *Plant Cell Rep.* 24, 629–641. doi: 10.1007/s00299-005-0004-6

Wijffels, R. H., Kruse, O., and Hellingwerf, K. J. (2013). Potential of industrial biotechnology with cyanobacteria and eukaryotic microalgae. *Curr. Opin. Biotechnol.* 24, 405–413. doi: 10.1016/j.copbio.2013.04.004

Wuang, S. C., Khin, M. C., Chua, P. Q. D., and Luo, Y. D. (2016). Use of Spirulina biomass produced from treatment of aquaculture wastewater as agricultural fertilizers. *Algal Res.* 15, 59–64. doi: 10.1016/j.algal.2016.02.009

Xiao, R., and Zheng, Y. (2016). Overview of microalgal extracellular polymeric substances (EPS) and their applications. *Biotechnol. Adv.* 34, 1225–1244. doi: 10.1016/j.biotechadv.2016.08.004

Yakhin, O. I., Lubyanov, A. A., Yakhin, I. A., and Brown, P. H. (2017). Biostimulants in plant science: a global perspective. *Front. Plant Sci.* 7:2049. doi: 10.3389/fpls.2016.02049

Zhang, J., Wang, X., and Zhou, Q. (2017). Co-cultivation of *Chlorella spp* and tomato in a hydroponic system. *Biomass. Bioenergy* 97, 132–138. doi: 10.1016/j.biombioe.2016.12.024

Conflict of Interest Statement: The authors declare that the research was conducted in the absence of any commercial or financial relationships that could be construed as a potential conflict of interest.

frontiers in Plant Science

ORIGINAL RESEARCH
published: 07 January 2019
doi: 10.3389/fpls.2018.01870

Effects of Two Doses of Organic Extract-Based Biostimulant on Greenhouse Lettuce Grown Under Increasing NaCl Concentrations

*Roberta Bulgari[1], Alice Trivellini[2] and Antonio Ferrante[1]**

[1] Department of Agricultural and Environmental Sciences – Production, Landscape, Agroenergy, University of Milan, Milan, Italy, [2] Institute of Life Sciences, Scuola Superiore Sant'Anna Pisa, Pisa, Italy

The enhancement of plant tolerance toward abiotic stresses is increasingly being supported by the application of biostimulants. Salinity represents a serious problem in the Mediterranean region. To verify the effects deriving from the application of biostimulants, trials on Romaine lettuce plants under salt exposure were performed, in greenhouse. Plants were subjected to three NaCl solutions with 0.8, 1.3, and 1.8 dS/m of electrical conductivity. The volume of the solution was 200 mL/plant and delivered every 3 days. Biostimulant treatments started after crop establishment and were: control (water) and two doses (0.1 or 0.2 mL/plant) of the commercial biostimulant Retrosal® (Valagro S.p.A), containing calcium, zinc, and specific active ingredients. Four Retrosal® treatments were applied, every 7 days, directly to the substrate. Non-destructive analyses were conducted to assess the effects on leaf photosynthetic efficiency. At harvest, plants fresh weight (FW) and dry weight were determined, as well as the concentration of chlorophylls, carotenoids, total sugars, nitrate, proline, and abscisic acid (ABA). The biostimulant tested increased significantly the FW of lettuce (+65% in the highest dose) compared to controls. Results indicate that treatments positively affected the chlorophyll content measured *in vivo* (+45% in the highest dose) and that a general positive effect was observable on net photosynthesis rate. Retrosal® seems to improve the gas exchanges under our experimental conditions. The total sugars levels were not affected by treatments. Biostimulant allowed maintaining nitrate concentration similar to the untreated and unstressed controls. The increasing levels of water salinity caused a raise in proline concentration in control plants (+85%); biostimulant treatments at 0.2 mL/plant dose kept lower the proline levels. All plants treated with the biostimulant showed lower value of ABA (−34%) compared to controls. Results revealed that Retrosal® is able to stimulate plant growth independently from the salinity exposure. However, treated plants reached faster the commercial maturity stage. The fresh biomass of control at the end of experiment, after 30 days, ranged from 15 to 42 g/head, while in biostimulant treated plants ranged from 45 to 94 g/head. The product applied at maximum dose seems to be the most effective in our experimental conditions.

Keywords: biostimulant, *Lactuca sativa* L., salinity, abiotic stress, non-destructive measurements, biochemical analyses

OPEN ACCESS

Edited by:
Youssef Rouphael, University of Naples Federico II, Italy

Reviewed by:
Francisco Perez-Alfocea, Spanish National Research Council (CSIC), Spain
Marios Costa Kyriacou, Agricultural Research Institute, Cyprus

****Correspondence:***
Antonio Ferrante
antonio.ferrante@unimi.it

Specialty section:
This article was submitted to Crop and Product Physiology, a section of the journal Frontiers in Plant Science

Received: *31 May 2018*
Accepted: *04 December 2018*
Published: *07 January 2019*

Citation:
Bulgari R, Trivellini A and Ferrante A (2019) Effects of Two Doses of Organic Extract-Based Biostimulant on Greenhouse Lettuce Grown Under Increasing NaCl Concentrations. Front. Plant Sci. 9:1870. doi: 10.3389/fpls.2018.01870

INTRODUCTION

Abiotic stresses are among the primary causes of crop losses worldwide, reducing average yields for most major crops by more than 50% (Bray et al., 2000; La Pena and Hughes, 2007). The reduction of yield under abiotic stress is mainly due to the energy that crops have to use for adaptation. These yield losses are usually known as "fitness cost" of the crops.

Several approaches have been employed to enhance abiotic stress tolerance. However, some of them are considered time-consuming. For instance, conventional breeding requires laborious selection and the process of several generations of crossing, selfing, and testing plants for tolerance (Ashkani et al., 2015). On the other hand, the new plant breeding techniques allow to develop new tolerance traits within a given species through genetic engineering in a short period of time, but they are currently forbidden in many countries (Savvides et al., 2016). An interesting and sustainable alternative can be provided by the application of biostimulants that strengthen the plants to more successfully tolerate future abiotic stress conditions. Biostimulants have been promoted for their ability to counteract abiotic stresses in plants and their mode of action is increasingly studied. These products are able to counteract environmental stress such as water deficit, soil salinization, and exposure to sub-optimal growth temperatures (du Jardin, 2015; Pokluda et al., 2016; Rouphael et al., 2017a; Van Oosten et al., 2017; Desoky et al., 2018; Di Stasio et al., 2018; Masondo et al., 2018; Ugena et al., 2018). Salinity, in particular, is considered one of the main environmental factor that affects plant growth and metabolism in many Mediterranean areas, leading to severe damage, turgor loss and severe inhibition of growth (Borgognone et al., 2014; Lucini et al., 2015; Taïbi et al., 2016; Rouphael et al., 2017b). It represents a serious problem for commercial horticulture with substantial loss of productivity (Xu and Mou, 2016; Orsini et al., 2018), especially in the Mediterranean region where the electric conductivity of water is often higher and overcome the crop threshold sensitivity (Colla et al., 2010). Sodium chloride (NaCl) is the main salt presents in saline environments along the seaside production areas (Viégas et al., 2001). Salinity stress can be induced by the salts accumulated in the soils that are distributed through the irrigation water. In the case of cultivations in open field along the coast, plants can also suffer from aerosol marine due to salt accumulation on the leaves. Plants exposure to salinity results in stunted growth, nutrient imbalance, and reduction in water potential (Munns and Termaat, 1986; Blaylock, 1994; Marschner, 1995; Maas and Grattan, 1999; Shaheen et al., 2013). Salt stress in plants induces similar effects of drought (Munns, 2002; Chaves et al., 2009); in fact, osmotic stress can be a consequence of either salt or drought (Forni et al., 2017). Plants have different degree of tolerance that depends from various adaptation methods and metabolic plasticity. Salt stress could also alter several metabolic processes in plants, such as photosynthesis (Agastian et al., 2000; Sayyad-Amin et al., 2016), respiration (Moud and Maghsoudi, 2008), phytohormone regulation, protein synthesis, nitrogen assimilation, and can also generate secondary oxidative stress (Flowers, 2004; Van Breusegem and Dat, 2006; Colla et al., 2010). Salinity stress induces a wide activation of the biosynthesis of bioactive compounds, which are able to reduce cell damage (Cavaiuolo et al., 2015). Several transcription factors have been identified and found differently expressed in stressed leaves. To reduce the interferences with cell physiology, salts are accumulated in vacuoles and in older leaves. Plants defense mechanisms are oriented to reduce the water uptake to avoid salts loading in the cells. Physiological alterations to enhance plant tolerance to salt stress involve the plant hormone abscisic acid (ABA) (Ferrante et al., 2011; Trivellini et al., 2016). For example, salt-induced ABA accumulation was reported to activate ABA-dependent signaling pathways (Zhu, 2002), which in turn led to adaptation.

To verify the effects deriving from the applications of biostimulants, trials on lettuce plants under salinity exposure were performed. Lettuce is in fact considered to be a moderately salt sensitive crop (Shannon and Grieve, 1998; Fernandez et al., 2016) and it is one of the most important leafy vegetable cultivated in the Mediterranean area, where saline water is frequently used for irrigation. The salinity threshold for this vegetable species is in average 1.3 dS/m (Ayers et al., 1951; Maas and Hoffman, 1977; Shannon and Grieve, 1998; Andriolo et al., 2005; Ünlükara et al., 2010), as observed in the majority of the cultivars or varieties. The effect of biostimulants can be ascribed to the improvement of the osmotic adjustment in cells by the accumulation of osmotic metabolites and the sequestration of salts in vacuoles, interfering with other compounds. The hypothesis of this work was to evaluate if an organic extract-based biostimulant, containing calcium, zinc, and specific active ingredients could enhance the tolerance against salinity in lettuce, since bioactive compounds and calcium can improve plant response and adaptation.

MATERIALS AND METHODS

Plant Material and Treatments

Romaine lettuce (*Lactuca sativa* 'Longifolia') plantlets were obtained from a local nursery. Two-week-old plantlets were transplanted in 22 cm diameter plastic pots (eight pots/treatment), on a peaty substrate, in a glasshouse of the Faculty of Agricultural and Food Sciences of Milan, under controlled conditions. The environmental conditions during the experimental period were 22–33°C, with a relative of humidity ranging from 70–80%, and 600–800 μmol m^{-2} s^{-1}. Nutrients were directly added to the substrate and were supplied by providing 14 g of slow-release fertilizer containing (NPK+MgO $+SO_3$: 14-7-17 + 2 +20). The first application, 7 g, was performed at transplanting, mixing the fertilizer with peat, and the second one (7 g) was carried out during cultivation. The density was 10 plants/m^2. Three NaCl solutions, with increasing concentration [0.8, 1.3, and 1.8 dS/m of electrical conductivity (EC)] were prepared in laboratory. These EC levels were obtained by adding 0.5 g L^{-1} (1.3 dS/m) or 0.8 g L^{-1} (1.8 dS/m) NaCl; the 0.8 dS/m was maintained using only tap water. The first saline solution can be considered not stressful for lettuce, the second one as a threshold of salinity tolerance, while the last one as stressful for the crop considered. The volume of the

saline solution was 200 mL/plant and delivered every 3 days. EC values of the substrate at harvest are reported in **Supplementary Table S2**. Treatments conditions were: control (water) and the commercial biostimulant Retrosal® (Valagro S.p.A) applied every 7 days at 10 or 20 L/ha dose, which correspond to 0.1 or 0.2 mL plant^{-1}. The biostimulant Retrosal® is an organic mix with high concentration of carboxylic acids, containing calcium oxide (CaO) 8.0% (w/w) soluble in water and 1.4% complexed by ammonium ligninsulfonate, Zinc (Zn) 0.2% (w/w) soluble in water and 0.2% (w/w) chelated by EDTA. Calcium complexed by ammonium ligninsulfonate is stable in the pH comprised from 3 to 9, while the Zn chelated with EDTA is stable in pH comprised from 4 to 11.

The irrigation was carried out considering the substrate moisture content and the amount of water was determined to maintain the 80% of substrate water availability.

Lettuce plants were harvested when the first treatment reached the commercial maturity stage, after 30 days of cultivation. Fresh weight (FW), dry biomass, and dry matter were determined weighting the whole lettuce head before and after an over-dry period (4 days) at 75°C in a ventilated over. At harvest, non-destructive analyses were conducted on leaves and then fresh leaf tissues were immediately stored at −80 or −20°C until use for biochemical analyses.

Destructive Analyses

Chlorophylls and Carotenoids

Chlorophyll *a*+*b* and total carotenoids concentrations were determined spectrophotometrically at harvest. Leaf tissue (30–50 mg) was extracted using 100% (v/v) methanol, for 24 h at 4°C in the dark; afterward quantitative determination of pigments was carried out. Absorbance readings were measured at 665.2 and 652.4 nm for chlorophylls and 470 nm for total carotenoids. Pigment levels were calculated by Lichtenthaler's (1987) formula and expressed on the basis of tissue FW.

Total Sugars

Leaf tissue (1 g) was homogenized in 3 mL of distilled water and centrifuged at 3000 × *g* (ALC centrifuge-model PK130R) for 15 min at room temperature (RT). Total sugars were assayed according to the anthrone assay. About 1 g of leaf tissue was homogenized in 3 mL of distilled water and centrifuged at 3000 × *g*, for 15 min, at RT. Anthrone (0.2 g) was melted in 100 mL of H_2SO_4 and shook for 30–40 min. 1 mL of the leaf tissue extract was added to 5 mL of anthrone solution, cooled in ice for 5 min and mixed thoroughly. Samples were incubated at 95°C for 5 min and then cooled on ice (Yemm and Willis, 1954). Absorbance was read at 620 nm and the levels were calculated referring to glucose calibration curve (Cocetta et al., 2015).

Leaf Nitrate Concentration

Nitrate concentration was measured by the salicylsulfuric acid method (Cataldo et al., 1975). One gram of fresh leaf tissue was homogenized (mortar and pestle) in 3 mL of distilled water. The extract was centrifuged at 3000 × *g* for 15 min at RT and the recovered supernatant was used for the colorimetric determination. Twenty μL of sample were added to 80 μL of 5% (w/v) salicylic acid dissolved in H_2SO_4 plus 3 mL of 1.5N NaOH. Samples were cooled at RT and absorbance at 410 nm was measured. Nitrate concentration was calculated referring to a KNO_3 standard calibration curve.

Proline

Proline was determined with a colorimetric assay, as described by Abrahám et al. (2010). Lettuce leaves (0.5 g) were ground in 10 mL of sulfosalicylic acid (3%). Tubes were kept on ice and, subsequently, samples were centrifuged for 5 min, at RT, at 3800 × *g* for 10 min. In a separate tube was prepared the reaction mixture: 100 μL of 3% sulfosalicylic acid, 200 μL of glacial acetic acid, 200 μL of acidic ninhydrin. Then 100 μL from the supernatant of the plant extract were added and the tubes were mixed well. Tubes were incubated at 96°C for 60 min. Then samples were put in ice. Afterward, 1 mL of toluene was added to the reaction mixture and samples were vortexed for 20 s. Tubes were left on the bench for 5 min to allow the separation of the organic and water phases. The chromophore containing toluene was removed into a fresh tube. Absorbance readings were performed at 520 nm using toluene as reference. Proline concentration was determined using a standard concentration curve and calculated based on the FW.

Abscisic Acid

Abscisic acid was determined by an indirect enzyme linked immuno-sorbent assay (ELISA) based on the use of DBPA1 monoclonal antibody, raised against S(+)-ABA (Vernieri et al., 1989). About 1 g of lettuce leaf was homogenized (mortar and pestle) in 3 mL of distilled water. The extract was centrifuged at 3000 × *g* for 15 min at RT and the recovered supernatant was used for the analysis. The ELISA was performed as described by Trivellini et al. (2016).

Mineral Element Determinations

About 300 mg dry weight (DW) was mineralized at 120°C in 5 mL 14.4 M HNO_3, clarified with 1.5 mL 33% H_2O_2 and finally dried at 80°C. The mineralized material was solubilized in 5 mL 1 M HNO_3 and filtered on a 0.45-μm nylon membrane. Mineral content (Na, Ca, Mg, K, Mn, Fe, Cu, Zn, Cd, and P) was measured by inductively-coupled plasma techniques (ICP-MS; Varian 820-MS, ICP Mass Spectrometer).

Non-destructive Measurements

Leaf Gas Exchange

Leaf gas exchange rates were measured using the portable infrared gas exchange system CIRAS-1 (PP Systems, Hitchin, United Kingdom), operated in open-configuration with controlled temperature, CO_2 concentration, and vapor pressure. Measurements were carried out on a fully expanded leaf between 09:00 and 13:00 h IT time. During the recording time, the light intensity in the cuvette was fixed to 1000 μmol m^{-2} s^{-1} and CO_2 concentration was set to 350 ppm.

Chlorophyll *a* Fluorescence

Chlorophyll *a* fluorescence was measured using a hand-portable fluorometer (Handy PEA, Hansatech, King's Lynn,

United Kingdom). Leaves were dark-adapted for 30 min. Using a leaf clip (4 mm diameter), a rapid pulse of high-intensity light of 3000 μmol m^{-2} s^{-1} (600 W m^{-2}) was administered to the leaf inducing fluorescence. The fluorescence parameters were calculated automatically by the used device. Modulated chlorophyll *a* fluorescence was also determined, to measure the fluorescence yield even in full sunlight, using a field portable pulse modulated chlorophyll fluorometer (FMS2, Hansatech, King's Lynn, United Kingdom).

Chlorophyll Measurements *in vivo*

Chlorophyll content was estimated *in vivo* with a chlorophyll meter (CL-01, Hansatech, United Kingdom). This device provides an indication of green color of leaves and it determines relative chlorophyll content using dual wavelength optical absorbance (620 and 940 nm wavelength).

Statistical Analysis

Statistical analysis was performed with GraphPad Prism 6. All data were compared by using two-way ANOVA, with Tukey's multiple comparison test. Where the interaction between the two factors BS treatments and EC levels (AxB) was significant, data were subjected to one-way ANOVA, comparing all treatments each other. On the contrary, where AxB interaction was not significant, the effect of BS treatments and EC levels was separately evaluated, comparing the respective means.

Each treatment was composed by eight plants randomly distributed on the greenhouse bench. The non-destructive analyses were performed on four biological replications, while destructive analyses on three biological replications. Additional information is reported in each figure legends.

RESULTS

Fresh Yield, Dry Biomass Production, and Percentage Dry Matter of Plants

The FW of the whole lettuce plants (g/head) was determined at harvest, when the first treatment reached the commercial development stage (about 80 g/head). Statistical analysis showed that the interaction between salinity and biostimulant treatments was significant for $p < 0.05$. The biostimulant factor had a significant effect for $p < 0.0001$. Therefore, all treatments were analyzed using one-way ANOVA (**Figure 1**). Biostimulant significantly increased the FW of treated lettuce plants compared to control in all EC levels. The application of Retrosal® at 0.2 mL/plant dose increased more than double this parameter, also under salinity (**Figure 1**). The DW of plants was statistically different among the biostimulant treatments for $p < 0.001$ (**Figure 2**). Statistical differences were found at 0.8 and 1.8 dS/m EC. The highest DW value was found in plants treated with 0.2 mL/plant dose in 1.8 dS/m salinity level. No significant difference was observed for the dry matter percentage that in average was 7% (**Supplementary Figure S1**).

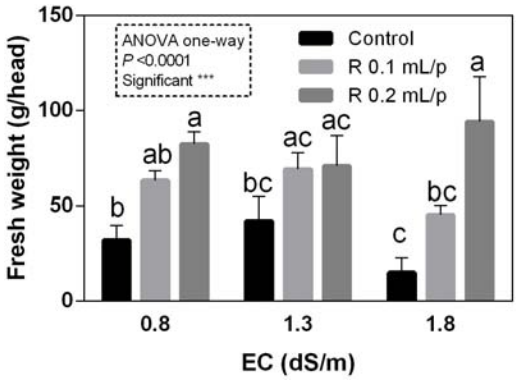


FIGURE 1 | Fresh weight (FW) (g/head) of Romaine lettuce plants, at harvest, subjected to different levels of salinity (0.8, 1.3, and 1.8 dS/m) and treated with water (control) or Retrosal® at 0.1 or 0.2 mL/plant dose. Values are means ± SE ($n = 3$). Data were subjected to two way ANOVA and Tukey's multiple comparison test was used for evaluating the differences among means at (*$p < 0.05$, ***$p < 0.001$). Since interaction was significant, data were also subjected to one-way ANOVA. Different letters indicate statistical differences for $p < 0.05$.

Leaf Gas Exchange Measurements

The statistical analysis showed that net photosynthesis data (A) had significant interaction ($p < 0.05$) between biostimulant and salinity while no significant differences were observed among biostimulant or salinity treatments. Subsequently, data were also analyzed using one-way ANOVA but no significant differences were found (**Figure 3A**). The stomatal conductance (**Figures 4A,B**), that indicates the degree of exchange of CO_2 and water vapor between environment and inner leaf, showed low values in control plants, mainly under salinity. Statistical analysis showed that there was not significant interaction between factors and in the salinity. Significant differences were observed for the biostimulant ($p < 0.001$). Stomatal conductance showed significant differences in plants subjected to 1.3 or 1.8 dS/m EC treated with Retrosal® 0.2 mL/plant (**Figures 4A,B**). The photosynthetic water use efficiency (pWUE) did not show significant interaction between factors, while significant differences were found among biostimulant treatments in the 0.8 dS/m salinity (**Figures 4C,D**). The pWUE was higher in controls and decreased after biostimulant applications; a noticeable reduction occurred in Retrosal® 0.2 mL/plant at 0.8 dS/m treated plants. Transpiration rate data (E) showed significant interaction ($p < 0.05$) between biostimulant and salinity (**Figure 3B**). Significant differences were also observed for biostimulant for $p < 0.001$. Since no significant interaction was found, data were also analyzed by one-way ANOVA. Results showed that E raised after biostimulant treatment at both doses; significant differences were observable in plants treated with Retrosal® at 0.2 mL/plant compared to control plants under 1.3 or 1.8 dS/m EC.

Chlorophyll *a* Fluorescence

Among the different JIP index, the performance index (PI) and the number of reaction centers per cross section (RC/CSm)

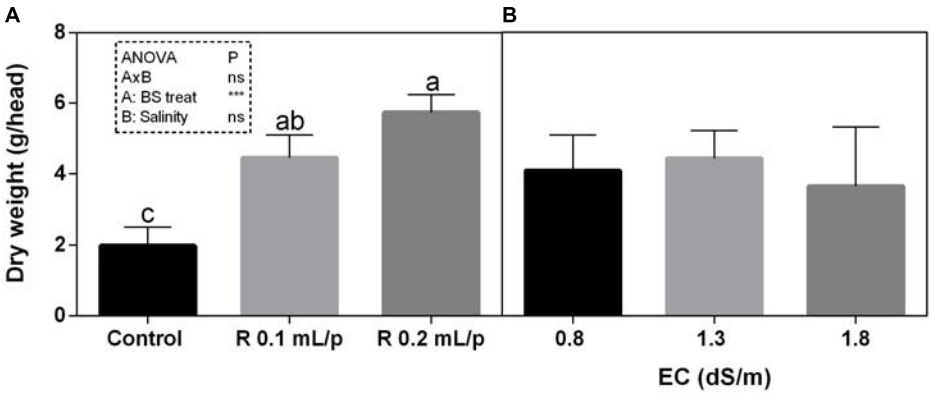


FIGURE 2 | Dry weight (DW) (g/head) of Romaine lettuce plants, at harvest, subjected to different levels of salinity (0.8, 1.3, and 1.8 dS/m) and treated with water (control) or Retrosal® at 0.1 or 0.2 mL/plant dose. Data were subjected to two way ANOVA and Tukey's multiple comparison test was used for evaluating the differences among means at (*$p < 0.05$, ***$p < 0.001$). Since there was not significant AxB interaction, the effect of BS treatments **(A)** and EC levels **(B)** was evaluated separately, comparing the respective means ± SE ($n = 3$). Different letters indicate statistical differences for $p < 0.05$.

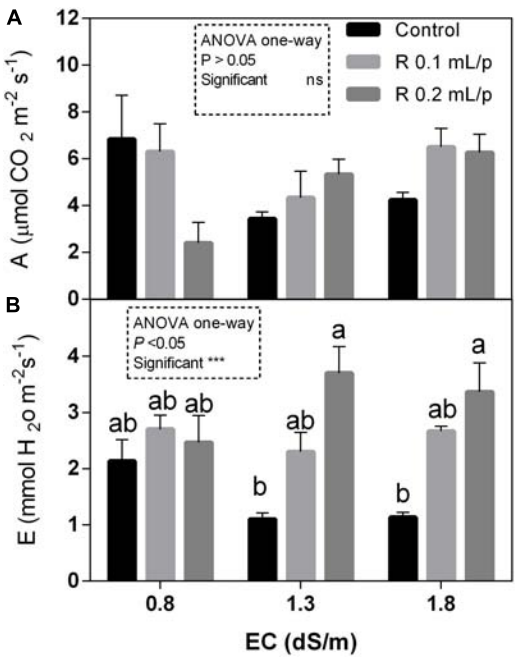


FIGURE 3 | Leaf gas exchanges parameters [net photosynthesis **(A)** and transpiration **(B)**] measured *in vivo* in Romaine lettuce plants, at harvest. Plants were subjected to different levels of salinity (0.8, 1.3, and 1.8 dS/m) and treated with water (control) or Retrosal® at 0.1 or 0.2 mL/plant dose. Values are means ± SE ($n = 4$). Data were compared by using two way ANOVA, with Tukey's multiple comparison test (*$p < 0.05$, ***$p < 0.001$). Since interaction was significant, data were also subjected to one-way ANOVA. Different letters indicate statistical differences for $p < 0.05$.

have been reported and both showed the same pattern (**Figure 5**). Statistical analysis showed significant differences for the biostimulant factor, while salinity and interaction between the two factors were not significant. In fact, treatments with the biostimulant at 0.2 mL/plant dose induced a slight increase in these parameters than controls. Significant differences were observed in plants under 1.3 dS/m salinity level. PI ranged from 0.78 in control plants to 2.12 in the Retrosal® 0.2 mL/plant subjected to 1.3 dS/m of salinity (**Figures 5A,B**). The RC/CSm were significantly different in plant at 1.3 dS/m EC, between control and the Retrosal® 0.2 mL/plant treatment (**Figures 5C,D**).

As regards the modulated chlorophyll *a* fluorescence measurements, the electron transport rate (ETR) and the quantum efficiency of the photosystem II (ΦPS2) are reported (**Figure 6**). The two-way ANOVA for ETR and ΦPS2 showed significant values for the biostimulant factor, while the interaction and the salinity were not significant. ETR showed significant increases after biostimulant applications at all salinity levels (**Figures 6A,B**) in particular at 0.2 mL/plant dose. The ΦPS2 (**Figures 6C,D**) was significantly higher in 0.2 mL/plant dose treated leaves with 0.8 dS/m salinity level.

Total Chlorophylls, Carotenoids, and Sugars

The two-way ANOVA analysis showed that the interaction between factors was not significant, as well as the salinity, for all the determinations considered. On the contrary, the biostimulant factor was significant ($p < 0.001$) for the chlorophyll *in vivo* and carotenoids. It is possible to notice that the chlorophyll content measured *in vivo* in lettuce leaves (**Figure 7**) showed similar values in controls. Retrosal® induced an increment of chlorophylls, confirmed by statistical analyses in leaves treated with the biostimulant 0.2 mL/plant at 1.3 or 1.8 dS/m salinity level. The destructive determinations showed the same pattern for chlorophylls $a+b$ concentration and carotenoids (**Figures 8A**, **9**). Total carotenoids showed values of 0.07 mg/g FW in controls and 0.14 mg/g FW in leaves of treated plants at 0.2 mL/plant dose. In fact, biostimulant treatment caused a slightly increment of the considered pigments, however the effect was not statistically relevant compared to controls.

The total sugars concentration in lettuce leaves did not show significant differences among treatments (**Figure 8B**).

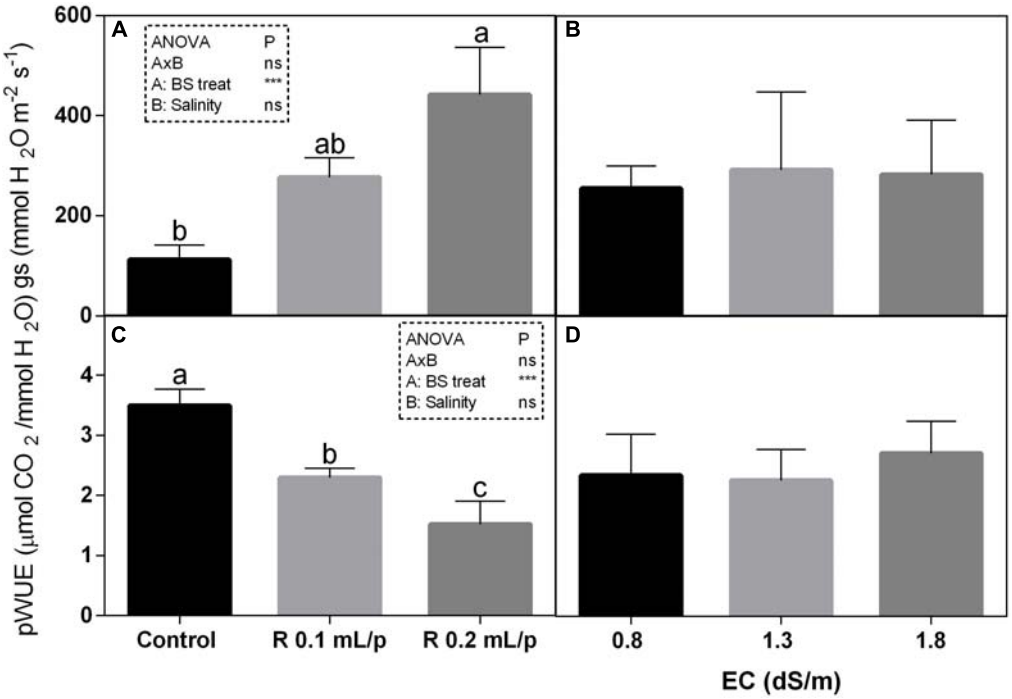


FIGURE 4 | Leaf gas exchanges parameters [stomatal conductance **(A,B)** and photosynthetic water use efficiency (pWUE) **(C,D)**] measured *in vivo* in Romaine lettuce plants, at harvest. Plants were subjected to different levels of salinity (0.8, 1.3, and 1.8 dS/m) and treated with water (control) or Retrosal® at 0.1 or 0.2 mL/plant dose. Data were compared by using two way ANOVA, with Tukey's multiple comparison test (*$p < 0.05$, ***$p < 0.001$). Since there was not significant AxB interaction, the effect of BS treatments and EC levels was evaluated separately, comparing the respective means ± SE ($n = 3$). Different letters indicate statistical differences for $p < 0.05$.

Nitrate Levels and Proline

The two-way ANOVA analysis for nitrate data showed that interaction and factors were statistically significant. Therefore, data of all treatments were analyzed using one-way ANOVA and results indicated that biostimulant significantly reduced the nitrate concentration at 1.3 and 1.8 EC levels. In fact, treated plants showed values similar to the control 0.8 dS/m EC, suggesting that Retrosal® allowed keeping lower nitrate levels. Nitrate values ranged from 83.7 to 248.7 mg kg^{-1} FW (**Figure 10A**). The graph shows that the increment of salinity caused a sensible increase of nitrate in leaves of control plants.

As observed for nitrate, the two-way ANOVA showed high significance ($p < 0.001$) for all factors and their interaction in the proline data (**Figure 10B**). In control plants it is possible to observe that the increasing levels of salinity caused a raise in proline concentration. Biostimulant treatment allowed maintaining proline levels lower, except in leaves treated with the biostimulant at 0.1 mL/plant dose at 0.8 dS/m EC, that showed the highest concentration observed. This result was also confirmed by one-way ANOVA analysis.

Abscisic Acid

The statistical analysis revealed that the biostimulant factor was significant for $p < 0.01$, while the interaction and salinity were not significant (**Figure 11**). All plants treated with Retrosal® showed lower values of ABA compared to control. A sensible decrease of ABA concentration was recorded after biostimulant application at 0.1 or 0.2 mL/plant dose at 0.8 and 1.8 dS/m EC.

Mineral Content

The mineral content was determined in leaves at harvest. Mineral concentrations are reported in the **Supplementary Table S1**. Since the work was focused on salinity exposure, the values of sodium (Na) and calcium (Ca) have been discussed in relation to biostimulant applications. Na was strongly affected by the salinity ($p < 0.0001$) and increased with the increment of EC levels. The interaction was not significant, while the biostimulant factor was significant for $p < 0.001$. The lowering effect of the biostimulant in the Na leaf accumulation was evident in plants grown under 1.3 dS/m EC, treated with 0.1 mL/plant dose (**Figure 12**). The Ca content in leaves (**Supplementary Table S1**) did not show significant differences, even if a slightly increment was noticeable in leaves after the application of 0.2 mL/plant dose, at 1.8 dS/m EC level.

DISCUSSION

Plant responses to salinity differ greatly among species and to a lesser extent among varieties (Shannon and Grieve, 1998;

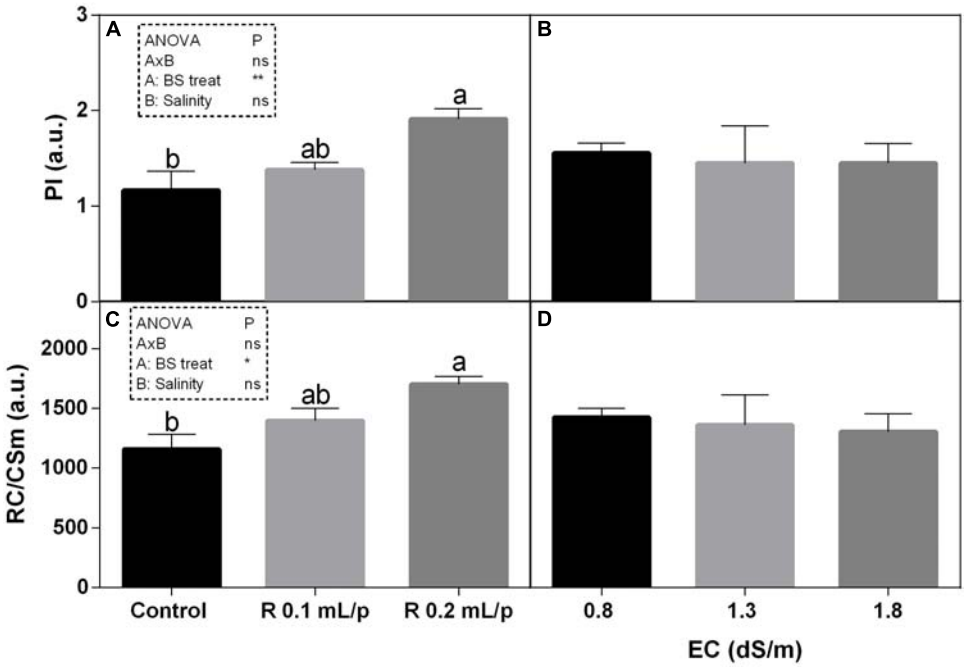


FIGURE 5 | Chlorophyll *a* fluorescence parameters [performance index (PI) **(A,B)** and number of reaction centers per cross section **(C,D)**] measured *in vivo* in Romaine lettuce plants, at harvest. Plants were subjected to different levels of salinity (0.8, 1.3, and 1.8 dS/m) and treated with water (control) or Retrosal® at 0.1 or 0.2 mL/plant dose. Data were compared by using two way ANOVA, with Tukey's multiple comparison test (*$p < 0.05$, **$p < 0.01$). Since there was not significant AxB interaction, the effect of BS treatments and EC levels was evaluated separately, comparing the respective means ± SE ($n = 3$). Different letters indicate statistical differences for $p < 0.05$.

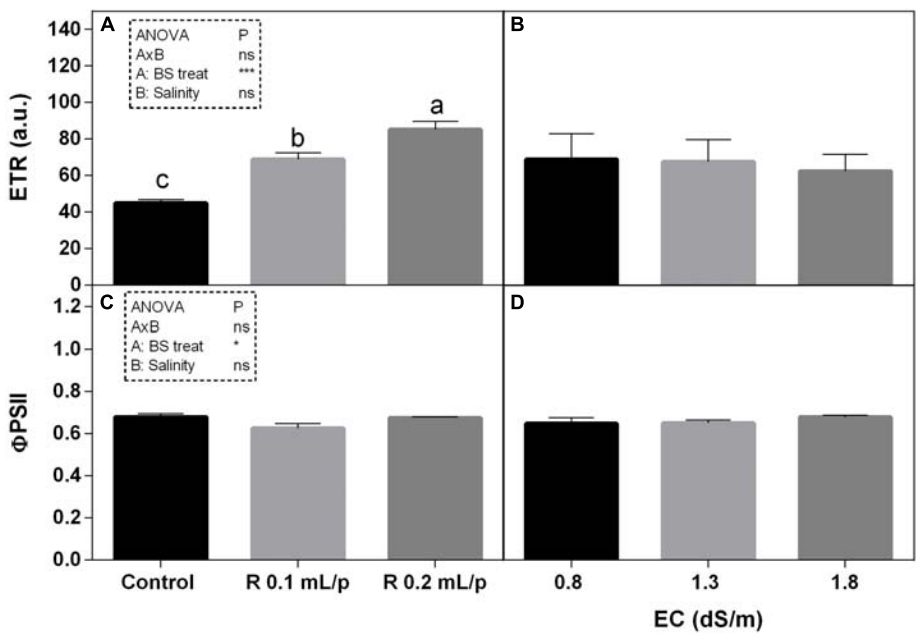


FIGURE 6 | Modulated chlorophyll *a* fluorescence parameters [electron transport rate (ETR) **(A,B)** and photosystem II quantum efficiency **(C,D)**] measured *in vivo* in Romaine lettuce plants, at harvest. Plants were subjected to different levels of salinity (0.8, 1.3, and 1.8 dS/m) and treated with water (control) or Retrosal® at 0.1 or 0.2 mL/plant dose. Data were compared by using two way ANOVA, with Tukey's multiple comparison test (*$p < 0.05$, ***$p < 0.001$). Since there was not significant AxB interaction, the effect of BS treatments and EC levels was evaluated separately, comparing the respective means ± SE ($n = 3$). Different letters indicate statistical differences for $p < 0.05$.

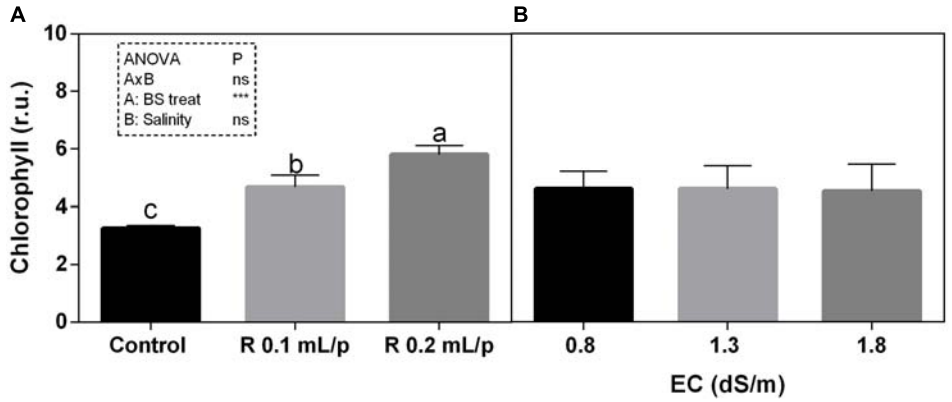


FIGURE 7 | Chlorophyll content, measured *in vivo*, of Romaine lettuce leaves at harvest, subjected to different levels of salinity (0.8, 1.3, and 1.8 dS/m) and treated with water (control) or Retrosal® at 0.1 or 0.2 mL/plant dose. Data were compared by using two way ANOVA, with Tukey's multiple comparison test (***$p < 0.001$). Since there was not significant AxB interaction, the effect of BS treatments **(A)** and EC levels **(B)** was evaluated separately, comparing the respective means ± SE ($n = 3$). Different letters indicate statistical differences for $p < 0.05$.

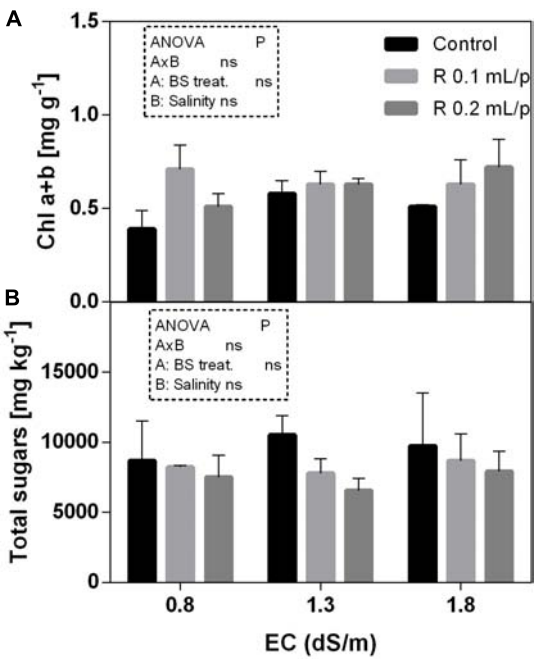


FIGURE 8 | Chlorophyll *a+b* **(A)** and total sugars **(B)** concentrations in Romaine lettuce leaves, at harvest, subjected to different levels of salinity (0.8, 1.3, and 1.8 dS/m) and treated with water (control) or Retrosal® at 0.1 or 0.2 mL/plant dose. Values are means ± SE ($n = 3$). Data were compared by using two way ANOVA, with Tukey's multiple comparison test ($p < 0.05$).

Parida and Das, 2005). Their sensitivity is higher during seedling and reproductive stage (Machado and Serralheiro, 2017; Negrão et al., 2017). Furthermore, several environmental factors (temperature, wind, relative humidity, radiation, air pollution) show significant interaction with salinity effects (Shannon, 1997). Another environmental hazard that can be aggravated by salinity is the root zone waterlogging; in fact, root zone salinity, and waterlogging greatly increase the salt uptake compared with normal soil conditions (Shannon, 1997). The negative effect of salt stress can be commonly observed on growth reduction of plants (Shannon and Grieve, 1998; Santos and Caldeira, 1999; Akram et al., 2012). A decrease in fresh mass was observed in lettuce plants cv. Vera by Andriolo et al. (2005) and in a work of De Pascale and Barbieri (1995) conducted on lettuce, endive, and fennel. As described by Ünlükara et al. (2008), the yield of plants of lettuce cv. Crispa maintained steady values up to a threshold of salinity tolerance and then decreased with the increment of the soil salinity. The biostimulant tested in this work increased significantly the FW of lettuce plants compared to control. The enhancement in the growth of lettuce plants, after treatments, could be attributed to an increased nutrient uptake, as reported by Türkmen et al. (2004), who used humic acids in combination with Ca to treat tomato seedlings. In recent years, the functions of Ca were studied in particular for its role as a second messenger in the signal conduction between environmental factors and plant responses, in terms of growth and development (Kaya et al., 2002; Hepler, 2005). Free Ca is directly involved in the activation of salt overlay sensitive (SOS) sodium channels. The Ca acts as an inhibitor of sodium channels and reduces the uptake in cells. These findings are in agreement with our results observed in plants treated with Retrosal® 0.2 mL/plant at the highest salinity. In fact, Na declined while Ca slightly increased, although not significant differences were observed.

Lucini et al. (2015) observed that applications of plant-derived protein hydrolysate mitigated the deleterious effects of salt stress (3.5 dS/m EC) on lettuce cv. Regina di Maggio. These results were consistent with a previous study of Ertani et al. (2013), who observed that a protein hydrolysate biostimulant derived from alfalfa increased maize plant biomass, even under salinity. The effect of biostimulants can be direct on the salt sensitivity but also indirect, increasing plant biomass and fastening the growing cycle. The application of Retrosal® significantly increased the development rate, indicating a clear biostimulant effect.

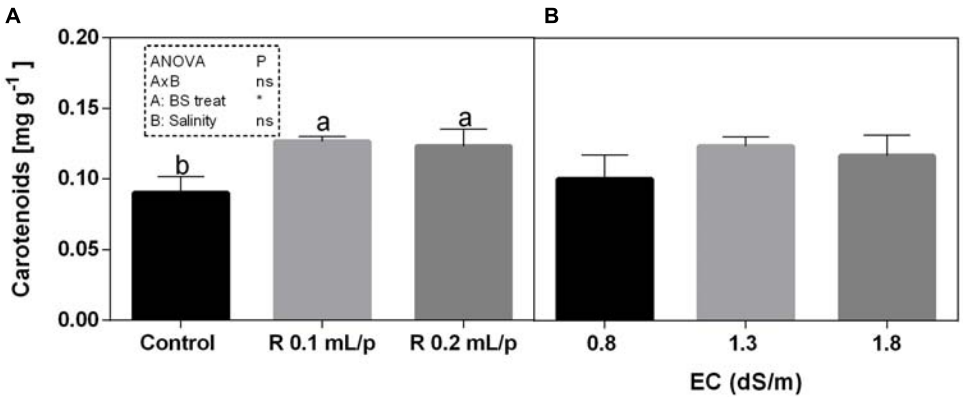


FIGURE 9 | Carotenoids concentrations in Romaine lettuce leaves, at harvest, subjected to different levels of salinity (0.8, 1.3, and 1.8 dS/m) and treated with water (control) or Retrosal® at 0.1 or 0.2 mL/plant dose. Data were compared by using two way ANOVA, with Tukey's multiple comparison test (*$p < 0.05$). Since there was not significant AxB interaction, the effect of BS treatments **(A)** and EC levels **(B)** was evaluated separately, comparing the respective means ± SE ($n = 3$). Different letters indicate statistical differences for $p < 0.05$.

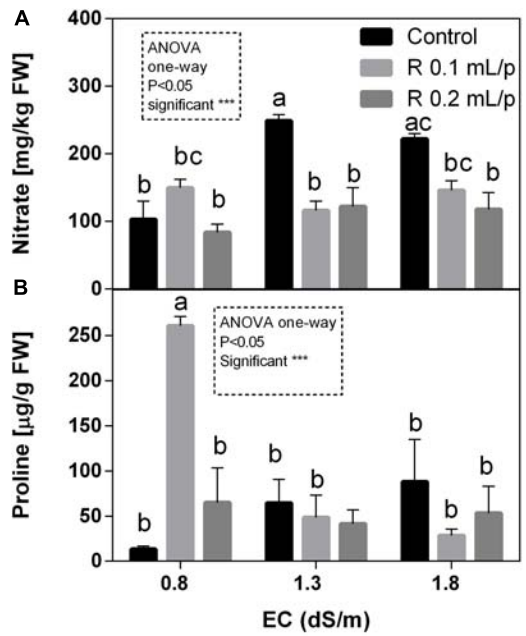


FIGURE 10 | Nitrate **(A)** and proline **(B)** concentration in Romaine lettuce leaves, at harvest, subjected to different levels of salinity (0.8, 1.3, and 1.8 dS/m) and treated with water (control) or Retrosal® at 0.1 or 0.2 mL/plant dose. Values are means ± SE ($n = 3$). Data were compared by using two way ANOVA, with Tukey's multiple comparison test (**$p < 0.01$, ***$p < 0.001$). Since interaction was significant, data were also subjected to one-way ANOVA. Different letters indicate statistical differences for $p < 0.05$.

Salt stress was demonstrated to affect negatively also the leaves photosynthetic pigment contents (Smirnoff, 1998; Türkmen et al., 2004). In fact, stress conditions led to an inhibition of chlorophyll biosynthesis, along with the activation of the pigments degradation by enzyme chlorophyllase (Santos, 2004). In the present work, results indicated that biostimulant treatments had a positive effect on the chlorophyll content measured *in vivo* compared to controls. Biostimulant applications preserved leaves pigments, contributing to maintain a good produce visual appearance and nutraceutical properties. Biostimulants are often able to increase leaf pigments concentrations; this concerns in particular products containing seaweed extracts, plant extracts, humic acid (Bulgari et al., 2015, 2017; Chbani et al., 2015; and references therein).

To evaluate the health-status of the photosynthetic apparatus in response to stress factors, the gas exchange analysis is a useful non-destructive method. Salinity has direct impact on the primary metabolism. The reduction of water uptake in salt stress conditions limits the photosynthesis. The excess of energy absorbed from the leaf must be dissipated to avoid leaf photo-damages. Among non-destructive methods, the chlorophyll *a* fluorescence and derived JIP indexes can be useful to monitor the progress of stress conditions, as well as leaf gas exchanges. These tools can be also used for evaluating the efficacy of biostimulant treatments (Bulgari et al., 2017). PI is an overall evaluation parameter of leaf functionality and it is associated to leaf health status. This index has been widely used for assessing plant performance under stress (Mehta et al., 2010) or to evaluate the effect of treatments (Misra et al., 2001; Cocetta et al., 2016). In our experiment, biostimulant treatment at 0.2 mL/plant dose induced a slight increase in PI than controls, and this increment was statistically significant at 1.3 dS/m EC level. Biostimulant also enhanced the ETR, indicating that higher electron flux was destined to the photosynthetic machinery. This higher energy use can be also explained by the higher number of active RC/CSm, which normally declined under salinity (Ferrante et al., 2011). Results suggested that, in our experimental conditions, a general positive effect deriving from the application of biostimulant was observable on net photosynthesis rate. Consistent results, regarding the effect of biostimulants on parameters of photosynthetic activity, were found, among others, in rocket treated with biostimulants of vegetable origin (Abdalla, 2013), in strawberry after seaweed extract application (Spinelli et al., 2010), in maize under drought treated with fulvic acid

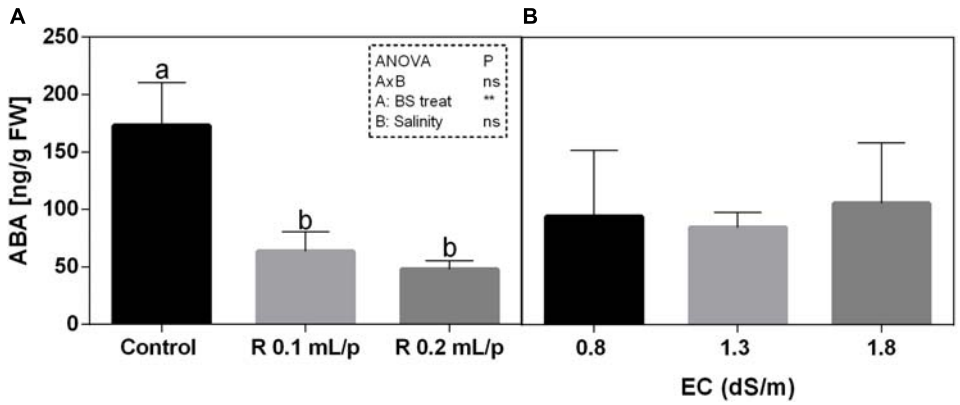


FIGURE 11 | Abscisic acid (ABA) concentration in Romaine lettuce leaves, at harvest, subjected to different levels of salinity (0.8, 1.3, and 1.8 dS/m) and treated with water (control) or Retrosal® at 0.1 or 0.2 mL/plant dose. Data were compared by using two way ANOVA, with Tukey's multiple comparison test (**$p < 0.01$). Since there was not significant AxB interaction, the effect of BS treatments **(A)** and EC levels **(B)** was evaluated separately, comparing the respective means ± SE ($n = 3$). Different letters indicate statistical differences for $p < 0.05$.

(Anjum et al., 2011), and also in ornamental plants after the application of a mix of plant extract rich in fulvic acids, humic acids, amino acids, and glycine betaine (Massa et al., 2016). To sum up, since photosynthesis has been measured at harvest it cannot be ruled out that it decreases at a later stage along with stomatal conductance. However, our data suggest that Retrosal® could stimulate the crop performance by keeping open stomata, maintaining photosynthesis, source-sink relations (growth), and thus protecting from possible photoinhibition/photooxidation effects (Castro et al., 2012; Massa et al., 2016). Generally speaking, soluble sugars tend to increase in plants under salt stress, while starch content decreases (Chaves, 1991; Baki et al., 2000). However, as reported by Ashraf and Harris (2004), the role of carbohydrates in the salinity tolerance is not clear and further investigations are needed to conclude that they are universally associated with salt tolerance, because the variations in the accumulation of these compounds could vary among species. In our material, the tissue levels of total sugars were not affected by salinity and treatment applications, in fact all plants showed similar concentrations. These results indicated that treated plants did not show salinity stress under the conditions applied.

On the contrary, nitrate levels were affected by salinity; a sensible increase of nitrate was observable in control plants. Biostimulant treatments allowed maintaining nitrate concentration similar to the controls. The reduction of nitrate concentration in leaves is probably due to the increase of the nitrate assimilation by the activation of the nitrate and nitrite reductase enzymes. A reduction of nitrate after biostimulant application was observed in several species of leafy vegetables (Vernieri et al., 2005; Liu et al., 2007; Dudaš et al., 2016). The capability to keep nitrates low and under the limits imposed by EU regulations (Reg. No. 1258/2011) is very interesting in this commercial sector. The low nitrate concentrations observed are commonly found in Romaine lettuce as previously reported by Bulgari et al. (2017). The low nitrate accumulation also depends by the

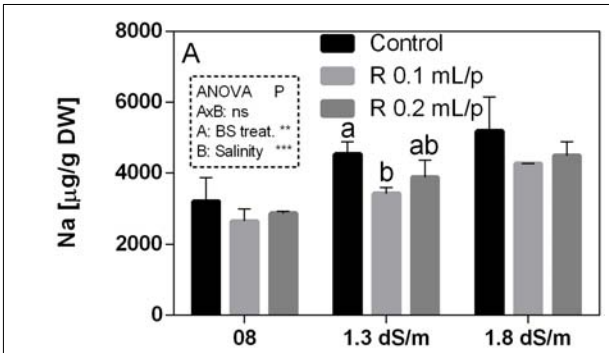


FIGURE 12 | Sodium (Na) concentration in Romaine lettuce leaves, at harvest, subjected to different levels of salinity (0.8, 1.3, and 1.8 dS/m) and treated with water (control) or Retrosal® at 0.1 or 0.2 mL/plant dose. Values are means ± SE ($n = 3$). Data were compared by using two way ANOVA, with Tukey's multiple comparison test (**$p < 0.01$, ***$p < 0.001$). Different letters indicate statistical differences for $p < 0.05$.

fertilizers application as well as from genotype, environmental, and management factors (Cometti et al., 2011). In our experiment, slow-release fertilizer was supplied at transplanting and during cultivation for satisfying the plant's requirements. This strategy avoided the high accumulation of nitrate in leaves.

Nitrate and salinity are inversely correlated, because under stress conditions the sodium is accumulated in vacuoles avoiding the storage of nitrates that are straightly assimilated or not absorbed from the soil or nutrient solution. However, this behavior occurs when the plants are under severe salinity stress, while at beginning of the stress plants can also counteract the salinity by increasing the osmotic potential, by accumulating osmolytes, including nitrates. The initial increase of nitrate concentration under 10–20 mM NaCl salinity exposure was observed in Lettuce (*Lactuca sativa* L. subsp. capitata) grown

in floating system, while declined at 30 mM (Tesi et al., 2003). Analogous results were also observed for lettuce grown in soilless cultivation, in which the nitrate concentration increased up to 2.5 dS/m and declined in the plants grown under 3.5 dS/m of salinity (Serio et al., 2001). Our results demonstrated that the nitrate content did not significantly change compared to the untreated and unstressed control after biostimulant applications, demonstrating the role of this product to alleviate the exposure to saline solutions.

Proline accumulates in many plant species under to a broad range of environmental stress conditions (Ashraf and Harris, 2004; Claussen, 2005; Rejeb et al., 2014; Xiong et al., 2014). Nowadays it is known that proline has multifunctional roles in plants (Szabados and Savouré, 2010). In addition, proline being an osmoprotectant, can act as a potent non-enzymatic antioxidant. In our treatments, we observed that the increasing levels of salinity caused a raise in proline concentration in control plants. Proline can play an important role in the osmotic adjustment and may participate to the scavenging of reactive oxygen species. Retrosal® treatments, in general, allowed maintaining lower the proline levels under salinity. On the contrary, the highest concentration was found in leaves treated with the biostimulant at 0.1 mL/plant dose at 0.8 dS/m. These results prove a kind of dose depending effect of treatments on lettuce and support the hypothesized positive role of biostimulants in protecting plants from saline exposure. Further investigation should be performed to better understand the role of this biostimulant in the proline metabolism.

Abscisic acid is an essential phytohormone that regulates various aspects of plant growth and development in response to abiotic stress (Fujita et al., 2011). In stressful conditions, such as salinity, ABA content increases and it triggers the expression of many genes encoding various proteins important for biochemical and physiological processes (Xiong et al., 2014 and references therein). When lettuce plants were harvested at commercial maturity stage, all plants treated with biostimulant showed lower values of ABA compared to controls and in some cases this decrease was statistically significant. A reduction in ABA content in salt-stressed and biostimulant-treated plants might be related to the de-activation of ABA signaling pathways which controls the stomata closure (Trivellini et al., 2016). In fact, in our experiment the biostimulant treatments enhanced the stomatal conductance and this behavior might be reflected by a slightly improvement of net photosynthetic rate and an enhancement of FW. Thus, the treatment of plants with selected biostimulant agents during their development was accompanied by significant amelioration of stress impacts on plant physiology and growth. Moreover, similar findings were observed in a field study with pistachio (*Pistacia vera*), in which biostimulant treatments ameliorated negative effects on plant growth resulting from irrigation with low to moderate rates of NaCl. This effect was related to a reduction in proline accumulation and decreased levels of ABA in leaves of treated plants compared to controls (Moghaddam and Soleimani, 2012). Additionally, in plants grown under different stressful conditions, a decreased level of free ABA after application of biostimulant has been shown (Przybysz et al., 2010, 2014), suggesting again that changes in ABA concentration by lowering its accumulation resulted in a general positive effect on leaf gas exchanges and stimulated growth under salinity, as it was recorded also in this work.

CONCLUSION

Crops are subjected to abiotic and artificial-induced stresses during their life span that greatly reduce productivity and also the quality of these commodities.

The preliminary results reported in this study indicate that the application of the biostimulant Retrosal® on lettuce confers enhanced tolerance when plants are exposed to NaCl treatments, due to its multifaceted action at both biochemical and physiological level. In particular, we noted a significant biostimulant effect on several variables examined, among which fresh yield, dry biomass, chlorophyll content *in vivo*, nitrate concentration and some leaf gas exchange parameters as well as chlorophyll *a* fluorescence parameters. Thus, this biostimulant represents an effective tool to employ in crop management to stimulate plant growth and productivity. Further experiments will be necessary in order to investigate in depth the effects of Retrosal® against salinity, subjecting lettuce plants to higher salinity concentrations, that could result more stressful for the considered crop.

AUTHOR CONTRIBUTIONS

RB performed the experiments and analytical determinations, and contributed to manuscript writing. AT contributed to ABA extraction and determination, and manuscript writing. AF was responsible for the research activities, experimental plan, and revision of the final manuscript.

FUNDING

This work was supported by University of Milan ID: 34265.

SUPPLEMENTARY MATERIAL

The Supplementary Material for this article can be found online at: https://www.frontiersin.org/articles/10.3389/fpls.2018.01870/full#supplementary-material

FIGURE S1 | Percentage dry matter of Romaine lettuce plants, at harvest. Plants were subjected to different levels of salinity (0.8, 1.3, and 1.8 dS/m) and treated with water (control) or Retrosal® at 0.1 or 0.2 mL/plant dose. Values are means ± SE (n = 3). Data were compared by using two way ANOVA, with Tukey's multiple comparison test.

TABLE S1 | Concentration of mineral elements in lettuce leaves treated with water (control) or Retrosal® 0.1 or 0.2 mL/plant. Data are means with standard deviations (n = 4).

TABLE S2 | Electrical conductivity measured from substrate extract (1:2.5 v/v) where lettuce plants treated with water (control) or Retrosal® 0.1 or 0.2 mL/plant were grown.

REFERENCES

Abdalla, M. M. (2013). The potential of *Moringa oleifera* extract as a biostimulant in enhancing the growth, biochemical and hormonal contents in rocket (*Eruca vesicaria* subsp. sativa) plants. *Int. J. Plant Physiol. Biochem.* 5, 42–49. doi: 10.5897/IJPPB2012.026

Abrahám, E., Hourton-Cabassa, C., Erdei, L., and Szabados, L. (2010). Methods for determination of proline in plants. *Plant Stress Tolerance* 639, 317–331. doi: 10.1007/978-1-60761-702-0_20

Agastian, P., Kingsley, S. J., and Vivekanandan, M. (2000). Effect of salinity on photosynthesis and biochemical characteristics in mulberry genotypes. *Photosynthetica* 38, 287–290. doi: 10.1023/A:1007266932623

Akram, N. A., Ashraf, M., and Al-Qurainy, F. (2012). Aminolevulinic acid-induced changes in some key physiological attributes and activities of antioxidant enzymes in sunflower (*Helianthus annuus* L.) plants under saline regimes. *Sci. Hortic.* 142, 143–148. doi: 10.1016/j.scienta.2012.05.007

Andriolo, J. L., Luz, G. L. D., Witter, M. H., Godoi, R. D. S., Barros, G. T., and Bortolotto, O. C. (2005). Growth and yield of lettuce plants under salinity. *Hortic. Bras.* 23, 931–934. doi: 10.1590/S0102-05362005000400014

Anjum, S. A., Wang, L., Farooq, M., Xue, L., and Ali, S. (2011). Fulvic acid application improves the maize performance under well-watered and drought conditions. *J. Agron. Crop Sci.* 197, 409–417. doi: 10.1111/j.1439-037X.2011.00483.x

Ashkani, S., Rafii, M. Y., Shabanimofrad, M., Miah, G., Sahebi, M., Azizi, P., et al. (2015). Molecular breeding strategy and challenges toward improvement of blast disease resistance in rice crops. *Front. Plant Sci.* 6:886. doi: 10.3389/fpls.2015.00886

Ashraf, M. P. J. C., and Harris, P. J. C. (2004). Potential biochemical indicators of salinity tolerance in plants. *Plant Sci.* 166, 3–16. doi: 10.1016/j.plantsci.2003.10.024

Ayers, A. D., Wadleigh, C. H., and Bernstein, L. (1951). Salt tolerance of 6 varieties of lettuce. *Proc. Am. Soc. Hortic. Sci.* 57, 237-242.

Baki, G. A. E., Siefritz, F., Man, H. M., Weiner, H., Kaldenhoff, R., and Kaiser, W. M. (2000). Nitrate reductase in *Zea mays* L. under salinity. *Plant Cell Environ.* 23, 515–521. doi: 10.1046/j.1365-3040.2000.00568.x

Blaylock, A. D. (1994). *Soil Salinity, Salt Tolerance, and Growth Potential of Horticultural and Landscape Plants*. Laramie, WY: University of Wyoming.

Borgognone, D., Cardarelli, M., Rea, E., Lucini, L., and Colla, G. (2014). Salinity source-induced changes in yield, mineral composition, phenolic acids and flavonoids in leaves of artichoke and cardoon grown in floating system. *J. Sci. Food Agric.* 94, 1231–1237. doi: 10.1002/jsfa.6403

Bray, E. A., Bailey-Serres, J., and Weretilnyk, E. (2000). "Response to abiotic stresses," in *Biochemistry and Molecular Biology of Plants*, eds W. Gruissem, B. Buchanan, and R. Jones (Rockville, MD: American Society of Plant Physiologists), 1158–1249.

Bulgari, R., Cocetta, G., Trivellini, A., Vernieri, P., and Ferrante, A. (2015). Biostimulants and crop responses: a review. *Biol. Agric. Hortic.* 31, 1–17. doi: 10.1080/01448765.2014.964649

Bulgari, R., Morgutti, S., Cocetta, G., Negrini, N., Farris, S., Calcante, A., et al. (2017). Evaluation of borage extracts as potential biostimulant using a phenomic, agronomic, physiological, and biochemical approach. *Front. Plant Sci.* 8:935. doi: 10.3389/fpls.2017.00935

Castro, J., Vera, J., González, A., and Moenne, A. (2012). Oligo-carrageenans stimulate growth by enhancing photosynthesis, basal metabolism, and cell cycle in tobacco plants (var. Burley). *J. Plant Growth Regul.* 31, 173–185. doi: 10.1007/s00344-011-9229-5

Cataldo, D. A., Haroon, M., Sehrader, L. E., and Youngs, V. L. (1975). Rapid colorimetric determination of nitrate in plant tissue by titration of salicylic acid. *Commun. Soil Sci. Plant. Anal.* 6, 71–80. doi: 10.1080/00103627509366547

Cavaiuolo, M., Cocetta, G., Bulgari, R., Spinardi, A., and Ferrante, A. (2015). Identification of innovative potential quality markers in rocket and melon fresh-cut produce. *Food Chem.* 188, 225–233. doi: 10.1016/j.foodchem.2015.04.143

Chaves, M. M. (1991). Effects of water deficits on carbon assimilation. *J. Exp. Bot.* 42, 1–16. doi: 10.1093/jxb/42.1.1

Chaves, M. M., Flexas, J., and Pinheiro, C. (2009). Photosynthesis under drought and salt stress: regulation mechanisms from whole plant to cell. *Ann. Bot.* 103, 551–560. doi: 10.1093/aob/mcn125

Chbani, A., Majed, S., Mawlawi, H., and Kammoun, M. (2015). The use of seaweed as a bio-fertilizer: Does it influence Proline and Chlorophyll concentration in plants treated? *Arab. J. Med. Aromat. Plants* 1, 67–77.

Claussen, W. (2005). Proline as a measure of stress in tomato plants. *Plant Sci.* 168, 241–248. doi: 10.1016/j.plantsci.2004.07.039

Cocetta, G., Ferrante, A., Trivellini, A., and Francini, A. (2016). Effect of washing treatments on chlorophyll a fluorescence and vitamin C content in minimally processed lamb's lettuce during storage. *Agrochimica* 60, 1–14.

Cocetta, G., Rossoni, M., Gardana, C., Mignani, I., Ferrante, A., and Spinardi, A. (2015). Methyl jasmonate affects phenolic metabolism and gene expression in blueberry (*Vaccinium corymbosum*). *Physiol. Plant* 153, 269–283. doi: 10.1111/ppl.12243

Colla, G., Rouphael, Y., Leonardi, C., and Bie, Z. (2010). Role of grafting in vegetable crops grown under saline conditions. *Sci. Hortic.* 127, 147–155. doi: 10.1016/j.scienta.2010.08.004

Cometti, N. N., Martins, M. Q., Bremenkamp, C. A., and Nunes, J. A. (2011). Nitrate concentration in lettuce leaves depending on photosynthetic photon flux and nitrate concentration in the nutrient solution. *Hortic. Brasil.* 29, 548–553. doi: 10.1590/S0102-05362011000400018

De Pascale, S., and Barbieri, G. (1995). Effects of soil salinity from long-term irrigation with saline-sodic water on yield and quality of winter vegetable crops. *Sci. Hortic.* 64, 145–157. doi: 10.1016/0304-4238(95)00823-3

Desoky, E. S. M., Merwad, A. R. M., and Rady, M. M. (2018). Natural biostimulants improve saline soil characteristics and salt stressed-sorghum performance. *Commun. Soil Sci. Plant Anal.* 49, 967–983. doi: 10.1080/00103624.2018.1448861

Di Stasio, E., Van Oosten, M. J., Silletti, S., Raimondi, G., Dell'Aversana, E., Carillo, P., et al. (2018). *Ascophyllum nodosum*-based algal extracts act as enhancers of growth, fruit quality, and adaptation to stress in salinized tomato plants. *J. Appl. Phycol.* 30, 2675–2686. doi: 10.1007/s10811-018-1439-9

du Jardin, P. (2015). Plant biostimulants: definition, concept, main categories and regulation. *Sci. Hort.* 196, 3–14. doi: 10.1016/j.scienta.2015.09.021

Dudaš, S., Šola, I., Sladonja, B., Erhatić, R., Ban, D., and Poljuha, D. (2016). The effect of biostimulant and fertilizer on "low input" lettuce production. *Acta Bot. Croat.* 75, 253–259. doi: 10.1515/botcro-2016-0023

Ertani, A., Schiavon, M., Muscolo, A., and Nardi, S. (2013). Alfalfa plant-derived biostimulant stimulate short-term growth of salt stressed *Zea mays* L. plants. *Plant Soil* 364, 145–158. doi: 10.1007/s11104-012-1335-z

Fernandez, J. A., Niñirola, D., Ochoa, J., Orsini, F., Pennisi, G., Gianquinto, G., et al. (2016). Root adaptation and ion selectivity affects the nutritional value of salt-stressed hydroponically grown baby-leaf *Nasturtium officinale* and *Lactuca sativa*. *Agric. Food Sci.* 25, 230–239. doi: 10.23986/afsci.58960

Ferrante, A., Trivellini, A., Malorgio, F., Carmassi, G., Vernieri, P., and Serra, G. (2011). Effect of seawater aerosol on leaves of six plant species potentially useful for ornamental purposes in coastal areas. *Sci. Hort.* 128, 332–341. doi: 10.1016/j.scienta.2011.01.008

Flowers, T. J. (2004). Improving crop salt tolerance. *J. Exp. Bot.* 55, 307–319. doi: 10.1093/jxb/erh003

Forni, C., Duca, D., and Glick, B. R. (2017). Mechanisms of plant response to salt and drought stress and their alteration by rhizobacteria. *Plant Soil* 410, 335–356. doi: 10.1007/s11104-016-3007-x

Fujita, Y., Fujita, M., Shinozaki, K., and Yamaguchi-Shinozaki, K. (2011). ABA-mediated transcriptional regulation in response to osmotic stress in plants. *J. Plant Res.* 124, 509–525. doi: 10.1007/s10265-011-0412-3

Hepler, P. K. (2005). Calcium: a central regulator of plant growth and development. *Plant Cell* 17, 2142–2155. doi: 10.1105/tpc.105.032508

Kaya, C., Kirnak, H., Higgs, D., and Saltali, K. (2002). Supplementary calcium enhances plant growth and fruit yield in strawberry cultivars grown at high (NaCl) salinity. *Sci. Hortic.* 93, 65–74. doi: 10.1016/S0304-4238(01)00313-2

La Pena, R. D., and Hughes, J. (2007). Improving vegetable productivity in a variable and changing climate. *J. SAT Agric. Res.* 4, 1–22.

Lichtenthaler, H. K. (1987). Chlorophylls and carotenoids: pigments of photosynthetic biomembranes. *Methods Enzymol.* 148, 350–382. doi: 10.1016/0076-6879(87)48036-1

Liu, X. Q., Ko, K. Y., Kim, S. H., and Lee, K. S. (2007). Effect of amino acid fertilization on nitrate assimilation of leafy radish and soil chemical properties

in high nitrate soil. *Commun. Soil Sci. Plant Anal.* 39, 269–281. doi: 10.1080/00103620701759301

Lucini, L., Rouphael, Y., Cardarelli, M., Canaguier, R., Kumar, P., and Colla, G. (2015). The effect of a plant-derived biostimulant on metabolic profiling and crop performance of lettuce grown under saline conditions. *Sci. Hort.* 182, 124–133. doi: 10.1016/j.scienta.2014.11.022

Maas, E. V., and Grattan, S. R. (1999). Crop yields as affected by salinity. *Agronomy* 38, 55–110.

Maas, E. V., and Hoffman, G. J. (1977). Crop salt tolerance–current assessment. *J. Irrig. Drain. Div.* 103, 115–134. doi: 10.1002/ece3.1060

Machado, R. M. A., and Serralheiro, R. P. (2017). Soil salinity: effect on vegetable crop growth. Management practices to prevent and mitigate soil salinization. *Horticulturae* 3:30. doi: 10.1002/jsfa.7989

Marschner, H. (1995). "Adaptation of plants to adverse chemical soil conditions," in *Mineral Nutrition of Higher Plants*, ed. P. Marschner (Amsterdam: Elsevier), 596–680. doi: 10.1016/B978-012473542-2/50018-3

Masondo, N. A., Kulkarni, M. G., Finnie, J. F., and Van Staden, J. (2018). Influence of biostimulants-seed-priming on *Ceratotheca triloba* germination and seedling growth under low temperatures, low osmotic potential and salinity stress. *Ecotoxicol. Environ. Saf.* 147, 43–48. doi: 10.1016/j.ecoenv.2017.08.017

Massa, D., Prisa, D., Montoneri, E., Battaglini, D., Ginepro, M., Negre, M., et al. (2016). Application of municipal biowaste derived products in hibiscus cultivation: effect on leaf gaseous exchange activity, and plant biomass accumulation and quality. *Sci. Hortic.* 205, 59–69. doi: 10.1016/j.scienta.2016.03.033

Mehta, P., Jajoo, A., Mathur, S., and Bharti, S. (2010). Chlorophyll a fluorescence study revealing effects of high salt stress on Photosystem II in wheat leaves. *Plant Physiol. Biochem.* 48, 16–20. doi: 10.1016/j.plaphy.2009.10.006

Misra, A. N., Srivastava, A., and Strasser, R. J. (2001). Utilization of fast chlorophyll a fluorescence technique in assessing the salt/ion sensitivity of mung bean and Brassica seedlings. *J. Plant Physiol.* 158, 1173–1181. doi: 10.1078/S0176-1617(04)70144-3

Moghaddam, A. R., and Soleimani, A. (2012). "Compensatory effects of humic acid on physiological characteristics of pistachio seedlings under salinity stress," in *Proceedings of the XXVIII International Horticultural Congress on Science and Horticulture for People (IHC2010): International Symposium on the Challenge for a Sustainable Production, Protection and Consumption of Mediterranean Fruits and Nuts* (Stellenbosch: ISHS Acta Horticulturae), 253–255. doi: 10.17660/ActaHortic.2012.940.35

Moud, A. M., and Maghsoudi, K. (2008). Salt stress effects on respiration and growth of germinated seeds of different wheat (*Triticum aestivum* L.) *cultivars. World J. Agric. Sci.* 4, 351–358.

Munns, R. (2002). Comparative physiology of salt and water stress. *Plant Cell Environ.* 25, 239–250. doi: 10.1046/j.0016-8025.2001.00808.x

Munns, R., and Termaat, A. (1986). Whole-plant responses to salinity. *Funct. Plant Biol.* 13, 143–160. doi: 10.1071/PP9860143

Negrão, S., Schmöckel, S. M., and Tester, M. (2017). Evaluating physiological responses of plants to salinity stress. *Ann. Bot.* 119, 1–11. doi: 10.1093/aob/mcw191

Orsini, F., Pennisi, G., Mancarella, S., Al Nayef, M., Sanoubar, R., Nicola, S., et al. (2018). Hydroponic lettuce yields are improved under salt stress by utilizing white plastic film and exogenous applications of proline. *Sci. Hortic.* 233, 283–293. doi: 10.1016/j.scienta.2018.01.019

Parida, A. K., and Das, A. B. (2005). Salt tolerance and salinity effects on plants: a review. *Ecotox Environ. Saf.* 60, 324–349. doi: 10.1016/j.ecoenv.2004.06.010

Pokluda, R., Sękara, A., Jezdinský, A., Kalisz, A., Neugebauerová, J., and Grabowska, A. (2016). The physiological status and stress biomarker concentration of *Coriandrum sativum* L. plants subjected to chilling are modified by biostimulant application. *Biol. Agric. Hort.* 32, 258–268. doi: 10.1080/01448765.2016.1172344

Przybysz, A., Gawronska, H., and Gajc-Wolska, J. (2014). Biological mode of action of a nitrophenolates-based biostimulant: case study. *Front. Plant Sci.* 5:713. doi: 10.3389/fpls.2014.00713

Przybysz, A., Janowiak, F., Słowinski, A., and Gawronska, H. (2010). Protective′role of Asahi SL against drought stress. *Zeszyty Problemowe Post,epów Nauk Rolniczych* 545, 199–223.

Rejeb, K. B., Abdelly, C., and Savouré, A. (2014). How reactive oxygen species and proline face stress together. *Plant Physiol. Biochem.* 80, 278–284. doi: 10.1016/j.plaphy.2014.04.007

Rouphael, Y., Cardarelli, M., Bonini, P., and Colla, G. (2017a). Synergistic action of a microbial-based biostimulant and a plant derived-protein hydrolysate enhances lettuce tolerance to alkalinity and salinity. *Front. Plant Sci.* 8:131. doi: 10.3389/fpls.2017.00131

Rouphael, Y., De Micco, V., Arena, C., Raimondi, G., Colla, G., and De Pascale, S. (2017b). Effect of *Ecklonia maxima* seaweed extract on yield, mineral composition, gas exchange, and leaf anatomy of zucchini squash grown under saline conditions. *J. Appl. Phycol.* 29, 459–470. doi: 10.1007/s10811-016-0937-x

Santos, C., and Caldeira, G. (1999). Comparative responses of *Helianthus annuus* plants and calli exposed to NaCl: I. Growth rate and osmotic regulation in intact plants and calli. *J. Plant Physiol.* 155, 769–777. doi: 10.1016/S0176-1617(99)80095-9

Santos, C. V. (2004). Regulation of chlorophyll biosynthesis and degradation by salt stress in sunflower leaves. *Sci. Hortic.* 103, 93–99. doi: 10.1016/j.scienta.2004.04.009

Savvides, A., Ali, S., Tester, M., and Fotopoulos, V. (2016). Chemical priming of plants against multiple abiotic stresses: mission possible? *Trends Plant Sci.* 21, 329–340. doi: 10.1016/j.tplants.2015.11.003

Sayyad-Amin, P., Jahansooz, M. R., Borzouei, A., and Ajili, F. (2016). Changes in photosynthetic pigments and chlorophyll-a fluorescence attributes of sweet-forage and grain sorghum cultivars under salt stress. *J. Biol. Phys.* 42, 601–620. doi: 10.1007/s10867-016-9428-1

Serio, F., Elia, A., Santamaria, P., and Rodriguez, G. R. (2001). Lettuce growth, yield and nitrate content as affected by electrical conductivity of nutrient solution. *Acta Hortic.* 559, 563–568. doi: 10.17660/ActaHortic.2001.559.82

Shaheen, S., Naseer, S., Ashraf, M., and Akram, N. A. (2013). Salt stress affects water relations, photosynthesis, and oxidative defense mechanisms in *Solanum melongena* L. *J. Plant Interact.* 8, 85–96. doi: 10.1080/17429145.2012.718376

Shannon, M. C. (1997). Adaptation of plants to salinity. *Adv. Agron.* 60, 75–120. doi: 10.1016/S0065-2113(08)60601-X

Shannon, M. C., and Grieve, C. M. (1998). Tolerance of vegetable crops to salinity. *Sci. Hortic.* 78, 5–38. doi: 10.1016/S0304-4238(98)00189-7

Smirnoff, N. (1998). Plant resistance to environmental stress. *Curr. Opin. Biotechnol.* 9, 214–219. doi: 10.1016/S0958-1669(98)80118-3

Spinelli, F., Fiori, G., Noferini, M., Sprocatti, M., and Costa, G. (2010). A novel type of seaweed extract as a natural alternative to the use of iron chelates in strawberry production. *Sci. Hortic.* 125, 263–269. doi: 10.1016/j.scienta.2010.03.011

Szabados, L., and Savouré, A. (2010). Proline: a multifunctional amino acid. *Trends Plant Sci.* 15, 89–97. doi: 10.1016/j.tplants.2009.11.009

Taïbi, K., Taïbi, F., Abderrahim, L. A., Ennajah, A., Belkhodja, M., and Mulet, J. M. (2016). Effect of salt stress on growth, chlorophyll content, lipid peroxidation and antioxidant defence systems in *Phaseolus vulgaris* L. *S. Afr. J. Bot.* 105, 306–312. doi: 10.1016/j.sajb.2016.03.011

Tesi, R., Lenzi, A., and Lombardi, P. (2003). Effect of salinity and oxygen level on lettuce grown in a floating system. *Acta Hortic.* 609, 383–387. doi: 10.17660/ActaHortic.2003.609.58

Trivellini, A., Lucchesini, M., Ferrante, A., Carmassi, G., Scatena, G., Vernieri, P., et al. (2016). Survive or die? A molecular insight into salt-dependant signaling network. *Environ. Exp. Bot.* 132, 140–153. doi: 10.1016/j.envexpbot.2016.07.007

Türkmen, Ö, Dursun, A., Turan, M., and Erdinç, Ç (2004). Calcium and humic acid affect seed germination, growth, and nutrient content of tomato (*Lycopersicon esculentum* L.) seedlings under saline soil conditions. *Acta Agric. Scand. Sec. B Soil Plant Sci.* 54, 168–174. doi: 10.1080/09064710310022014

Ugena, L., Hylova, A., Podlešáková, K., Humplík, J. F., Dolezal, K., De Diego, N., et al. (2018). Characterisation of biostimulant mode of action using novel multi-trait high-throughput screening of Arabidopsis germination and rosette growth. *Front. Plant Sci.* 9:1327. doi: 10.3389/fpls.2018.01327

Ünlükara, A., Cemek, B., Karaman, S., and Erşahin, S. (2008). Response of lettuce (*Lactuca sativa* var. *crispa*) to salinity of irrigation water. *New Zealand J. Crop Hortic. Sci.* 36, 265–273. doi: 10.1080/01140670809510243

Ünlükara, A., Kurunç, A., Kesmez, G. D., Yurtseven, E., and Suarez, D. L. (2010). Effects of salinity on eggplant (*Solanum melongena* L.) growth and evapotranspiration. *Irrig. Drain.* 59, 203–214. doi: 10.3389/fpls.2018.01327

Van Breusegem, F., and Dat, J. F. (2006). Reactive oxygen species in plant cell death. *Plant Physiol.* 141, 384–390.

Van Oosten, M. J., Pepe, O., De Pascale, S., Silletti, S., and Maggio, A. (2017). The role of biostimulants and bioeffectors as alleviators of abiotic stress in crop plants. *Technol. Agric.* 4:5. doi: 10.1186/s40538-017-0089-5

Vernieri, P., Borghesi, E., Ferrante, A., and Magnani, G. (2005). Application of biostimulants in floating system for improving rocket quality. *JFAE* 3:86. doi: 10.1186/s40538-017-0089-5

Vernieri, P., Perata, P., Armellini, D., Bugnoli, M., Presentini, R., Lorenzi, R., et al. (1989). Solid phase radioimmunoassay for the quantitation of abscisic acid in plant crude extracts using a new monoclonal antibody. *J. Plant Physiol.* 134, 441–446.

Viégas, R. A., da Silveira, J. A., Lima Junior, A. R. D., Queiroz, J. E., and Fausto, M. J. (2001). Effects of NaCl-salinity on growth and inorganic solute accumulation in young cashew plants. *Rev. Bras. Eng. Agríc. Ambient.* 5, 216–222. doi: 10.1016/S0176-1617(89)80007-0

Xiong, H., Li, J., Liu, P., Duan, J., Zhao, Y., Guo, X., et al. (2014). Overexpression of OsMYB48-1, a novel MYB-related transcription factor, enhances drought and salinity tolerance in rice. *PLoS One* 9:e92913. doi: 10.1371/journal.pone.0092913

Xu, C., and Mou, B. (2016). Responses of spinach to salinity and nutrient deficiency in growth, physiology, and nutritional value. *J. Am. Soc. Hortic. Sci.* 141, 12–21. doi: 10.1371/journal.pone.0092913

Yemm, E. W., and Willis, A. J. (1954). The estimation of carbohydrates in plant extracts by anthrone. *Biochem. J.* 57, 508–514. doi: 10.3389/fpls.2016.02049

Zhu, J. K. (2002). Salt and drought stress signal transduction in plants. *Annu. Rev. Plant Biol.* 53, 247–273. doi: 10.1042/bj0570508

Conflict of Interest Statement: The authors declare that the research was conducted in the absence of any commercial or financial relationships that could be construed as a potential conflict of interest.

frontiers in Plant Science

ORIGINAL RESEARCH
published: 14 January 2019
doi: 10.3389/fpls.2018.01956

Bacteria Associated With a Commercial Mycorrhizal Inoculum: Community Composition and Multifunctional Activity as Assessed by Illumina Sequencing and Culture-Dependent Tools

*Monica Agnolucci[1], Luciano Avio[1], Alessandra Pepe[1], Alessandra Turrini[1], Caterina Cristani[2], Paolo Bonini[3], Veronica Cirino[4], Fabrizio Colosimo[4], Maurizio Ruzzi[5] and Manuela Giovannetti[1]**

[1] Department of Agriculture, Food and Environment, University of Pisa, Pisa, Italy, [2] "E. Avanzi" Research Center, University of Pisa, Pisa, Italy, [3] NGAlab La Riera de Gaia, Tarragona, Spain, [4] ATENS - Agrotecnologias Naturales SL, La Riera de Gaia, Tarragona, Spain, [5] Department for Innovation in Biological, Agrofood and Forest Systems, University of Tuscia, Viterbo, Italy

OPEN ACCESS

Edited by:
Youssef Rouphael,
University of Naples Federico II, Italy

Reviewed by:
Alessandra Salvioli,
Università degli Studi di Torino, Italy
Giovanni Vallini,
University of Verona, Italy

***Correspondence:**
Manuela Giovannetti
manuela.giovannetti@unipi.it

Specialty section:
This article was submitted to Crop and Product Physiology, a section of the journal Frontiers in Plant Science

Received: 27 September 2018
Accepted: 17 December 2018
Published: 14 January 2019

Citation:
Agnolucci M, Avio L, Pepe A, Turrini A, Cristani C, Bonini P, Cirino V, Colosimo F, Ruzzi M and Giovannetti M (2019) Bacteria Associated With a Commercial Mycorrhizal Inoculum: Community Composition and Multifunctional Activity as Assessed by Illumina Sequencing and Culture-Dependent Tools. Front. Plant Sci. 9:1956. doi: 10.3389/fpls.2018.01956

The implementation of sustainable agriculture encompasses practices enhancing the activity of beneficial soil microorganisms, able to modulate biogeochemical soil cycles and to affect soil fertility. Among them, arbuscular mycorrhizal fungi (AMF) establish symbioses with the roots of most food crops and play a key role in nutrient uptake and plant protection from biotic and abiotic stresses. Such beneficial services, encompassing improved crop performances, and soil resources availability, are the outcome of the synergistic action of AMF and the vast communities of mycorrhizospheric bacteria living strictly associated with their mycelium and spores, most of which showing plant growth promoting (PGP) activities, such as the ability to solubilize phosphate and produce siderophores and indole acetic acid (IAA). One of the strategies devised to exploit AMF benefits is represented by the inoculation of selected isolates, either as single species or in a mixture. Here, for the first time, the microbiota associated with a commercial AMF inoculum was identified and characterized, using a polyphasic approach, i.e., a combination of culture-dependent analyses and metagenomic sequencing. Overall, 276 bacterial genera were identified by Illumina high-throughput sequencing, belonging to 165 families, 107 orders, and 23 phyla, mostly represented by Proteobacteria and Bacteroidetes. The commercial inoculum harbored a rich culturable heterotrophic bacterial community, whose populations ranged from 2.5 to 6.1 $\times$ 10^6 CFU/mL. The isolation of functional groups allowed the selection of 36 bacterial strains showing PGP activities. Among them, 14 strains showed strong IAA and/or siderophores production and were affiliated with Actinomycetales (*Microbacterium trichotecenolyticum*, *Streptomyces deccanensis/scabiei*), Bacillales (*Bacillus litoralis*, *Bacillus megaterium*), Enterobacteriales (*Enterobacter*), Rhizobiales (*Rhizobium radiobacter*). This work demonstrates for the first time that an AMF inoculum, obtained following industrial production processes, is home of a large and diverse

community of bacteria with important functional PGP traits, possibly acting in synergy with AMF and providing additional services and benefits. Such bacteria, available in pure culture, could be utilized, individually and/or in multispecies consortia with AMF, as biofertilizers and bioenhancers in sustainable agroecosystems, aimed at minimizing the use of chemical fertilizers and pesticides, promoting primary production, and maintaining soil health and fertility.

Keywords: arbuscular mycorrhizal symbionts, mycorrhizosphere, plant-growth promoting bacteria, siderophores production, indole acetic acid production, metagenomics

INTRODUCTION

Worldwide, a major shift is taking place in agriculture, in order to meet the growing global demand for a safe production of high-quality food, able to maintain or enhance environmental quality and to conserve natural resources for future generations. The implementation of sustainable agriculture encompasses practices enhancing the activity of soil biogeochemical cycles, at the basis of long-term soil productivity and health. The most important players of soil biological fertility are represented by beneficial soil microorganisms, able to modulate biochemical and physiological soil processes, and to affect its biological and nutritional characteristics (Barea et al., 2005). Among them, arbuscular mycorrhizal fungi (AMF, Glomeromycota) are recognized as ecologically and economically important elements of sustainable food production systems, given the key role played in plant nutrition and health, by reducing the input of chemical fertilizers and pesticides (Smith and Read, 2008).

AMF are obligate mutualistic biotrophs, establishing symbioses with the roots of most land plants, including the major food and feed crops, from cereals and legumes to fruits and vegetables, including also important industrial plants, such as sunflower, tobacco, cotton, and medicinal plants (Smith and Read, 2008). AMF symbionts facilitate plant nutrient uptake, mainly phosphorus (P), nitrogen (N), sulfur (S) potassium (K), calcium (Ca), copper (Cu), and zinc (Zn), by means of a large network of extraradical hyphae spreading from colonized roots to the surrounding soil and functioning as a supplementary absorbing system (Giovannetti et al., 2001; Avio et al., 2006). Moreover, they protect plants from biotic and abiotic stresses (Augé, 2001; Evelin et al., 2009; Sikes et al., 2009), provide essential ecosystem services (Gianinazzi et al., 2010), and affect the biosynthesis of beneficial plant secondary metabolites, contributing to the production of safe and high quality food (Sbrana et al., 2014; Avio et al., 2018). However, such beneficial services, encompassing improved crop performances and soil resources availability, are the outcome of the synergistic action of AMF and the vast communities of mycorrhizospheric bacteria living strictly associated with their mycelium and spores (Hildebrandt et al., 2006; Agnolucci et al., 2015). AMF-associated microbiota has been reported to promote mycorrhizal activity (Mayo et al., 1986; Xavier and Germida, 2003; Horii and Ishii, 2006; Giovannetti et al., 2010), to protect plants from soilborne pathogens (Citernesi et al., 1996; Budi et al., 1999; Li et al., 2007; Bharadwaj et al., 2008a,b) and to provide nutrients and growth factors (Barea et al., 2002; Xavier and Germida, 2003), thus being considered as plant growth promoting (PGP) bacteria (PGPB) (Philippot et al., 2013). Molecular investigations allowed the description of the complexity and diversity of bacterial communities associated to AMF spores belonging to different species and isolates, suggesting that their differential occurrence may affect the performance of the relevant taxa in terms of infectivity and efficiency, given their important functional roles as PGPB (Roesti et al., 2005; Long et al., 2008; Agnolucci et al., 2015). Other studies, aimed at isolating and functionally characterizing spore associated bacteria, reported the occurrence of bacteria showing antagonistic activity against plant pathogens (Budi et al., 1999; Bharadwaj et al., 2008a), phosphate-solubilizing and nitrogenase activity (Cruz et al., 2008; Cruz and Ishii, 2011), and indole acetic acid (IAA) production (Bharadwaj et al., 2008a). A recent work, using a culture-dependent approach, showed that bacterial strains isolated in pure culture from *Rhizophagus intraradices* spores were able to solubilize P from phytate and inorganic sources (69.7 and 49.2%, respectively), produce siderophores (65.6%), and IAA (42.6%) (Battini et al., 2016). The last two molecules are very important for plant growth and nutrition. Actually, IAA, a phytohormone of the auxin class, affects the morphology and physiology of roots, enhancing cell division and elongation, and the formation of lateral roots, thus improving water and nutrient uptake and playing a key role in the regulation of plant development (Khalid et al., 2004; Aloni et al., 2006; Duca et al., 2014). Siderophores are low molecular weight, high-affinity iron-chelating compounds able to bind soluble Fe_3, even at high pH when Fe solubility decreases (Mimmo et al., 2014), thus making it available to bacteria and plants (Colombo et al., 2014). Given the essential role played by iron in plant biochemical processes, such as photosynthesis and respiration (Kobayashi and Nishizawa, 2012), bacterial siderophores, facilitating plant Fe acquisition, represent important factors of plant growth and development (Crowley et al., 1988; Duijff et al., 1994a,b; Walter et al., 1994; Yehuda et al., 1996; Siebner-Freibach et al., 2003; Jin et al., 2006; Vansuyt et al., 2007; Robin et al., 2008). Moreover, siderophores have been reported to possess biocontrol activity against soilborne diseases, by means of iron competition (Thomashow et al., 1990; Glick, 1995; Whipps, 2001), inhibiting the development of deleterious plant pathogens (Davison, 1988; Arora et al., 2001).

Although the individual roles of AMF and their associated bacteria in optimizing plant performance are still to be

completely dissected, AMF are progressively more considered among the main factors of sustainable food (primary) production (Philippot et al., 2013; Rouphael et al., 2015). Two main strategies have been devised to exploit the benefits deriving from the mycorrhizal symbionts: the adoption of specific management practices and the use of AMF inoculation. The first one focuses on the improvement of the activity of native AMF, pursued by using crop rotation and mycotrophic cover crops, able to raise soil mycorrhizal potential and to shape native AMF communities (Kabir and Koide, 2002; Karasawa and Takebe, 2012; Lehman et al., 2012; Njeru et al., 2014, 2015; Turrini et al., 2016, 2017), and by reducing tillage intensity or chemical fertilizations, which affect AMF species composition, spore abundance and mycorrhizal colonization (Douds et al., 1995; Jansa et al., 2003; Oehl et al., 2004; Castillo et al., 2006; Brito et al., 2012; Avio et al., 2013). The second strategy focuses on the inoculation of selected AMF, either as single species or in a mixture, reported as efficient root colonizers and plant nutrition enhancers (Jeffries et al., 2003; Gianinazzi and Vosatka, 2004; Lekberg and Koide, 2005; Rouphael et al., 2015).

Many types of commercial AMF inoculum are available on the market, including sterile products obtained *in vitro* using genetically modified Ri T-DNA roots and the species *Rhizoglomus irregulare* (synonym *Rhizophagus irregularis*, basionym *Glomus irregulare*). However, most of the commercial products are obtained from greenhouse multiplication on mycotrophic trap plants and represent a multipartite symbiosis, where a rich community of bacteria may thrive, associated with AMF propagules, and exert important functional activities, as PGPB. Here, for the first time, we explored the bacterial metagenome of a commercially available AMF inoculum by Illumina high-throughput sequencing, a method able to provide information about culturable and unculturable members of the inoculum microbiota. Moreover, we isolated and functionally selected culturable bacteria showing important PGP traits, as the ability to produce IAA and siderophores, to be utilized, individually and/or in multispecies consortia with AMF as beneficial biofertilizers/bioenhancers in sustainable agroecosystems.

MATERIALS AND METHODS

Biological Activity of the Commercial Inoculum

The commercial inoculum utilized consisted of the substrate where trap plants (*Allium ampeloprasum* var. *porrum* L.) were grown and of mycorrhizal root fragments, AMF spores, and extraradical mycelium of *Rhizoglomus irregulare* BEG72 (synonym *Rhizophagus irregularis*, basionym *Glomus irregulare*). The substrate (vermiculite) and the seeds utilized for the inoculum production were sterilized prior to their utilization. The only microbial input in the commercial product arised from the AM fungus and its associated microbiota. The corresponding AMF inoculum is available on the market under the name "AEGIS" (Atens, Agrotecnologias Naturales S.L.). The percentage of mycorrhizal colonization of the roots contained within the inoculum was assessed on three 5 g samples by the gridline intersect method, after clearing with 10% KOH and staining with 0.05% Trypan blue in lactic acid (Giovannetti and Mosse, 1980). The mycorrhizal potential of the commercial inoculum was assessed by using the Mycorrhizal Inoculum Potential (MIP) bioassay, as described in Njeru et al. (2014). Briefly, three replicate inoculum samples were sown with *Cichorium intybus* L. cv. Zuccherina di Trieste, put in sun-transparent bags and maintained in a growth chamber at 27°C and 16/8 h light/dark daily cycle until harvest. Roots were harvested 30 days after sowing, cleaned with tap water and cleared, stained, and examined for AMF colonization assessment, as described above.

Illumina MySeq Analysis of Bacteria Associated With the Inoculum

DNA Extraction

The composition of the bacterial community of three commercial inoculum samples was determined by Next-generation high-throughput DNA sequencing (NGS; Ansorge, 2009). Total community DNA was extracted from each sample using DNeasy PowerSoil Kit (Qiagen, Hilden, Germany). In brief, 50 g of sample and 0.1 mL Tween 20 were suspended in saline phosphate buffer (100 mL) and homogenized in a paddle blender (BagMixer® 400, Interscience, Saint Nom, France) for three min at maximum speed. Substrate soil and root fragments were removed by low speed (1,000 g) centrifugation, then, for DNA extraction, cells were collected after centrifugation and lysed using the DNeasy PowerSoil reagents and Qiagen spin columns on a QIAcube automated station (Qiagen, Hilden, Germany).

Library Preparation

Three 16S rRNA gene amplicon libraries were prepared by PCR amplification of an approximate 630 bp region within the hypervariable (V3-V4) region of the 16S rRNA gene according to the Illumina 16S metagenomic sequencing library protocol. PCR amplification was performed with broad spectrum 16S rRNA primers (forward primer: 5′-TCGTCGGCAGCGTCA GATGTGTAT AAGAGACAGCCTACGGGNGGCWGCAG-3′, reverse primer: 5′-GTCTCGTGGGCTCGGAGATGTGTA TAA GAGACAGGACTACHVGGGTAT CTAATCC-3′) (Klindworth et al., 2013), using Kapa HiFi HotStart 2 × ReadyMix DNA polymerase (Kapa Biosystems Ltd., London, UK). Cycle conditions were: an initial step at 95°C for 3 min; 25 cycles of 95°C (30 s), 55°C (30 s), 72°C (30 s); a final extension of 5 min at 72°C. Libraries were purified using AMPure XP beads (LABPLAN; Naas, Ireland) according to the Illumina 16S metagenomic sequencing library protocol. Dual indices and Illumina sequencing adapters from the Illumina Nextera XT index kits v2 B and C (Illumina, San Diego, USA) were added to the target amplicons in a second Index PCR step using Kapa HotStart HiFi 2 × ReadyMix DNA polymerase (Kapa Biosystems Ltd.). Cycle conditions were: 95°C (3 min); 9 cycles of 95°C (30 s), 55°C (30 s), 72°C (30 s); a final extension of 5 min at 72°C. Libraries were again purified using AMPure XP beads (LABPLAN; Naas, Ireland) according to the Illumina 16S metagenomic sequencing library protocol. Libraries were quantified using a Qubit fluorometer (Life Technologies, Paisley, UK) and pooled in equal concentrations (4 nM) into a single pool,

according to their Qubit quantification measurement. The library pool was diluted and denatured according to the Illumina MiSeq library preparation guide. The amplicon library (8 pM) was spiked with 10% denatured and diluted PhiX Illumina control library (12.5 pM). The sequencing run was conducted on the Illumina MiSeq using the 600 cycle MiSeq reagent kit (version 3) with paired 300 bp reads.

Sequencing

Illumina sequencing was performed using MiSeq (Illumina, San Diego, CA). Paired-end sequencing used custom primers and a 600-cycle sequencing kit (V3), according to manufacturer instructions. Amplicon sequencing was carried out in the presence of 10% PhiX control (Illumina, San Diego, CA) to allow proper focusing and matrix calculations.

Bionformatics

Raw data processing, run de-multiplexing and operational taxonomic unit (OTU) analysis were performed using the CLC Genomics Workbench (Version 11.0.1) with CLC Microbial Genomics Module (Version 3.5) (Qiagen Bioinformatics, Hilden, Germany). Such programme was used also for the estimation of alpha diversity (total richness in OTUs). Taxonomy attribution was performed against SILVA 16S v132 at the identity level of 97%.

Isolation and Characterization of Beneficial Bacteria Associated With the Inoculum

Bacterial Isolation

Three 40 g samples of the commercial inoculum were suspended in 360 mL of sterile physiological solution added with Tween 80 (0.36 μL). The suspension was shaken for 30 min using a multi wrist shaker (Labline Instruments, Illinois, USA). Hundred microliter suspension for each sample were plated in triplicate onto Petri dishes containing different agar media. Culturable heterotrophic bacteria were isolated on Tryptic Soil Agar (TSA, 30 g L^{-1} tryptic soy broth, 20 g L^{-1} bacteriological agar, Oxoid, Milan, Italy), a medium which, given its non-selectivity, allows the recovery of a wide range of aerobic and facultative anaerobic gram-negative and gram-positive bacteria. In order to isolate specific functional bacterial groups, two additional selective media were used. The selective N-free Winogradsky medium (N-free W) (Tchan, 1984) was utilized for the isolation of putative nitrogen-fixing bacteria, able to grow on N-free medium. For the isolation of bacteria able to solubilize inorganic phosphate the National Botanical Research Institute's Phosphate growth (NBRIP) medium was used (Nautiyal, 1999). The three culture media were supplemented with 100 mg L^{-1} of cyclohexymide and 500 UI L^{-1} of nystatin (Sigma–Aldrich, Milan, Italy) to inhibit possible fungal development. The number of colony forming units (CFU) was assessed after 2 and 7 days of incubation at 28°C for TSA and the other two media, respectively. Bacteria grown on N-free W and those showing halo zones formation on NBRIP, were selected and purified by streaking four times onto the same medium used for the isolation. In addition, bacteria grown in TSA medium were randomly selected on the basis of phenotypic colony characteristics, i.e., shape, size, edge morphology, surface and pigment and inoculated onto N-free W and NBRIP media and then purified by streaking four times onto the same medium. The purified strains were maintained at −80°C in cryovials with 20% (v/v) of glycerol in the collection IMA (International Microbial Archives) of the Department of Agriculture, Food and Environment, University of Pisa.

Screening of the Selected Bacteria for PGP Traits

All the bacterial strains isolated and selected as described above, were screened *in vitro* for two functional traits linked to the promotion of plant growth and performance, i. e., the ability to produce IAA and siderophores. The production of IAA was assessed using Luria–Bertani Broth (LBB) (Bharadwaj et al., 2008a) and following the method described by Battini et al. (2016). Briefly, strains were inoculated in 4 mL of LBB amended with 1 mg mL^{-1} of l-tryptophan (Sigma–Aldrich, Milan, Italy), incubated at 20°C until exponential growth phase was reached. They were centrifugated (7500 rpm for 10 min) and 1mL of supernantant was transferred in a 24-well plate, mixed with 2 mL of Salkowski reagent (1.2% $FeCl_3$ in 37% sulfuric acid). The non-inoculated medium represented the negative control, and the medium amended with pure IAA the positive one. Development of red–purple color after 3 min incubation in the dark indicated positive strains for IAA production. Strains were classified using a rating scale as follows (**Figure 1**): –, no production (no color development); +/–, low production (pale pink); +, production (light purple); ++, moderate production (bright purple); +++, high production (dark purple), considering color intensity of the positive controls, IAA (66 μg/mL) representing the maximum value (10+) and IAA 1:2 the half (5+). The test was replicated three times. The ability to produce siderophores was investigated using the overlay Chrome Azurol S assay (CAS) described by Pérez-Miranda et al. (2007). CAS agar was prepared following the procedure described by Louden et al. (2011). Siderophore-producing bacterial strains showed a change in color, from blue to yellow/orange, in the overlaid medium around the colonies. After 7 days the radius of the halo was measured (mm) from the colony edge to the edge of the colored halo. Strains were classified using a rating scale as follows: no production (halo = 0 mm), +/– = low production (halo < 2 mm), + = production (3 mm ≤ halo ≤8 mm), ++ = moderate production (9 mm < halo < 14 mm), +++ = high production (halo > 15 mm).

Molecular Identification of Cultured PGP Bacteria

The purified bacterial strains showing the best ability to produce IAA and siderophores were identified based on 16S rDNA sequencing. Genomic DNA was extracted from bacterial liquid cultures grown overnight at 28°C using "MasterPure™ Yeast DNA Purification Kit" (Epicenter®), following the manufacturer's protocol. Bacterial 16S rRNA gene was amplified using the primers 27F (5′-GAGAGTTTGATC CTG GCT CAG-3′) e 1495R (5′-CTA CGG CTA CCT TGT TAC GA-3′) (Lane, 1991; Weisburg et al., 1991). The amplification reaction was carried out in a final volume of 25 μL, containing: 5

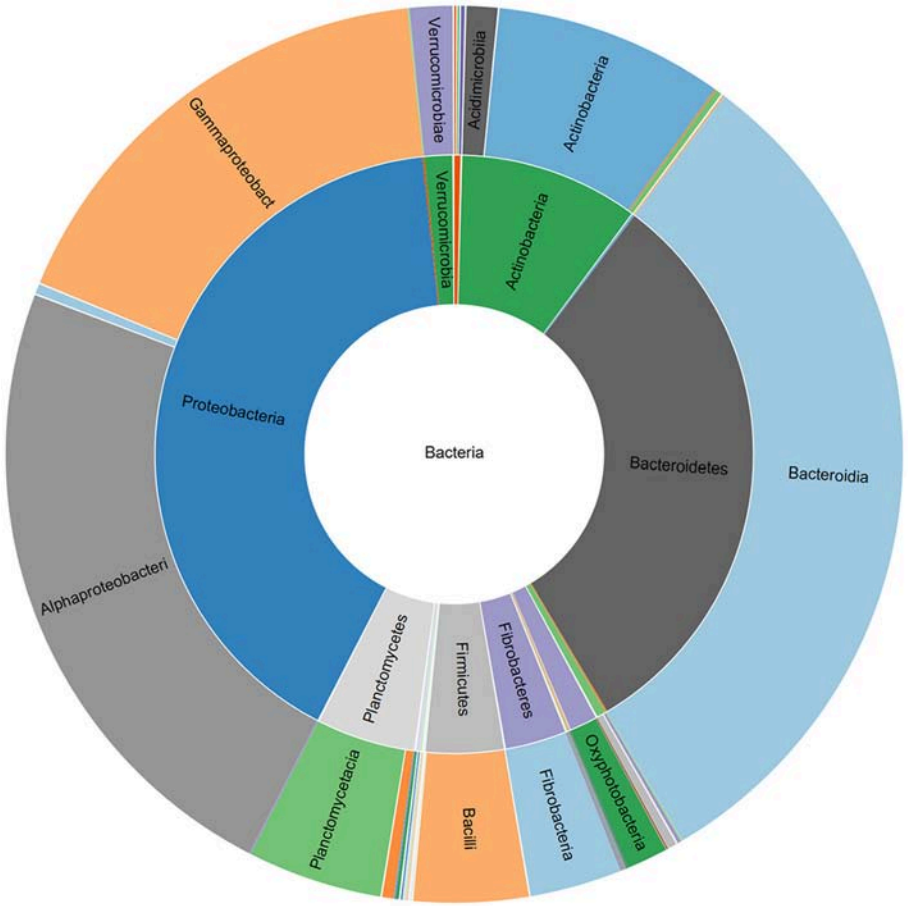


FIGURE 1 | Relative abundance of bacterial phyla and classes associated with a commercial AMF inoculum.

μL of DyNAzyme buffer 10X (Finnzymes), 0.2 μM of each primer, 0.2 mM of each dNTPs (EuroClone), 0.625 U of Taq DyNAzyme II DNA polymerase (Finnzymes) and 10–20 ng of DNA. The samples were amplified using an iCycleriQ Multicolor Real-Time PCR Detection System (BIORAD), with the following PCR protocol: 95°C 2 min; 94°C 1 min and 20 s, 54°C 1 min, 72°C 1 min, and 30 s for 35 cycles; 72°C 5 min. PCR amplicons were analyzed by 1.5% agarose gel electrophoresis, stained with ethidium bromide, visualized and captured as TIFF format files by the UVITEC UV1-1D program for UVITEC Gel Documentation system Essential V6 (Cambridge, UK). The amplification products were purified by the Clean PCR CleanUp kit" (CABRU), quantified and 5′ sequenced by Eurofins Genomics (Ebersberg, Germany), as reported in Palla et al. (2017). Sequences were analyzed using BLAST on the NCBI web (http://blast.ncbi.nlm.nih.gov/Blast.cgi). The sequences were aligned using MUSCLE, and phylogenetic trees were constructed using the Neighbor-Joining method based on Tamura 3-parameter method in MEGA10 (Kumar et al., 2018) software with 1,000 bootstrap replicates. The sequences were submitted to the European Nucleotide Archive under the accession numbers from LS999506 to LS999519.

RESULTS

Biological Activity of the Inoculum

The percentage of colonized length of the root fragments contained within the inoculum was 77±0.7%. The mycorrhizal potential of the inoculum ranged from 20 to 30%.

Illumina MySeq Analysis of Bacteria Associated With the Inoculum

The V3-V4 region of 16S rRNA gene was sequenced to analyze the composition of the bacterial microbiota associated with three different lots of AMF inoculum. NGS analysis allowed us to generate a number of reads per sample comprised between 3.1 and 3.9 million (**Supplementary Material 1**). Approximately 88% of raw reads per sample passed merging, trimming and chimera filtering steps and were analyzed for OTU search. The clustering produced a mean of reads in OTUs of 386,899 ± 25,087 with an average read length after trim of 232 bp. Alpha diversity (OTUs richness) value was 1485 ± 14 (**Supplementary Material 1**). In total, 23 phyla, 107 orders, 165 families, and 276 bacterial genera were identified in the samples. Nine phyla accounted for 95.8% of the sequence

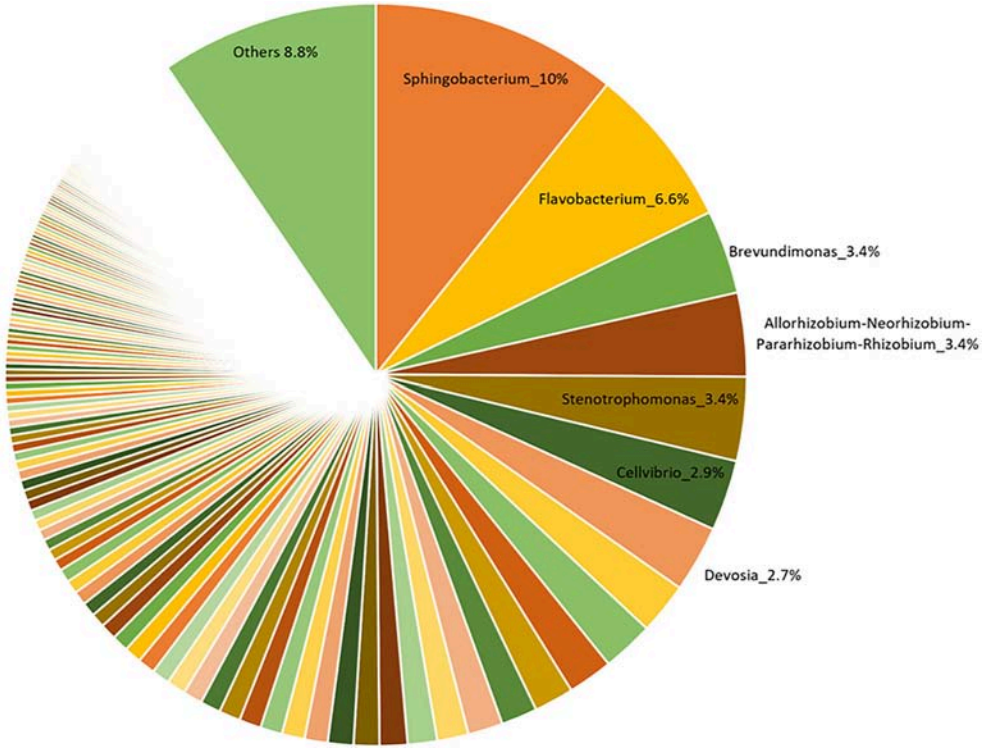


FIGURE 2 | Relative abundance of bacterial genera associated with a commercial AMF inoculum.

reads across all samples with the majority being Proteobacteria (36.9%) and Bacteroidetes (29.3%; **Figure 1**). Other phyla that comprised ≥2.5% of the bacterial communities were: Actinobacteria (8.4%), Planctomycetes (6.3%), Verrucomicrobia (3.7%), Firmicutes (3.3%), Patescibacteria (3.1%), Deinococcus-Thermus (2.6%), and Fibrobacteres (2.5%). The predominant orders were: Rhizobiales (23.6%), Caulobacterales (12.9%), Sphingomonadales (12.1%), and Cellvibrionales (9.0%) among Proteobacteria; Sphingobacteriales (45.1%) and Flavobacteriales (34.7%) among Bacteroidetes. A deeper phylogenetic classification of the reads revealed that the most represented genera were *Sphingobacterium* (10% of total bacteria), *Flavobacterium* (6.6%), *Brevundimonas* (3.4%), *Allorhizobium/Neorhizobium/Pararhizobium/Rhizobium* group (3.4%), *Stenotrophomonas* (3.4%), *Cellvibrio* (2.9%), and *Devosia* (2.7%) (**Figure 2**).

Isolation and Characterization of Beneficial Bacteria Associated With the Inoculum

Microbiological analyses allowed the determination of the bacterial cells associated with the inoculum. The CFU/ml number of heterotrophic bacteria ranged from 2.5 ± 0.2 to $6.1 \pm 1.4 \times 10^6$, while putative N-fixers and P-solubilizers ranged from $9.7 \pm 0.8 \times 10^5$ to $2.2 \pm 0.4 \times 10^6$ and from $9.2 \pm 0.3 \times 10^5$ to $2.4 \pm 0.9 \times 10^6$, respectively (**Table 1**). A total of 26 putative N-fixers and 9 P-solubilizers were obtained in pure culture. As an additional strain showed both characteristics, the total strains successively tested for their PGP traits were 36.

Among the 36 strains analyzed for IAA production, 6 showed the red/orange color similar to the positive controls. Such IAA producers were the isolates N-67 and N-92 within the putative N-fixers, and P-30, P-36, P-42, and P-57 within the P-solubilizers (**Supplementary Material 2**). The other isolates produced lower levels of IAA, as indicated by the golden yellow color of the substrate (**Supplementary Table S1**).

As to siderophores production, most strains showed the indicative clarification halo around the colonies, 5 of them producing a halo with a diameter higher than 5 mm, i.e. isolates N-21, N-75, N-78, and N-87 within the putative N-fixers and the isolate P-24 within P-solubilizers (**Supplementary Material 2**).

Three additional strains, P-23, N-P-27 and N-64, showed a moderate siderophore production, together with IAA production.The 14 bacterial isolates showing the best combination of PGP traits (production of IAA and siderophores) were 16S rDNA sequenced and affiliated to bacterial genera and species. Sequences were affiliated with Actinomycetales (*Microbacterium trichotecenolyticum*, *Streptomyces deccanensis/scabiei*), Bacillales (*Bacillus litoralis*, *Bacillus megaterium*), Enterobacteriales (*Enterobacter*), Rhizobiales (*Rhizobium radiobacter,* syn *Agrobacterium radiobacter/tumefaciens*) (**Table 2**, **Figure 3**).

DISCUSSION

In this work, for the first time, the microbiota associated with a commercial AMF inoculum was identified and characterized, using a polyphasic approach, i.e., a combination of traditional

TABLE 1 | Number of culturable bacteria isolated from three 40 g samples (A, B, C) of the AMF commercial inoculum (mean CFU/mL ± SE) isolated from three different microbiological substrates.

Medium	A	B	C
TSA	$6.1 \pm 1.4 \times 10^6$	$3.8 \pm 0.6 \times 10^6$	$2.5 \pm 0.2 \times 10^6$
N-free W	$2.2 \pm 0.1 \times 10^6$	$9.7 \pm 0.8 \times 10^5$	$2.2 \pm 0.4 \times 10^6$
NBRIP	$1.6 \pm 0.6 \times 10^6$	$2.4 \pm 0.9 \times 10^6$	$9.2 \pm 0.3 \times 10^5$

Each value represents the mean of three replicates. TSA, Tryptic Soil Agar; N-free W, N-free Winogradsky medium; NBRIP, National Botanical Research Institute's Phosphate growth medium.

TABLE 2 | Phylogenetic identification of the 14 best performing plant growth promoting bacteria isolated from the mycorrhizal commercial inoculum (sequence accession numbers from LS999506 to LS999519).

Isolate	Identification	Identity (%)	Most closely related Genebank sequence
N-21	*Microbacterium trichotecenolyticum* DSM 8608	99	NR044937
P-23	*Bacillus megaterium* NBRC 15308	99	NR112636
P-24	*Bacillus megaterium* DSM 32	99	KJ476721
N-P-27	*Bacillus megaterium* XJGJ9	99	KR708952
P-30	*Enterobacter* sp. WP7	99	KU523560
P-36	*Enterobacter* sp. WP7	99	KU523560
P-42	*Enterobacter* sp. AJ2	99	KJ913658
P-57	*Streptomyces* sp. SCY301	99	GU045544
N-64	*Bacillus litoralis* KUDC 1714	99	KC414705
N-67	*Rhizobium radiobacter* (syn *Agrobacterium radiobacter/tumefaciens*) N70a	99	KM894180
N-75	*Microbacterium trichotecenolyticum* DSM 8608	99	NR044937
N-78	*Microbacterium trichotecenolyticum* DSM 8608	99	NR044937
N-87	*Microbacterium trichotecenolyticum* DSM 8608	99	KJ767329
N-92	*Enterobacter* sp. WP7	99	KU523560

Strains isolated from N-free Winogradsky and NBRIP, National Botanical Research Institute's Phosphate growth media are preceded by N and P, respectively.

microbiological culture-dependent analyses and metagenomic sequencing. A complex and highly diverse bacterial community was identified by Illumina high-throughput sequencing and several bacteria showing important PGP traits, as the ability to produce IAA and siderophores, were isolated and identified.

The assessment of mycorrhizal colonization of the roots contained in the inoculum and of the MIP was the necessary prerequisite for the feasibility of our study, given the recent data on the poor colonization of plant roots by a commercial AMF inoculum (Berruti et al., 2013). In our material, both roots contained in commercial inoculum and those of the plants used for the MIP bioassay were well colonized, showing that the commercial inoculum was highly infective and able to rapidly establish the mycorrhizal symbiosis.

The crude inoculum analyzed, consisting of the substrate where trap plants were grown (mycorrhizal root fragments, AMF spores and mycelium) harbored a rich culturable heterotrophic bacterial community, whose populations ranged from 2.5 to 6.1×10^6 CFU/mL. Such values are high, when considering the origin of the sampled material, which did not derive from living roots, but from a dry inoculum, and show that the rich bacterial community thriving in the particular ecological niche, rich in nutrients and exudates, represented by trap plants during AMF inoculum production, is able to maintain its vitality and activity through the different phases leading to the production of the commercial AMF inoculum, from plant harvest to substrate drying. Moreover, present data confirm previous molecular findings which detected large and complex bacterial communities associated with AMF spores (Roesti et al., 2005; Long et al., 2008; Agnolucci et al., 2015).

The culture-independent approach revealed the occurrence of 7 most represented bacterial genera known to include species isolated from a variety of environments that can be subjected to different environmental stresses. For example, bacteria belonging to *Sphingobacterium*, the most represented genus in the commercial inoculum, can survive at temperatures lower than 5°C (Shivaji et al., 1992) and higher than 65°C (Yoo et al., 2007), or can survive in soil contaminated with herbicides (Lü et al., 2006) or solvents (Mohammad et al., 2006). Some species of this genus have been reported to have PGP activities, such as inorganic phosphate solubilization, surfactant and IAA production (Marques et al., 2010; Ahmad et al., 2014; Ali et al., 2017), that can improve the efficacy of AMF inocula. Plant growth-promoting traits were also reported in bacteria belonging to other genera associated with the inoculum, including *Flavobacterium* (phosphate solubilization, production of phytohormones and antimicrobial compounds, Nishioka et al., 2016), *Brevundimonas* (production of IAA and ammonia, Kumar and Gera, 2014), *Stenotrophomonas* (production of antibiotics and plant growth regulators, Messiha et al., 2007) and *Devosia* (development of a nitrogen-fixing root-nodule symbiosis, Rivas et al., 2002). The potential contribution of these bacteria to the efficacy of AMF inocula is supported by recent findings reporting that inoculation with PGPB *Flavobacterium* and *Stenotrophomonas* can be effective in promoting plant growth under draft (Gontia-Mishra et al., 2016) or salinity stress (Singh and Jha, 2017). Interestingly, several sequences (2.9%) were assigned to *Cellvibrio*, a genus known for its cellulose and complex carbohydrate degradation potential, which was previously retrieved from AMF spores, where it was supposed to feed on components of the spore walls, thus facilitating AMF spore germination (Roesti et al., 2005). Many other genera were represented in the bacterial community associated with the commercial inoculum (**Supplementary Material 1**). Among them, several sequences occurring at low frequencies were ascribed to *Streptomyces* (0.22%), *Enterobacter* (0.24%), *Bacillus* (0.66%), *Microbacterium* (0.83%), genera to which our selected strains belonged.

Here, the inoculation and successive purification on selective media allowed the initial isolation of 36 bacterial strains, and their subsequent screening allowed the selection of the 14 best performing strains showing important PGP traits. Six and five strains were strong producers of IAA and siderophores,

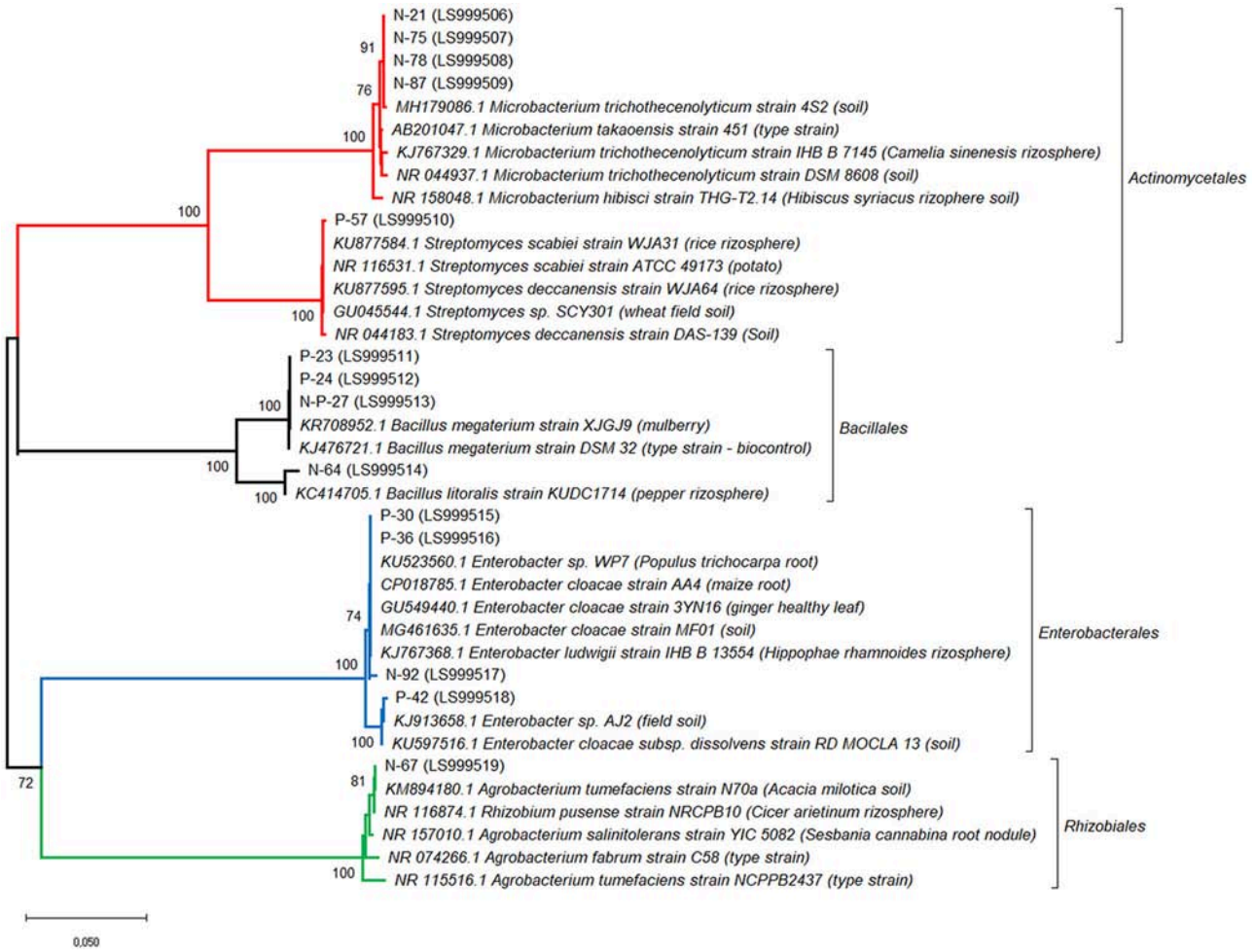


FIGURE 3 | Affiliation of the sequences of the 14 bacterial strains showing the best PGP traits with the existing 16S rRNA gene sequences. Phylogenetic analysis was inferred by using the Neighbor-Joining method. The evolutionary distances were computed using the Tamura 3-parameter method. The rate variation among sites was modeled with a gamma distribution (shape parameter = 1). Bootstrap (1,000 replicates) values below 70 are not shown. Evolutionary analyses were conducted in MEGA10.

respectively, while two of them (N-67 and N-92), displayed at high levels the two PGP traits. The occurrence of such bacterial functional groups in the commercial inoculum further supports our previous evidence that the beneficial microbiota associated with AMF maintains not only its vitality and activity, but also its functional properties during the different phases of the life cycle (Battini et al., 2016). The ability of such strains to produce IAA, a hormone enhancing cell division and boosting the development of plant root systems (Patten and Glick, 2002) and siderophores, able to facilitate plant acquisition of Fe, thus acting as potential biocontrol agents against soilborne plant pathogens (Glick, 1995; Arora et al., 2001; Whipps, 2001; Battini et al., 2016), confirms the need and utility of adopting culture-dependent methods in order to gain knowledge on functional traits of AMF-associated bacteria. The availability of such beneficial bacteria in pure culture allows their use in ecological studies aimed at investigating their mycorrhizospheric competence and role in plant growth promotion.

Fourteen bacterial strains showing the best combination of PGP traits were identified by 16S rDNA sequencing. Interestingly, 5 out of 14 strains (36%) belonged to Actinomycetales: among them, the *Microbacterium trichotecenolyticum* strains N-21, N-75, N-78, N-87, and the *Streptomyces* sp. strain P-57 were strong siderophores and IAA producers, respectively. Actinobacteria are ubiquitous in the soil and able to produce many biologically active secondary metabolites, including antibacterial, antifungal, antiparasitic, anticancer and immunosuppressant drugs (Wolf and Zähner, 1972; Weitnauer et al., 2001; Ritacco and Eveleigh, 2008; Qin et al., 2014) and/or to utilize a wide range of complex compounds (Vandera et al., 2015). They were previously reported to live in strict association with spores and hyphae of different AMF, including *F. coronatum*, *F. mosseae,* and *R. intraradices* (Walley and Germida, 1996; Andrade et al., 1997; Bharadwaj et al., 2008b; Agnolucci et al., 2015; Battini et al., 2016). Many Actinobacteria showed PGP traits, acting as antagonists against

plant pathogens, and mycorrhizal helper traits, enhancing mycorrhizal colonization and AMF functionality (Bharadwaj et al., 2008a; Hamdali et al., 2008; Giovannetti et al., 2010).

Members of the genus *Microbacterium* are ubiquitous in many environments and considered important players of biogeochemical cycles, due to their diazotrophic properties and endophytic behavior (Miliute et al., 2015). Consistent with our findings a *M. trichotecenolyticum* strain isolated from roots of wild *Dodonaea viscosa* L. was reported to possess multiple plant growth promoting activities, such as siderophore and IAA production (Afzal et al., 2017).

The genus *Streptomyces* is one of the main component of soil bacterial communities and is considered within the promising taxa to be investigated for PGP activity, given its ability to solubilize phosphates and produce growth regulators (Mohandas et al., 2013; Hamedi and Mohammadipanah, 2015), two activities shown also by our strain P-57. Actually, two *Streptomyces* strains, W94 and W77, isolated from the spores of the AM fungus *R. irregularis* IMA6, significantly increased the uptake and translocation of ^{33}P in maize plants, and hyphal length specific ^{33}P uptake, respectively, compared with control plants (Battini et al., 2017). On the other hand, other IAA-producing bacteria isolated from AMF propagules were able to increase AMF development (Bidondo et al., 2011), in agreement with previous data reporting that *Streptomyces* spp. boosted AMF spore germination and hyphal growth (Mugnier and Mosse, 1987; Tylka et al., 1991; Carpenter-Boggs et al., 1995), thus showing mycorrhizal helper traits.

Four out of 14 strains (28%) were affiliated with Bacillales, and belonged to the species *Bacillus megaterium* and *Bacillus litoralis*. All of them produced siderophores, activity previously reported in other members of the order (Battini et al., 2016), known for their ability to control soilborne pathogens (Jeong et al., 2014) and to act as PGP and mycorrhizal helper bacteria, facilitating mycorrhizal establishment and improving plant growth (Budi et al., 2013; Pérez-Montaño et al., 2014; Zhao et al., 2014). The isolation of *Bacillus* species from our commercial inoculum represents a further confirmation of previous data obtained by culture-independent methods (Agnolucci et al., 2015).

One strain, *Rhizobium radiobacter* (syn. *Agrobacterium radiobacter/tumefaciens*) N-67, was affiliated to the Rhizobiales, an order thoroughly investigated for the ability of its members to fix nitrogen. This isolate was one of the two only strains able to produce both IAA and siderophores, confirming previous data on PGP ability of some rhizobia to boost plant nutritional status by producing phytohormones (Zahir et al., 2003; Chandra et al., 2007; Dodd et al., 2010). Its persistence in the AMF inoculum may be ascribed to the formation of biofilms containing exopolysaccharides which allow an efficient colonization of roots and mycorrhizal hyphae (Bianciotto et al., 1996; Toljander et al., 2006).

A very interesting finding is represented by the isolation of 4 strains, P-30, P-36, P-42, N-92, affiliated with Enterobacteriales (*Enterobacter cloacae/ludwigii*), which were strong producers of IAA, confirming previous data on the capacity of a strain of *E. cloacae* to produce as much IAA as a *Pseudomonas* strain (Imen et al., 2013). Recent works reported that a few strains of the genus *Enterobacter*, isolated from legume plants, possessed multiple plant-growth promoting characteristics, such as phosphate solubilisation activity and IAA production, thus affecting plant growth and development (Ghosh et al., 2015; Khalifa et al., 2016). On the other hand, one of our isolates, N-92, produced also siderophores, activity already reported for members of the genus *Enterobacter* (Tian et al., 2009).

In conclusion, this work demonstrates for the first time that an AMF inoculum, produced following industrial production processes, is home of a large and diverse community of bacteria with important functional PGP properties, possibly acting in synergy with AMF and providing new services and benefits. The commercial AMF product could be enriched with the selected beneficial bacterial isolates utilized as an additional inoculum, further boosting plant growth, nutrition and health, in order to optimize plant performance in sustainable food production systems. Indeed, our findings imply a new perspective of AM symbiosis, that of a multipartite association - host plants, AMF and bacteria - where different microbial functional groups are active: for example, specific mycorrhizospheric bacteria, by solubilizing P and fixing N, may improve the availability of key mineral nutrients, then absorbed and translocated to the host plant by AMF extraradical hyphae, while other bacteria, by producing siderophores and IAA, may control plant pathogens and promote plant growth. Notwithstanding, so far only few works have been carried out either on the isolation and functional characterization of mycorrhizospheric microbiota, or on their occurrence and significance in AMF inocula. Yet, these studies are necessary and urgent, in the perspective of developing new strategies for sustainable intensification in agriculture, aimed at minimizing the use of chemical fertilizers and pesticides, promoting primary production and maintaining soil health and fertility. To this aim, the most diverse combinations of AMF and bacteria should be studied, in model experimental systems and in the field, to discover possible synergistic effects on different host plants, in order to select the best performing ones for their targeted use in sustainable food production systems in the years to come.

AUTHOR CONTRIBUTIONS

All authors listed have made a substantial, direct and intellectual contribution to the work, and approved it for publication.

FUNDING

The authors gratefully acknowledge the financial support by the University of Pisa (Fondi di Ateneo) and by ATENS-Agrotecnologias Naturales SL, La Riera de Gaia, Tarragona, Spain.

SUPPLEMENTARY MATERIAL

The Supplementary Material for this article can be found online at: https://www.frontiersin.org/articles/10.3389/fpls.2018.01956/full#supplementary-material

REFERENCES

Afzal, I., Iqrar, I., Shinwari, Z. K., and Yasmin, A. (2017). Plant growth-promoting potential of endophytic bacteria isolated from roots of wild *Dodonaea viscosa* L. *Plant Growth Regul.* 81, 399–408. doi: 10.1007/s10725-016-0216-5

Agnolucci, M., Battini, F., Cristani, C., and Giovannetti, M. (2015). Diverse bacterial communities are recruited on spores of different arbuscular mycorrhizal fungal isolates. *Biol. Fertil. Soils* 51, 379–389. doi: 10.1007/s00374-014-0989-5

Ahmad, M., Zahir, Z. A., Jamil, M., Nazli, F., Latif, M., and Akhtar, M. F. (2014). Integrated use of plant growth promoting rhizobacteria, biogas slurry and chemical nitrogen for sustainable production of maize under salt–affected conditions. *Pak. J. Bot* 46, 375–382.

Ali, L., Khalid, M., Asghar, H. N., and Asgher, M. (2017). Scrutinizing of rhizobacterial isolates for improving drought resilience in maize (*Zea mays*). *Int. J. Agric. Biol.* 19, 1054–1064. doi: 10.17957/IJAB/15.0387

Aloni, R., Aloni, E., Langhans, M., and Ullrich, C. I. (2006). Role of cytokinin and auxin in shaping root architecture: regulating vascular differentiation, lateral root initiation, root apical dominance and root gravitropism. *Ann. Bot.* 97, 883–893. doi: 10.1093/aob/mcl027

Andrade, G., Mihara, K. L., Linderman, R. G., and Bethlenfalvay, G. J. (1997). Bacteria from rhizosphere and hyphosphere soils of different arbuscular-mycorrhizal fungi. *Plant Soil* 192, 71–79. doi: 10.1023/A:1004249629643

Ansorge, W. J. (2009). Next-generation DNA sequencing techniques. *New Biotechnol.* 25, 195–203. doi: 10.1016/j.nbt.2008.12.009

Arora, N. K., Kang, S. C., and Maheshwari, D. K. (2001). Isolation of siderophore-producing strains of *Rhizobium meliloti* and their biocontrol potential against *Macrophomina phaseolina* that causes charcoal rot of groundnut. *Curr. Sci.* 81, 673–677.

Augé, R. M. (2001). Water relations, drought and vesicular–arbuscular mycorrhizal symbiosis. *Mycorrhiza* 11, 3–42. doi: 10.1007/s005720100097

Avio, L., Castaldini, M., Fabiani, A., Bedini, S., Sbrana, C., Turrini, A., et al. (2013). Impact of nitrogen fertilization and soil tillage on arbuscular mycorrhizal fungal communities in a Mediterranean agroecosystem. *Soil Biol. Biochem.* 67, 285–294. doi: 10.1016/j.soilbio.2013.09.005

Avio, L., Pellegrino, E., Bonari, E., and Giovannetti, M. (2006). Functional diversity of arbuscular mycorrhizal fungal isolates in relation to extraradical mycelial networks. *New Phytol.* 172, 347–357. doi: 10.1111/j.1469-8137.2006.01839.x

Avio, L., Turrini, A., Giovannetti, M., and Sbrana, C. (2018). Designing the ideotype mycorrhizal symbionts for the production of healthy food. *Front. Plant Sci.* 9:1089. doi: 10.3389/fpls.2018.01089

Barea, J. M., Azcón, R., and Azcón-Aguilar, C. (2002). Mycorrhizosphere interactions to improve plant fitness and soil quality. *Anton. Van Leeuw.* 81, 343–351. doi: 10.1023/A:1020588701325

Barea, J. M., Pozo, M. J., Azcón, R., and Aczón-Aguilar, C. (2005). Microbial cooperation in the rhizosphere. *J. Exp. Bot.* 56, 1761–1778. doi: 10.1093/jxb/eri197

Battini, F., Cristani, C., Giovannetti, M., and Agnolucci, M. (2016). Multifunctionality and diversity of culturable bacterial communities strictly associated with spores of the plant beneficial symbiont *Rhizophagus intraradices*. *Microb. Res.* 183, 68–79. doi: 10.1016/j.micres.2015.11.012

Battini, F., Grønlund, M., Agnolucci, M., Giovannetti, M., and Jakobsen, I. (2017). Facilitation of phosphorus uptake in maize plants by mycorrhizosphere bacteria. *Sci. Rep.* 7:4686. doi: 10.1038/s41598-017-04959-0

Berruti, A., Borriello, R., Della Beffa, M. T., Scariot, V., and Bianciotto, V. (2013). Application of nonspecific commercial AMF inocula results in poor mycorrhization in *Camellia japonica* L. *Symbiosis* 61, 63–76. doi: 10.1007/s13199-013-0258-7

Bharadwaj, D. P., Lundquist, P. O., and Alström, S. (2008a). Arbuscular mycorrhizal fungal spore-associated bacteria affect mycorrhizal colonization, plant growth and potato pathogens. *Soil Biol. Biochem.* 40, 2494–2501. doi: 10.1016/j.soilbio.2008.06.012

Bharadwaj, D. P., Lundquist, P. O., Persson, P., and Alström, S. (2008b). Evidence for specificity of cultivable bacteria associated with arbuscular mycorrhizal fungal spores. *FEMS Microbiol. Ecol.* 65, 310–322. doi: 10.1111/j.1574-6941.2008.00515.x

Bianciotto, V., Bandi, C. D., Minerdi, M., Sironi, H., Tichy, V., and Bonfante, P. (1996). An obligately endosymbiotic mycorrhizal fungus itself harbors obligately intracellular bacteria. *Appl. Environ. Microbiol.* 62, 3005–3010.

Bidondo, L. F., Silvani, V., Colombo, R., Pérgola, M., Bompadre, J., and Godeas, A. (2011). Pre-symbiotic and symbiotic interactions between *Glomus intraradices* and two *Paenibacillus* species isolated from AM propagules. *In vitro* and *in vivo* assays with soybean (AG043RG) as plant host. *Soil Biol. Biochem.* 43, 1866–1872. doi: 10.1016/j.soilbio.2011.05.004

Brito, I., Goss, M. J., De Carvalho, M., Chatagnier, O., and van Tuinen, D. (2012). Impact of tillage system on arbuscular mycorrhiza fungal communities in the soil under Mediterranean conditions. *Soil Tillage Res.* 121, 63–67. doi: 10.1016/j.still.2012.01.012

Budi, S. W., Bakhtiar, Y., and May, N. L. (2013). Bacteria associated with arbuscula mycorrhizal spores *Gigaspora margarita* and their potential for stimulating root mycorrhizal colonization and neem (*Melia azedarach* Linn) seedling growth. *Microbiol. Indones.* 6, 180–188. doi: 10.5454/mi.6.4.6

Budi, S. W., van Tuinen, D., Martinotti, G., and Gianinazzi, S. (1999). Isolation from *Sorghum bicolor* mycorrhizosphere of a bacterium compatible with arbuscular mycorrhiza development and antagonistic towardssoil-borne fungal pathogens. *Appl. Environ. Microbiol.* 65, 5148–5150.

Carpenter-Boggs, L., Loynachan, T. E., and Stahl, P. D. (1995). Spore germination of *Gigaspora margarita* stimulated by volatiles of soil-isolated actinomycetes. *Soil Boil. Biochem.* 27, 1445–1451. doi: 10.1016/0038-0717(95)00075-P

Castillo, C. G., Rubio, R., Rouanet, J. L., and Borie, F. (2006). Early effects of tillage and crop rotation on arbuscular mycorrhizal fungal propagules in an ultisol. *Biol. Fertil. Soils* 43, 83–92. doi: 10.1007/s00374-005-0067-0

Chandra, S., Choure, K., Dubey, R. C., and Maheshwari, D. K. (2007). Rhizosphere competent *Mesorhizobium loti* MP6 induces root hair curling, inhibits *Sclerotinia sclerotiorum* and enhances growth of Indian mustard (*Brassica campestris*). *Braz. J. Microbiol.* 38, 124–130. doi: 10.1590/S1517-83822007000100026

Citernesi, A. S., Fortuna, P., Filippi, C., Bagnoli, G., and Giovannetti, M. (1996). The occurrence of antagonistic bacteria in *Glomus mosseae* pot cultures. *Agronomie* 16, 671–677.

Colombo, C., Palumbo, G., He, J. Z., Pinton, R., and Cesco, S. (2014). Review on iron availability in soil: interaction of Fe minerals, plants, and microbes. *J. Soil Sed.* 14, 538–548. doi: 10.1007/s11368-013-0814-z

Crowley, D. E., Reid, C. P. P., and Szaniszlo, P. J. (1988). Utilization of microbial siderophores in iron acquisition by oat. *Plant Physiol.* 87, 680–685. doi: 10.1104/pp.87.3.680

Cruz, A. F., Horii, S., Ochiai, S., Yasuda, A., and Ishii, T. (2008). Isolation and analysis of bacteria associated with spores of *Gigaspora margarita*. *J. Appl. Microbiol.* 104, 1711–1717. doi: 10.1111/j.1365-2672.2007.03695.x

Cruz, A. F., and Ishii, T. (2011). Arbuscular mycorrhizal fungal spores host bacteria that affect nutrient biodynamics and biocontrol of soil-borne plant pathogens. *Biol. Open* 1, 52–57. doi: 10.1242/bio.2011014

Davison, J. (1988). Plant beneficial bacteria. *Nat. Biotechnol.* 6, 282–286. doi: 10.1038/nbt0388-282

Dodd, I. C., Zinovkina, N. Y., Safronova, V. I., and Belimov, A. A. (2010). Rhizobacterial mediation of plant hormone status. *Ann. Appl. Biol.* 157, 361–379. doi: 10.1111/j.1744-7348.2010.00439.x

Douds, D. D., Galvez, L., Janke, R. R., and Wagoner, P. (1995). Effect of tillage and farming system upon populations and distribution of vesicular-arbuscular mycorrhizal fungi. *Agric. Ecosyst. Environ.* 52, 111–118. doi: 10.1016/0167-8809(94)00550-X

Duca, D., Lorv, J., Patten, C. L., Rose, D., and Glick, B. R. (2014). Indole-3-acetic acid in plant–microbe interactions. *Anton. Van Leeuw.* 106, 85–125. doi: 10.1007/s10482-013-0095-y

Duijff, B., Bakker, P. A. H. M., and Schippers, B. (1994a). Ferric pseudobactin 358 as an iron source for carnation. *J. Plant Nutr.* 17, 2069–2078. doi: 10.1080/01904169409364866

Duijff, B., De Kogel, W. J., Bakker, P. A. H. M., and Schippers, B. (1994b). Influence of pseudobactin 358 on the iron nutrition of barley. *Soil Biol. Biochem.* 26, 1681–1688. doi: 10.1016/0038-0717(94)90321-2

Evelin, H., Kapoor, R., and Giri, B. (2009). Arbuscular mycorrhizal fungi in alleviation of salt stress: a review. *Ann. Bot.* 104, 1263–1280. doi: 10.1093/aob/mcp251

Ghosh, P. K., Sen, S. K., and Maiti, T. K. (2015). Production and metabolism of IAA by *Enterobacter* spp. *(Gammaproteobacteria)* isolated from root nodules of a legume *Abrus precatorius L. Biocat. Agric. Biotech.* 4, 296–303. doi: 10.1016/j.bcab.2015.04.002

Gianinazzi, S., Gollotte, A., Binet, M. N., van Tuinen, D., Redecker, D., and Wipf, D. (2010). Agroecology the key role of arbuscular mycorrhizas in ecosystem services. *Mycorrhiza* 20, 519–530. doi: 10.1007/s00572-010-0333-3

Gianinazzi, S., and Vosatka, M. (2004). Inoculum of arbuscular mycorrhizal fungi for production systems, science meets business. *Can. J. Bot.* 82, 1264–1271. doi: 10.1139/b04-072

Giovannetti, M., Avio, L., and Sbrana, C. (2010). "Fungal spore germination and pre-symbiotic mycelial growth–physiological and genetic aspects," in *Arbuscular Mycorrhizas: Physiology and Function*, eds H. Koltai and Y. Kapulnik (Dordrecht: Springer), 3–32.

Giovannetti, M., Fortuna, P., Citernesi, A. S., Morini, S., and Nuti, M. P. (2001). The occurrence of anastomosis formation and nuclear exchange in intact arbuscular mycorrhizal networks. *New Phytol.* 151, 717–724. doi: 10.1046/j.0028-646x.2001.00216.x

Giovannetti, M., and Mosse, B. (1980). An evaluation of techniques for measuring vesicular-arbuscular infection in roots. *New Phytol.* 84, 489–500. doi: 10.1111/j.1469-8137.1980.tb04556.x

Glick, B. R. (1995). The enhancement of plant growth by free-living bacteria. *Can. J. Microbiol.* 41, 109–117. doi: 10.1139/m95-015

Gontia-Mishra, I., Sapre, S., Sharma, A., and Tiwari, S. (2016). Amelioration of drought tolerance in wheat by the interaction of plant growth-promoting rhizobacteria. *Plant Biol.* 18, 992–1000. doi: 10.1111/plb.12505

Hamdali, H., Hafidi, M., Virolle, M. J., and Ouhdouch, Y. (2008). Growth promotion and protection against damping-off of wheat by two rock phosphate solubilizing actinomycetes in a P-deficient soil under greenhouse conditions. *Appl. Soil Ecol.* 40, 510–517. doi: 10.1016/j.apsoil.2008.08.001

Hamedi, J., and Mohammadipanah, F. (2015). Biotechnological application and taxonomical distribution of plant growth promoting actinobacteria. *J. Ind. Microbiol. Biotechnol.* 42, 157–171. doi: 10.1007/s10295-014-1537-x

Hildebrandt, U., Ouziad, F., Marner, F.-J. J., and Bothe, H. (2006). The bacterium *Paenibacillus validus* stimulates growth of the arbuscular mycorrhizal fungus *Glomus intraradices* up to the formation of fertile spores. *FEMS Microbiol. Lett.* 254, 258–267. doi: 10.1111/j.1574-6968.2005.00027.x

Horii, S., and Ishii, T. (2006). Identification and function of *Gigaspora margarita* growth-promoting microorganisms. *Symbiosis* 41, 135–141.

Imen, C. F., Manel, C., Omar, S., Moez, J., and Salwa, H. J. (2013). Phytostabilization of moderate copper contaminated soils using co-inoculation of *Vicia faba* with plant-growth-promoting bacteria. *J. Basic Microbiol.* 53, 1–9. doi: 10.1002/jobm.201300323

Jansa, J., Mozafar, A., Kuhn, G., Anken, T., Ruh, R., Sanders, I. R., et al. (2003). Soil tillage affects the community structure of mycorrhizal fungi in maize roots. *Ecol. Appl.* 13, 1164–1176. doi: 10.1890/1051-0761(2003)13[1164:STATCS]2.0.CO;2

Jeffries, P., Gianinazzi, S., Perotto, S., Turnau, K., and Barea, J. M. (2003). The contribution of arbuscular mycorrhizal fungi in sustainable maintenance of plant health and soil fertility. *Biol. Fertil. Soils* 37, 1–16. doi: 10.1007/s00374-002-0546-5

Jeong, H., Choi, S. K., Kloepper, J. W., and Ryu, C. M. (2014). Genome sequence of the plant endophyte *Bacillus pumilus* INR7, triggering induced systemic resistance in field crops. *Genome Announc.* 2:e01093–1114. doi: 10.1128/genomeA.01093-14

Jin, C. W., He, Y. F., Tang, C. X., Wu, P., and Zheng, S. J. (2006). Mechanisms of microbially enhanced Fe acquisition in red clover (*Trifolium pratense* L.). *Plant Cell Environ.* 29, 888–897. doi: 10.1111/j.1365-3040.2005.01468.x

Kabir, Z., and Koide, R. T. (2002). Effect of autumn and winter mycorrhizal cover crops on soil properties, nutrient uptake and yield of sweet corn in Pennsylvania, USA. *Plant Soil* 238, 205–215. doi: 10.1023/A:1014408723664

Karasawa, T., and Takebe, M. (2012). Temporal or spatial arrangements of cover crops to promote arbuscular mycorrhizal colonization and P uptake of upland crops grown after nonmycorrhizal crops. *Plant Soil* 353, 355–366. doi: 10.1007/s11104-011-1036-z

Khalid, A., Arshad, M., and Kahir, Z. A. (2004). Screening plant growth promoting rhizobacteria for improving growth and yield of wheat. *J. Appl. Microbiol.* 96, 473–480. doi: 10.1046/j.1365-2672.2003.02161.x

Khalifa, A. Y., Alsyeeh, A. M., Almalki, M. A., and Saleh, F. A. (2016). Characterization of the plant growth promoting bacterium, *Enterobacter cloacae* MSR1, isolated from roots of non-nodulating *Medicago sativa*. *Saudi J Biol. Sci.* 23, 79–86. doi: 10.1016/j.sjbs.2015.06.008

Klindworth, A., Pruesse, E., Schweer, T., Peplies, J., Quast, C., Horn, M., et al. (2013). Evaluation of general 16S ribosomal RNA gene PCR primers for classical and next-generation sequencing-based diversity studies. *Nucleic Acids Res.* 41:e1. doi: 10.1093/nar/gks8

Kobayashi, T., and Nishizawa, N. K. (2012). Iron uptake, translocation, and regulation in higher plants. *Ann. Rev. Plant Biol.* 63, 131–152. doi: 10.1146/annurev-arplant-042811-105522

Kumar, S., Stecher, G., Li, M., Knyaz, C., and Tamura, K. (2018). MEGA X: Molecular evolutionary genetics analysis across computing platforms. *Molec. Biol. Evol.* 35, 1547–1549. doi: 10.1093/molbev/msy096

Kumar, V., and Gera, R. (2014). Isolation of a multi-trait plant growth promoting *Brevundimonas* sp. and its effect on the growth of Bt-cotton. *3 Biotech* 4, 97–101. doi: 10.1007/s13205-013-0126-4

Lane, D. J. (1991). "16S/23S rRNA sequencing," in *Nucleic Acid Techniques in Bacterial Systematic*, eds E. Stackebrandt and M. Goodfellow (New York, NY: Wiley), 115–175.

Lehman, R. M., Taheri, W. I., Osborne, S. L., Buyer, J. S., and Douds, D. D. Jr (2012). Fall cover cropping can increase arbuscular mycorrhizae in soils supporting intensive agricultural production. *Appl. Soil Ecol.* 61, 300–304. doi: 10.1016/j.apsoil.2011.11.008

Lekberg, Y., and Koide, R. T. (2005). Is plant performance limited by abundance of arbuscular mycorrhizal fungi? A meta-analysis of studies published between 1988 and 2003. *New Phytol.* 168, 189–204. doi: 10.1111/j.1469-8137.2005.01490.x

Li, B., Ravnskov, S., Xie, G., and Larsen, J. (2007). Biocontrol of *Pythium* damping-off in cucumber by arbuscular mycorrhiza-associated bacteria from the genus *Paenibacillus*. *Biocontrol* 52, 863–875. doi: 10.1007/s10526-007-9076-2

Long, L., Zhu, H., Yao, Q., and Ai, Y. (2008). Analysis of bacterial communities associated with spores of *Gigaspora margarita* and *Gigaspora rosea*. *Plant Soil* 310, 1–9. doi: 10.1007/s11104-008-9611-7

Louden, B. C., Haarmann, D., and Lynne, A. M. (2011). Use of blue agar CAS assay for siderophore detection. *J. Microbiol. Biol. Ed.* 12, 51–53. doi: 10.1128/jmbe.v12i1249

Lü, Z., Min, H., Li, N., Shao, T., and Ye, Y. (2006). Variations of bacterial community structure in flooded paddy soil contaminated with herbicide quinclorac. *J. Environ. Sci. Health Part B* 41, 821–832. doi: 10.1080/03601230600805873

Marques, A. P., Pires, C., Moreira, H., Rangel, A. O., and Castro, P. M. (2010). Assessment of the plant growth promotion abilities of six bacterial isolates using *Zea mays* as indicator plant. *Soil Biol. Biochem.* 42, 1229–1235. doi: 10.1016/j.soilbio.2010.04.014

Mayo, K., Davis, R. E., and Motta, J. (1986). Stimulation of germination of spores of *Glomus versiforme* by spore-associated bacteria. *Mycologia* 78, 426–431. doi: 10.2307/3793046

Messiha, N. A. S., Van Diepeningen, A. D., Farag, N. S., Abdallah, S. A., Janse, J. D., and Van Bruggen, A. H. C. (2007). *Stenotrophomonas maltophilia*: a new potential biocontrol agent of *Ralstonia solanacearum*, causal agent of potato brown rot. *Eur. J. Plant Phatol.* 118, 211–225. doi: 10.1007/s10658-007-9136-6

Miliute, I., Buzaite, O., Baniulis, D., and Stanys, V. (2015). Bacterial endophytes in agricultural crops and their role in stress tolerance: a review. *Zemdirbyste* 102, 465–478. doi: 10.13080/z-a.2015.102.060

Mimmo, T., Del Buono, D., Terzano, R., Tomasi, N., Vigani, G., Crecchio, C., et al. (2014). Rhizospheric organic compounds in the soil microorganism-plant system: their role in iron availability. *Eur. J. Soil Sci.* 65, 629–642. doi: 10.1111/ejss.12158

Mohammad, B. T., Wright, P. C., and Bustard, M. T. (2006). Bioconversion of isopropanol by a solvent tolerant *Sphingobacterium mizutae* strain. *J. Industr. Microbiol. Biotechnol.* 33, 975–983. doi: 10.1007/s10295-006-0143-y

Mohandas, S., Poovarasan, S., Panneerselvam, P., Saritha, B., Upreti, K. K., Kamal, R., et al. (2013). Guava (*Psidium guajava* L.) rhizosphere *Glomus mosseae* spores harbor actinomycetes with growth promoting and antifungal attributes. *Sci. Hortic.* 150, 371–376. doi: 10.1016/j.scienta.2012.11.019

Mugnier, J., and Mosse, B. (1987). Spore germination and viability of a vesicular arbuscular mycorrhizal fungus, *Glomus mosseae*. *Trans. Br. Mycol. Soc.* 88, 411–413. doi: 10.1016/S0007-1536(87)80018-9

Nautiyal, C. S. (1999). An efficient microbiological growth medium for screening phosphate solubilizing microorganisms. *FEMS Microbiol. Lett.* 170, 265–270. doi: 10.1111/j.1574-6968.1999.tb13383.x

Nishioka, T., Elsharkawy, M. M., Suga, H., Kageyama, K., Hyakumachi, M., and Shimizu, M. (2016). Development of culture medium for the Isolation of *Flavobacterium* and *Chryseobacterium* from rhizosphere soil. *Microb. Environ.* 31, 104–110. doi: 10.1264/jsme2.ME15144

Njeru, E. M., Avio, L., Bocci, G., Sbrana, C., Turrini, A., Bàrberi, P., et al. (2015). Contrasting effects of cover crops on 'hot spot' arbuscular mycorrhizal fungal communities in organic tomato. *Biol. Fertil. Soils* 51, 151–166. doi: 10.1007/s00374-014-0958-z

Njeru, E. M., Avio, L., Sbrana, C., Turrini, A., Bocci, G., Bàrberi, P., et al. (2014). First evidence for a major cover crop effect on arbuscular mycorrhizal fungi and organic maize growth. *Agron. Sustain. Dev.* 34, 841–848. doi: 10.1007/s13593-013-0197-y

Oehl, F., Sieverding, E., Mäder, P., Dubois, D., Ineichen, K., Boller, T., et al. (2004). Impact of long-term conventional and organic farming on the diversity of arbuscular mycorrhizal fungi. *Oecologia* 138, 574–583. doi: 10.1007/s00442-003-1458-2

Palla, M., Cristani, C., Giovannetti, M., and Agnolucci, M. (2017). Identification and characterization of lactic acid bacteria and yeasts of PDO Tuscan bread sourdough by culture dependent and independent methods. *Int. J. Food Microbiol.* 250, 19–26. doi: 10.1016/j.ijfoodmicro.2017.03.015

Patten, C. L., and Glick, B. R. (2002). Role of *Pseudomonas putida* indoleacetic acid in development of the host plant root system. *Appl. Environ. Microbiol.* 68, 3795–3801. doi: 10.1128/AEM.68.8.3795-3801.2002

Pérez-Miranda, S., Cabirol, N., George-Téllez, R., Zamudio-Rivera, L. S., and Fernández, F. J. (2007). O-CAS, a fast and universal method for siderophore detection. *J. Microbiol. Method* 70, 127–131. doi: 10.1016/j.mimet.2007.03.023

Pérez-Montaño, F., Alías-Villegas, C., Bellogín, R. A., Del Cerro, P., Espuny, M. R., Jiménez-Guerrero, I., et al. (2014). Plant growth promotion in cereal and leguminous agricultural important plants: from microorganism capacities to crop production. *Microbiol. Res.* 169, 325–336. doi: 10.1016/j.micres.2013.09.011

Philippot, L., Raaijmakers, J. M., Lemanceau, P., and van der Putten, W. H. (2013). Going back to the roots: the microbial ecology of the rhizosphere. *Nat. Rev. Microbiol.* 11, 789–799. doi: 10.1038/nrmicro3109

Qin, Z., Wang, X., Rateb, M. E., Ass'ad, L. A., Jaspars, M., Deng, Z., et al. (2014). Disruption of a methyltransferase gene in actinomycin G gene cluster in *Streptomyces iakyrus* increases the production of phenazinomycin. *FEMS Microbiol. Lett.* 352, 62–68. doi: 10.1111/1574-6968.12370

Ritacco, F. V., and Eveleigh, D. E. (2008). Molecular and phenotypic comparison of phaeochromycin-producing strains of *Streptomyces phaeochromogenes* and *Streptomyces ederensis*. *J. Ind. Microbiol. Biotechnol.* 35, 931–945. doi: 10.1007/s10295-008-0367-0

Rivas, R., Velázquez, E., Willems, A., Vizcaíno, N., Subba-Rao, N. S., Mateos, P. F., et al. (2002). A new species of *Devosia* that forms a unique nitrogen-fixing root-nodule symbiosis with the aquatic legume *Neptunia natans* (Lf) Druce. *Appl. Environ. Microbiol.* 68, 5217–5222. doi: 10.1128/AEM.68.11.5217-5222.2002

Robin, A., Vansuyt, G., Hinsinger, P., Meyer, J. M., Briat, J. F., and Lemanceau, P. (2008). Iron dynamics in the rhizosphere: consequences for plant health and nutrition. *Adv. Agron.* 99, 183–225. doi: 10.1016/S0065-2113(08)00404-5

Roesti, D., Ineichen, K., Braissant, O., Redecker, D., Wiemken, A., and Aragno, M. (2005). Bacteria associated with spores of the arbuscular mycorrhizal fungi *Glomus geosporum* and *Glomus constrictum*. *Appl. Environ. Microbiol.* 71, 6673–6679. doi: 10.1128/AEM.71.11.6673-6679.2005

Rouphael, Y., Franken, P., Schneider, C., Schwarz, D., Giovannetti, M., Agnolucci, M., et al. (2015). Arbuscular mycorrhizal fungi act as biostimulants in horticultural crops. *Sci. Hortic.* 196, 91–108. doi: 10.1016/j.scienta.2015.09.002

Sbrana, C., Avio, L., and Giovannetti, M. (2014). Beneficial mycorrhizal symbionts affecting the production of health-promoting phytochemicals. *Electrophoresis* 35, 1535–1546. doi: 10.1002/elps.201300568

Shivaji, S., Ray, M. K., Rao, N. S., Saisree, L., Jagannadham, M. V., Kumar, G. S., et al. (1992). Sphingobacterium antarcticus sp. *nov.,* a psychrotrophic bacterium from the soils of *Schirmacher Oasis,* Antarctica. *Int. J. Sys. Evol.Microbiol.* 42, 102–106. doi: 10.1099/00207713-42-1-102

Siebner-Freibach, H., Hadar, Y., and Chen, Y. (2003). Siderophores sorbed on Ca-montmorillonite as an iron source for plants. *Plant Soil* 251, 115–124. doi: 10.1023/A:1022984431626

Sikes, B. A., Kottenie, K., and Klironomos, J. N. (2009). Plant and fungal identity determines pathogen protection of plant roots by arbuscular mycorrhizas. *J. Ecol.* 97, 1274–1280. doi: 10.1111/j.1365-2745.2009.01557.x

Singh, R. P., and Jha, P. N. (2017). The PGPR *Stenotrophomonas maltophilia* SBP-9 augments resistance against biotic and abiotic stress in wheat plants. *Front. Microbiol.* 8:1945. doi: 10.3389/fmicb.2017.01945

Smith, S. E., and Read, D. J. (2008). *Mycorrhizal Symbiosis*. London: Academic Press.

Tchan, Y. T. (1984). "Azotobacteraceae," in *Bergey's Manual of Systematic Bacteriology*, vol. 1, eds N. Krieg and J. G. Holt (London: Williams and Wikins), 219–225.

Thomashow, L. S., Weller, D. M., Bonsall, R. F., and Pierson, L. S. (1990). Production of the antibiotic phenazine-1-carbox-ylic acid by fluorescent *Pseudomonas* species in the rhizosphere of wheat. *Appl. Environ. Microbiol.* 56, 908–912.

Tian, F., Ding, Y., Zhu, H., Yao, L., and Du, B. (2009). Genetic diversity of siderophore-producing bacteria of tobacco rhizosphere. *Braz. J. Microbiol.* 40, 276–284. doi: 10.1590/S1517-83822009000200013

Toljander, J. F., Artursson, V., Paul, L. R., Jansson, J. K., and Finlay, R. D. (2006). Attachment of different soil bacteria to arbuscular mycorrhizal fungal extraradical hyphae is determined by hyphal vitality and fungal species. *FEMS Microbiol. Lett.* 254, 34–40. doi: 10.1111/j.1574-6968.2005.00003.x

Turrini, A., Caruso, G., Avio, L., Gennai, C., Palla, M., Agnolucci, M., et al. (2017). Protective green cover enhances soil respiration and native mycorrhizal potential compared with soil tillage in a high-density olive orchard in a long term study. *Appl. Soil Ecol.* 116, 70–78. doi: 10.1016/j.apsoil.2017.04.001

Turrini, A., Sbrana, C., Avio, L., Njeru, E. M., Bocci, G., Bàrberi, P., et al. (2016). Changes in the composition of native root arbuscular mycorrhizal fungal communities during a short-term cover crop-maize succession. *Biol. Fertil. Soils* 52, 643–653. doi: 10.1007/s00374-016-1106-8

Tylka, G. L., Hussey, R. S., and Roncadori, R. W. (1991). Axenic germination of vesicular–arbuscular mycorrhizal fungi: effects of selected *Streptomyces* species. *Phytopathology* 81, 754–759.

Vandera, E., Samiotaki, M., Parapouli, M., Panayotou, G., and Koukkou, A. I. (2015). Comparative proteomic analysis of *Arthrobacter phenanthrenivorans* Sphe3 on phenanthrene, phthalate and glucose. *J. Proteomics* 113, 73–89. doi: 10.1016/j.jprot.2014.08.018

Vansuyt, G., Robin, A., Briat, J., Curie, C., and Lemanceau, P. (2007). Iron acquisition from Fe-pyoverdine by *Arabidopsis thaliana*. *Mol. Plant-Micr. Interac.* 20, 441–447. doi: 10.1094/MPMI-20-4-0441

Walley, F. L., and Germida, J. J. (1996). Failure to decontaminate *Glomus clarum* NT4spores is due to spore wall-associated bacteria. *Mycorrhiza* 6, 43–49. doi: 10.1007/s005720050104

Walter, A., Römheld, V., Marschner, H., and Crowley, D. E. (1994). Iron nutrition of cucumber and maize: effect of *Pseudomonas putida* YC3 and its siderophore. *Soil Biol. Biochem.* 26, 1023–1031. doi: 10.1016/0038-0717(94)90117-1

Weisburg, W. G., Barns, S. M., Pelletier, D. A., and Lane, D. J. (1991). 16S ribosomal DNA amplification for phylogenetic study. *J. Bacteriol.* 173, 697–703. doi: 10.1128/jb.173.2.697-703.1991

Weitnauer, G., Mühlenweg, A., Trefzer, A., Hoffmeister, D., Süssmuth, R. D, Jung, G., et al. (2001). Biosynthesis of the orthosomycin antibiotic avilamycin A: deductions from the molecular analysis of the avi biosynthetic gene cluster of *Streptomyces viridochromogenes* Tü57 and production of new antibiotics. *Chem. Biol.* 8, 569–581. doi: 10.1016/S1074-5521(01)00040-0

Whipps, J. M. (2001). Microbial interactions and biocontrol in the rhizosphere. *J. Exp. Bot.* 52, 487–511. doi: 10.1093/jexbot/52.suppl_1.487

Wolf, H., and Zähner, H. (1972). Metabolic products of microorganism. *Arch. Mikrobiol.* 83, 147–154.

Xavier, L. J. C., and Germida, J. J. (2003). Bacteria associated with *Glomus clarum* spores influence mycorrhizal activity. *Soil Biol. Biochem.* 35, 471–478. doi: 10.1016/S0038-0717(03)00003-8

Yehuda, Z., Shenker, M., Romheld, V., Marschner, H., Hadar, Y., and Chen, Y. (1996). The role of ligand exchange in the uptake of iron from microbial siderophores by gramineous plants. *Plant Physiol.* 112, 1273–1280. doi: 10.1104/pp.112.3.1273

Yoo, S. H., Weon, H. Y., Jang, H. B., Kim, B. Y., Kwon, S. W., Go, S. J., et al. (2007). *Sphingobacterium composti* sp. nov., isolated from cotton-waste composts. *Int. J. Sys. Evol.Microbiol.* 57, 1590–1593. doi: 10.1099/ijs.0.64948-0

Zahir, Z. A., Arshad, M., Frankenberger, W. T. Jr. (2003). "Plant growth promoting rhizobacteria: applications and perspectives in agriculture," in *Advances in Agronomy* vol. 81, ed D. L. Sparks (Newark, NY: Academic Press), 97–168.

Zhao, L., Wu, X. Q., Ye, J. R., Li, H., and Li, G. E. (2014). Isolation and characterization of a mycorrhiza helper bacterium from rhizosphere soils of poplar stands. *Biol. Fertil. Soils* 50, 593–601. doi: 10.1007/s00374-013-0880-9

Conflict of Interest Statement: MG received funding from and VC and FC are employees of Atens - Agrotecnologias Naturales SL, La Riera de Gaia, Tarragona, Spain.

The remaining authors declare that the research was conducted in the absence of any commercial or financial relationships that could be construed as a potential conflict of interest.

frontiers in Plant Science

ORIGINAL RESEARCH
published: 31 January 2019
doi: 10.3389/fpls.2019.00048

Polysaccharides Derived From the Brown Algae *Lessonia nigrescens* Enhance Salt Stress Tolerance to Wheat Seedlings by Enhancing the Antioxidant System and Modulating Intracellular Ion Concentration

Ping Zou[1], Xueli Lu[1], Hongtao Zhao[2], Yuan Yuan[1], Lei Meng[2], Chengsheng Zhang[1]* and Yiqiang Li[1]**

[1] Marine Agriculture Research Center, Tobacco Research Institute of Chinese Academy of Agricultural Sciences, Qingdao, China, [2] State Key Laboratory of Bioactive Seaweed Substances, Qingdao, China

OPEN ACCESS

Edited by:
Giuseppe Colla,
Università degli Studi della Tuscia, Italy

Reviewed by:
Petronia Carillo,
Università degli Studi della Campania
Luigi Vanvitelli, Italy
Fernanda Fidalgo,
Universidade do Porto, Portugal

***Correspondence:**
Ping Zou
zouping@caas.cn
Chengsheng Zhang
zhangchengsheng@caas.cn
Yiqiang Li
liyiqiang@caas.cn

Specialty section:
This article was submitted to Crop and Product Physiology, a section of the journal Frontiers in Plant Science

Received: 28 September 2018
Accepted: 14 January 2019
Published: 31 January 2019

Citation:
Zou P, Lu X, Zhao H, Yuan Y, Meng L, Zhang C and Li Y (2019) Polysaccharides Derived From the Brown Algae Lessonia nigrescens Enhance Salt Stress Tolerance to Wheat Seedlings by Enhancing the Antioxidant System and Modulating Intracellular Ion Concentration. Front. Plant Sci. 10:48. doi: 10.3389/fpls.2019.00048

Soil salinity reduces plant growth and is a major factor that causes decreased agricultural productivity worldwide. Seaweed polysaccharides promote crop growth and improve plant resistance to abiotic stress. In this study, polysaccharides from brown seaweed *Lessonia nigrescens* polysaccharides (LNP) were extracted and further separated and fractionated. Two acidic polysaccharides (LNP-1 and LNP-2) from crude LNP were obtained and characterized. The latter had a lower molecular weight (MW) (40.2 kDa) than the former (63.9 kDa), but had higher uronic acid and sulfate content. Crude LNP and LNP-2 were composed of mannose, glucuronic acid, fucose, and xylose, whereas LNP-1 has little mannose. Moreover, the effects of the three polysaccharides on plant salt tolerance were investigated. The results showed that crude LNP, LNP-1, and LNP-2 promoted the growth of plants, decreased membrane lipid peroxidation, increased the chlorophyll content, improved antioxidant activities, and coordinated the efflux and compartmentation of intracellular ion. All three polysaccharides could induce plant resistance to salt stress, but LNP-2 was more effective than the other two. The present study allowed to conclude that both MW and sulfate degree contribute to salt resistance capability of polysaccharides derived from *L. nigrescens*.

Keywords: salt tolerance, seaweed polysaccharide, *Lessonia nigrescens*, molecular weight, sulfate content

INTRODUCTION

Soil salinization is a major environmental problem that restricts crop growth and yield worldwide, mainly due to osmotic stress and ion toxicity (Rivero et al., 2014). Salinization is predicted to degrade approximately 30% of all cultivated land by 2050 (Van Oosten et al., 2017). High concentrations of salts in the soil decrease the capacity of roots to extract water, and high concentrations of salts in the plant can be toxic (Munns and Tester, 2008; Annunziata et al., 2017). High salt concentration in the soil has a devastating effect on plant metabolism, disrupting cellular homeostasis and uncoupling major physiological and biochemical processes. Salinity stress also

results in production of reactive oxygen species (ROS) in plants causing oxidative stress. Along evolution, plants have evolved a ubiquitous mechanism of salinity resistance, involving synthesis and accumulation of compatible compounds, sodium sequestration in vacuoles, and enhancing antioxidant enzymes activities. These specific strategies regulate cell penetration and control ion and water homeostasis to minimize stress damage and sustain growth.

Salinity resistance mechanisms can be further improved through the application of exogenous biostimulants (Bose et al., 2014). A plant biostimulant is a substance or microorganism that is beneficial to plants and can enhance nutrient uptake and nutrient use efficiency, abiotic stress tolerance, and crop quality, regardless of its own nutrient content. Considering that crops are exposed to extreme temperatures, drought and soil salinization, biostimulants may contribute more and more to global crop yields and quality as growth promoters and stress protectors. Various biostimulants have been used in commercial agriculture, such as humic and fulvic acids, protein hydrolysates and other N-containing compounds, seaweed extracts and botanicals, chitosan and other biopolymers, inorganic compounds, beneficial fungi, and bacteria (Jardin, 2015). Seaweed extracts have long been used in agriculture as sources of organic matter and as fertilizer. The beneficial constituents contributing to the plant growth promotion include the polysaccharides, micro- and macronutrients, sterols, N-containing compounds like betaines, and hormones. Many studies have demonstrated that polysaccharide from seaweed is a kind of plant physiological stimulants and stress-resistance elicitors. In tobacco plants, for example, carrageenan application can activate defense systems involving ethylene, jasmonic acid, and salicylic acid pathways in tobacco plants, while weekly spraying of 1 mg/ml oligocarrageenans stimulated growth, photosynthesis, and basal metabolism (Castro et al., 2011). The bioactive components in *Ulva lactuca* (Ibrahim et al., 2014) and *Fucus spiralis* (Latique et al., 2017) extract could enhance in the percentage of seed germination and growth parameters. Chernane et al. (2015)'s study suggested that seaweed extract of *Ulva rigida* can improve salt stress tolerance and contribute to protection of wheat plant against oxidative deterioration. Currently, the primary algal polysaccharides on the phytosanitary market are laminarans, derived from brown algae [e.g., *Laminaria digitata* (Hudson) J.V.]. Laminarans can induce various defense responses in tobacco and grapevine cell suspensions, including protein kinase activation, Ca^{2+} influx, oxidative outburst, extracellular-media alkalinization, and phytoalexin production. When sprayed on tobacco and grapevine plants, laminarans stimulate phytoalexin accumulation and expression of PR-proteins (Klarzynski et al., 2000; Aziz et al., 2003). The ability of these algal polysaccharides to activate multiple plant defenses is likely to benefit the development of novel resistance inducers. Economically important algae can be found in rocky intertidal and shallow subtidal zones, contain numerous bioactive compounds (e.g., fucans and phlorotannins) (Gonzalez et al., 2012). One of these species, *Lessonia nigrescens* Bory de Saint-Vincent grows quickly and produces large biomass, indicating its potential for agricultural application. However, the effectiveness of *L. nigrescens* compounds for stimulating the resistance of cultivated plants remains unclear. The purpose of the present study was to assess the effects of *L. nigrescens* polysaccharides (LNP) on wheat seedlings under salt stress. Moreover, we aimed to contribute to the understanding of the regulatory mechanism of LNPs in the improvement of plant salt stress resistance in terms of osmotic regulation, ion transport, and redox homeostasis. This study provides a simple, efficacious, and sustainable approach to ameliorate salt stress in commercially important crops.

MATERIALS AND METHODS

Samples and Reagents

Dried *L. nigrescens* was supplied by State Key Laboratory of Bioactive Seaweed Substances (Qingdao, China). After being ground, the seaweed was sieved through a 0.45 mm sifter and stored in a desiccator. Standard sugars were purchased from Sigma (United States). All other chemicals and reagents were of analytical grade.

Extraction of Crude Polysaccharides

Lessonia nigrescens (100 g) was extracted with 80% ethanol (2 l) under mechanical stirring at room temperature for 24 h to remove pigments, proteins, salts, and other small molecules. Next, 50 g of the dried residue was extracted with 1.5 l 0.1 M HCl in a 3 l flask at 100°C for 2 h. The precipitate was removed using gauze, and the remaining supernatant was filtered using siliceous earth. Subsequently, 2% (w/v) $CaCl_2$ solution was added to the liquid fraction, and the mixture was maintained overnight at 4°C for alginate removal and was then separated by centrifugation. The filtrate was dialyzed against distilled water for 48 h and concentrated under reduced pressure to approximately one-fourth of its original volume. Finally, polysaccharides were precipitated using fourfold volume of ethanol and were then lyophilized to yield LNP.

Purification of LNP Fraction

Crude polysaccharide (10 mg) solution (10 mg/ml) was loaded onto a DEAE-52 anion exchange column (2.6 × 30 cm). The column was eluted with a stepwise gradient of distilled water, followed by 0.1, 0.2, 0.3, 0.4, and 0.5 M NaCl solution at a flow rate of 1.0 ml/min. The eluate (10 ml/tube) was collected automatically (BSZ-100, Shanghai QingpuHuxi Instrument Factory Co., Ltd., P.R. China). Polysaccharide fractions were analyzed using the phenol–sulfuric acid method, eventually yielding two fractions.

These fractions were then re-dissolved in distilled water and loaded onto a Sephadex G-100 gel column (1.6 cm × 100 cm) for a second elution (0.1 M NaCl at a flow rate of 20 ml/h). As before, the eluent (5 ml/tube) was collected automatically and analyzed. The two purified fractions (LNP-1 and LNP-2) were concentrated, dialyzed, and lyophilized for further analyses.

Chemical Composition

Determination of Carbohydrate, Protein, Sulfate, and Uronic Acid Content

Total carbohydrate content of polysaccharides was determined via the phenol–sulfuric acid method, using D-glucose as a standard (Chen C.F. et al., 2018). Protein content was determined by the method of Bradford, with bovine serum albumin as a standard (Chen G. et al., 2018). The sulfate content was quantified using the $BaCl_2$ gelation method (standard: K_2SO_4) (Palanisamy et al., 2018). Finally, uronic acid content was determined using the carbazole method, with glucuronic acid as a standard (Palanisamy et al., 2018).

Determination of Monosaccharide Composition

Polysaccharides (1 mg) were hydrolyzed in 0.5 ml of 2 M trifluoroacetic acid (TFA) at 120°C for 2 h. The resulting solution was dried on a nitrogen blowing apparatus. After TFA was removed, the hydrolysate was derivatized with 1-phenyl-3-methyl-5-pyrazolone (PMP) at 70°C for 30 min. The solution was analyzed using an Agilent 1260 Infinity HPLC instrument with a Thermo ODS-2 C18 (4.6 × 250 mm, 5 μm) column at 25°C, with a flow rate of 1 ml/min. Separated monosaccharides were quantified through external calibration with an equimolar mixture of nine monosaccharide standards (mannose, rhamnose, fucose, glucose, galactose, glucuronic acid, galacturonic acid, arabinose, and xylose) (Yuan and Macquarrie, 2015).

Determination of Average Molecular Weight

The average polysaccharide molecular weight (MW) was determined using an Agilent 1260 gel permeation chromatograph (Agilent Technologies, United States) fitted with a refractive index detector (Zou et al., 2018). Chromatography was run on a TSK G4000-PW_{xl} column with 0.05 M aqueous $NaNO_3$ as the mobile phase. The flow rate was 0.5 ml/min and the column temperature was 30°C. Standards used to calibrate the column were dextrans with MWs of 1000, 5000, 12,000, 25,000, 50,000, 80,000, 270,000, and 670,000 Da (Sigma, United States).

Infrared Spectroscopy of Polysaccharides

Fourier transformed infrared (FT-IR) spectra of polysaccharides were plotted using a Thermo Fisher Scientific Nicolet iS10 FT-IR spectrometer (Thermo Fisher Scientific, United States) with KBr disks.

Determination of Salt-Defense Elicitor Activity

Plant Material and Treatments

Wheat (*Triticum aestivum* L. Jimai 22) seeds were surface-sterilized with a 1% (v/v) sodium hypochlorite solution for 10 min and thoroughly rinsed with distilled water. Seeds were germinated for 24 h at 25°C and then sown on nylon mesh in Petri dishes containing Hoagland solution. Dishes were placed in a growth incubator under the following controlled conditions: 14 h light /10 h dark photoperiod, 25°C/20°C day/night cycle, 65% relative humidity, and photosynthetic photon flux intensity of 800 mmol m^{-2} s^{-1}. Wheat seedlings with fully expanded second leaves were randomly divided into five groups. The experiment included five treatments: control (neither LNPs nor NaCl), negative control (150 mM NaCl), and three different LNP–NaCl mixtures. Each group contained three petri dishes, each with 30 plants. Nutrient solutions were renewed daily.

Growth Parameters

After 10 days of salt stress, three samples were randomly selected from each group and their physiological indices were determined. Next, 30 plants were randomly chosen and harvested to measure shoot length, root length, and fresh weight (FW). Samples were dried at 105°C for 2 h to determine dry weight (DW).

Determination of Lipid Peroxidation and Electrolyte Leakage

Membrane permeability was assessed by measuring relative electric leakage (REL) (Li et al., 2017). Fully expanded second leaves (1.0 g) were cut into 0.5-cm pieces and placed in a 50-ml test tube containing 30 ml distilled water. Leaf samples were then vacuumed for 30 min, immersed and vibrated for 20 min, before solution conductivity (EC1) was measured (DDSJ-308A, Shanghai Instrument and Electrical Scientific Instrument Ltd., Shanghai, China). Samples were then boiled for 30 min and cooled to room temperature. After this, conductivity (EC2) was measured again to yield REL (EC1/EC2 × 100%).

Malondialdehyde (MDA) content in plants indicates lipid peroxidation levels, and thiobarbituric acid (TBA) reactions were used to determine MDA content according to the previous method with certain modifications (Buono et al., 2011). 0.5 g leaf samples were homogenized in 10% (w/v) TCA before centrifugation at 4000 × *g* for 10 min. Two milliliters of 0.6% (w/v) TBA was added to 2 ml of the supernatant, and the mixture was then heated in boiling water for 15 min cooled, then centrifuged at 10,000 × *g* for 15 min. Absorbance (optical densities) were read at 450, 532, and 600 nm. MDA content was recorded as mg MDA/g FW.

Chlorophyll Contents

After 10 days of NaCl treatment, chlorophyll a (Chl a), chlorophyll b (Chl b), and total chlorophyll (Chla+b) content in seedlings were measured spectrophotometrically (665 and 649 nm), following previously published procedures (Ma et al., 2017). The procedure was performed under low light to avoid chlorophyll degradation.

Soluble Sugar Content and Proline Content

Leaf samples (0.5 g) were chopped and heated at 100°C in 5 ml distilled water for 30 min. Extracts were diluted fivefold. Extracts (500 ml), 5% (v/v) phenol (1 ml), and sulfuric acid (5 ml) were mixed, then left to stand for 5 min before absorbance was read at 485 nm (Zou et al., 2018). Soluble sugar concentration was quantified through comparisons against a glucose standard curve.

To determine proline content, 0.2 g leaf samples were ground in liquid nitrogen and homogenized in 5 ml of 3% (w/v) sulfosalicylic acid (Misra and Saxena, 2009). The sample was centrifuged at 18,000 × *g* for 10 min 500 μl of filtrate and made up 1 ml with distilled water and thereafter 1 ml glacial acetic acid

and 1 ml ninhydrin reagent added and then heated at 100°C for 10 min, cooled to room temperature, and centrifuged at 5000 × *g* for 4 min. Proline content in the supernatant was determined spectrophotometrically at 520 nm.

Antioxidant Enzyme Activities

After salt stress for 10 days, fully expanded second leaves (0.5 g) were used to extract enzymes. Samples were homogenized in liquid nitrogen and brought up to a volume of 5 ml with 0.2 mol/l cold sodium phosphate buffer solution (pH 7.8). Homogenates were centrifuged at 12,000 × *g* and 4°C for 15 min. Supernatants were immediately used to determine enzyme activities. The Bradford method was used to determine total soluble protein (Bradford and Williams, 1976). Superoxide dismutase (SOD) activity was assayed by the extent to which it inhibited the photochemical reduction of b-nitro blue tetrazolium chloride (NBT) (Zhang et al., 2016). Catalase (CAT) activity was determined based on the rate of disappearance of H_2O_2, as measured by decline in absorbance at 240 nm (Ma et al., 2017). Peroxidase activity (POD) was calculated from the rate of the formation of the guaiacol dehydrogenation product and was expressed as mmol GDHP min^{-1} mg^{-1} protein (Marta et al., 2016).

Measurement of Na^+ and K^+ Concentrations

Na^+ and K^+ concentration were measured as described previously (Zhang et al., 2018). Plant tissues, leaves, sheaths, and roots were dried at 60°C overnight. Dry samples (0.5 g) were incinerated in a muffle furnace at 500°C for 6 h. The ashes were dissolved in 5 ml of concentrated nitric acid with 500 ml distilled water. Ion concentrations were determined using an Atomic Absorption Spectrometer 900T (PerkinElmer, United States).

Expression Analysis of Genes Encoding Na^+/K^+ Transporter

Total RNA was extracted from leaves, sheaths, and roots of wheat seedlings using a Plant RNA Extraction Kit (Takara, Dalian, China). Total RNA was quantified using a UV spectrophotometer. First-strand cDNA was synthesized from 1 μg mRNA using a PrimeScript™ RT Reagent Kit with gDNA Eraser (Takara, Dalian, China). Real-time quantitative PCR was performed using an ABI 7500 (Life Tech Applied Biosystems, United States) with a TB Green™ Premix Ex TaqTM (Takara, Dalian, China). RT-PCR was performed in a total volume of 20 μl containing 100 ng of the first strand cDNA reaction products. Amplicons were subjected to melting curve analysis. Relative expression was analyzed using the comparative threshold cycle method ($2^{-\Delta\Delta Ct}$), with β-actin as a housekeeping gene. Primers are listed in **Supplementary Table S1**.

Statistical Analyses

All data are represented as means ± SD of three independent replicates. Analysis of variance (ANOVA) and *post hoc* Duncan's tests ($P < 0.05$) were used to compare means across treatments.

RESULTS

Purification of LNPs

The two LNP fractions (**Figure 1A**) detected during purification generated only one symmetrical peak, indicating relative homogeneity (**Figures 1B,C**). The two purified polysaccharides were termed LNP-1 (**Figure 1B**) and LNP-2 (**Figure 1C**). No fractions were obtained by elution with distilled water, which indicated the absence of neutral laminaran-type polysaccharides. This is consistent with the results of Nancy who found no laminaran-type polysaccharides in most species of the genus *Lessonia* (Nancy et al., 2001).

Preliminary Characterization of LNPs

The MWs of LNP, LNP-1, and LNP-2 were 45.4, 63.9, and 40.2 kDa, respectively (**Supplementary Figure S1**). The three polysaccharides differed in the amount of carbohydrate, protein, uronic acid, and sulfate content (**Table 1**), with LNP showing a significantly lower carbohydrate content (67.4%) than either LNP-1 (87.3%) or LNP-2 (84.0%). This result indicates that impurities were removed from crude polysaccharides. Protein contents of the three polysaccharides were similarly low. Total uronic acid content was lower in LNP-1 (16.4%) and LNP-2 (20.1%) than in LNP (22.1%). In comparison, uronic acid content was slightly higher than that of the polysaccharides extracted with diluted HCl from *Lessonia* sp. (Phaeophyceae) (14.7%) (Leal et al., 2018). Sulfate content in LNP, LNP-1, and LNP-2 was 33.7, 36.9, and 40.5%, respectively. Similarly, extractions from *L. vadosa* with 2% aqueous $CaCl_2$ produced fucoidan with 37.7% sulfate (Nancy et al., 2004).

The three polysaccharide fractions had different monosaccharide profiles (**Table 1**). Both LNP and LNP-2 contained mannose, glucuronic acid, fucose, xylose, galactose, and glucose. However, LNP contained more galactose, while LNP-2 contained more glucose. In contrast, LNP-1 was mainly composed of glucose, glucuronic acid, fucose, and xylose, with very little mannose. Notably, glucose content was highest in LNP-1. Previous studies on *Lessonia* sp. (Phaeophyceae) found that extracted polysaccharides were mainly fucose, with trace amounts of galactose and xylose (Leal et al., 2018).

Typical carbohydrate absorptions at 4000–500 cm^{-1} were observed in all three polysaccharides (**Figure 2**). Strong peaks around 3350–3380 cm^{-1} were assigned to hydroxyl stretching vibration. Weak peaks around 2930 cm^{-1} and 1420 cm^{-1} were characteristic of –CH stretching vibration and $–CH_2$ bending vibration, respectively. Additionally, the strong extensive absorption around 1100–1000 cm^{-1} was due to C–O–C and C–OH stretching vibration. Bands around 1600 cm^{-1}, corresponding to C–O double-bond asymmetric stretching vibration, were assigned to uronic acid carbonyl groups. A major peak at 1248–1300 cm^{-1} was attributed to the asymmetric stretching of S=O and suggested the presence of an ester sulfate (Yuan and Macquarrie, 2015). Previous studies have reported that sulfate groups at equatorial C-2 and C-3 positions

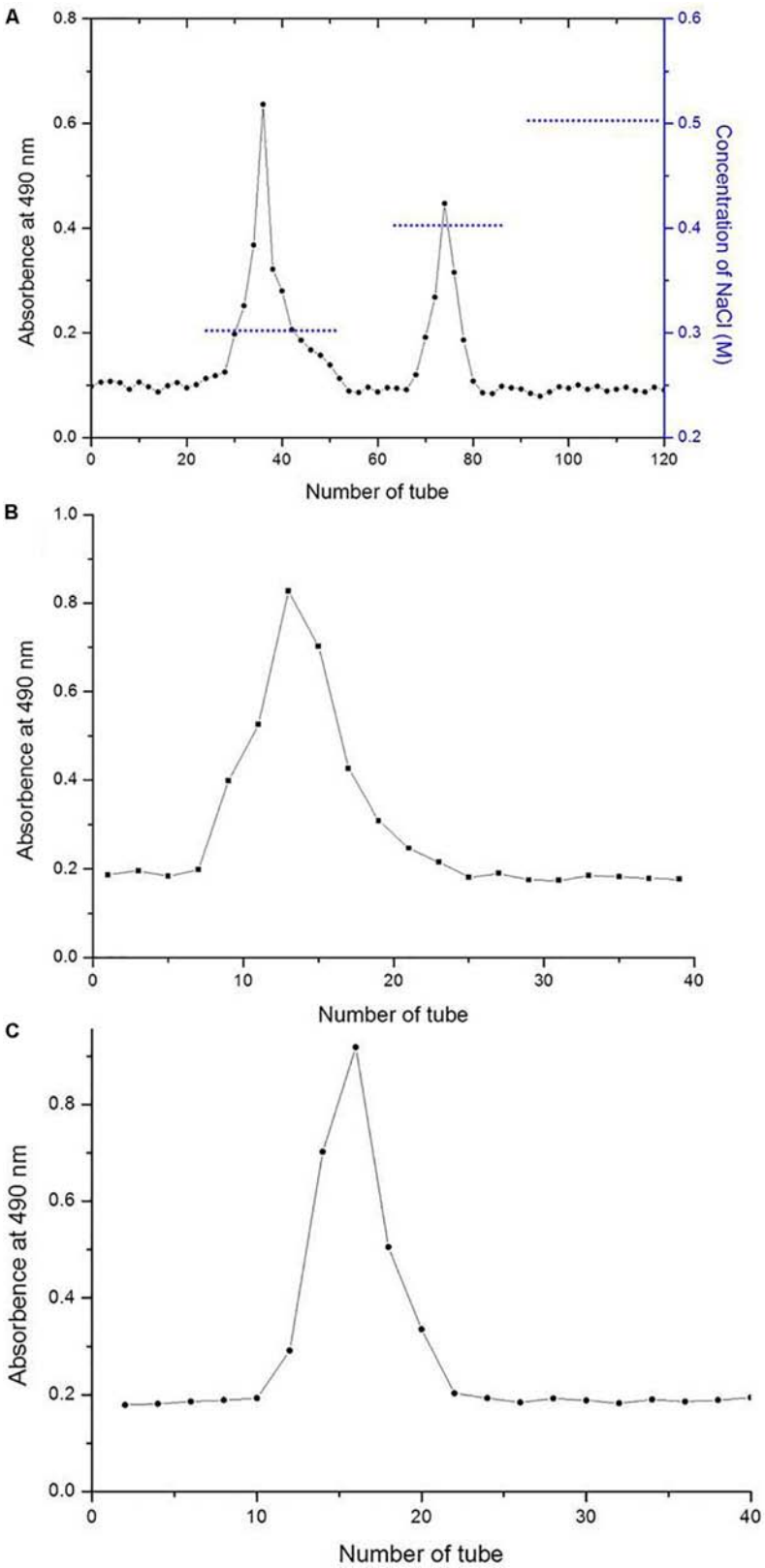


FIGURE 1 | Elution profiles of *Lessonia nigrescens* polysaccharide (LNP) fractions on a DEAE-cellulose column **(A)** and Sephadex G-100 gel chromatography column (**B** and **C**).

TABLE 1 | Preliminary characterization of crude LNP, LNP-1, and LNP-2.

Item	LNP	LNP-0.3	LNP-0.4
Molecular weight (kDa)	45.4	63.9	40.2
Carbohydrate (%)	67.4	87.3	84.0
Protein (%)	6.7	7.0	6.9
Uronic acid (%)	22.1	16.4	20.1
Sulfate	33.7	36.9	40.5
Sugar components (%)			
Mannose	0.24	0.05	0.29
Glucuronic acid	0.22	0.15	0.21
Galacturonic acid	0.02	0.06	0.03
Glucose	0.05	0.33	0.11
Galactose	0.13	0.09	0.06
Xylose	0.13	0.17	0.13
Fucose	0.21	0.15	0.17

produced bands around 820 cm^{-1} (Foley et al., 2011), and there is such a shoulder in the three polysaccharides at 815–820 cm^{-1}. Absorption peaks at 876–891 cm^{-1} indicated the presence of primarily β-glycosidic linkages (Ye et al., 2016).

Effect of Polysaccharides on Wheat Seedlings Under Salt Stress

Plant Growth and Biomass Accumulation

Under NaCl stress, the shoot length, root length, FW, and DW of wheat seedlings were all significantly lower than in control (**Table 2**). In contrast, all of these parameters increased due to treatment with LNP, LNP-1, and LNP-2.

Compared to the NaCl stress treatment, exogenous application of LNP, LNP-1, and LNP-2 increased wheat seedling shoot lengths by 6.1, 4.7, and 12.0%, respectively. Treatment with LNP, LNP-1, and LNP-2 significantly increased wheat seedling root length by 33.5, 29.0, and 37.3%, respectively, compared to the negative control. No statistically significant differences of wheat seedling shoot length were observed among polysaccharide treatments. The three polysaccharide treatments also increased the fresh and DWs of wheat seedlings under NaCl stress. LNP, LNP-1, and LNP-2 increased wheat seedling FWs by 18.1, 8.1, and 17.7% and their DWs by 25.0, 11.8, and 30.9%, respectively, compared to the NaCl stress treatment. The fresh and dry seedling weight in the LNP and LNP-2 groups were significantly higher than those in the LNP-1 group. These results suggest that the application of any of the LNPs can improve wheat seedling growth parameters (**Supplementary Figure S2**). Of the three applied polysaccharides, LNP and LNP-2 were more effective than LNP-1 for improving the growth parameters of plants under salt stress.

Lipid Peroxidation

Increased soil salinity can cause cell membrane impairment in plants by elevating the amount of ROS. A significant increase was observed in MDA content in wheat seedling leaves under salt stress (**Figure 3A**), and this oxidative damage can be alleviated by exogenous application of LNPs. LNP, LNP-1, and LNP-2 reduced the MDA content to 45.1, 36.2, and 52.4% compared to NaCl-stressed plants. Similarly, the REL significantly increased

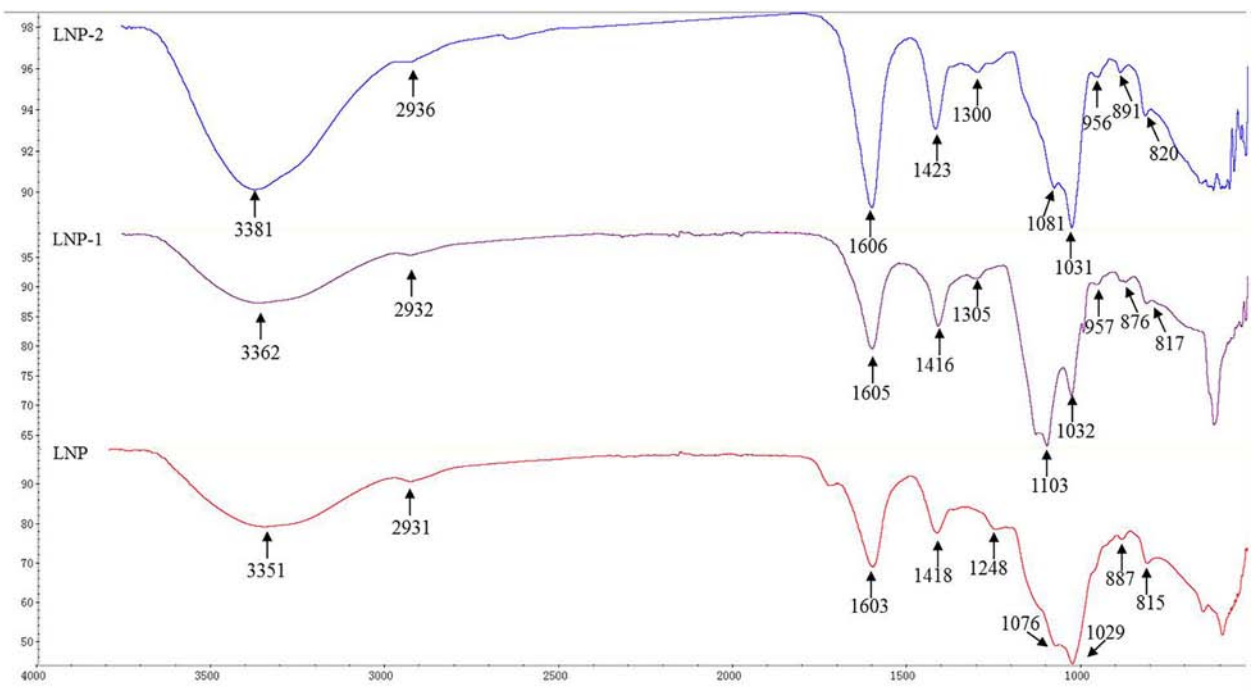


FIGURE 2 | FT-IR spectra of LNP, LNP-1, and LNP-2.

TABLE 2 | Effects of LNP, LNP-1, and LNP-2 on growth parameters of wheat seedlings.

	Shoot length (cm)	Root length (cm)	Fresh weight (g)	Dry weight (g)
control	28.6 ± 1.8^{a}	19.8 ± 4.2^{b}	0.89 ± 0.11^{a}	0.096 ± 0.014^{bc}
NaCl stress	20.8 ± 1.6^{d}	17.0 ± 3.7^{c}	0.74 ± 0.11^{c}	0.088 ± 0.011^{c}
LNP+NaCl stress	22.0 ± 1.5^{c}	22.7 ± 3.4^{a}	0.87 ± 0.11^{a}	0.110 ± 0.012^{a}
LNP-1+NaCl stress	21.8 ± 1.3^{c}	21.9 ± 4.0^{ab}	0.80 ± 0.09^{b}	0.098 ± 0.018^{b}
LNP-2+NaCl stress	23.3 ± 1.3^{b}	23.3 ± 4.0^{a}	0.87 ± 0.11^{a}	0.115 ± 0.017^{a}

Values are mean ± SD of 30 replicates. Different letters indicate significant differences at $P < 0.05$.

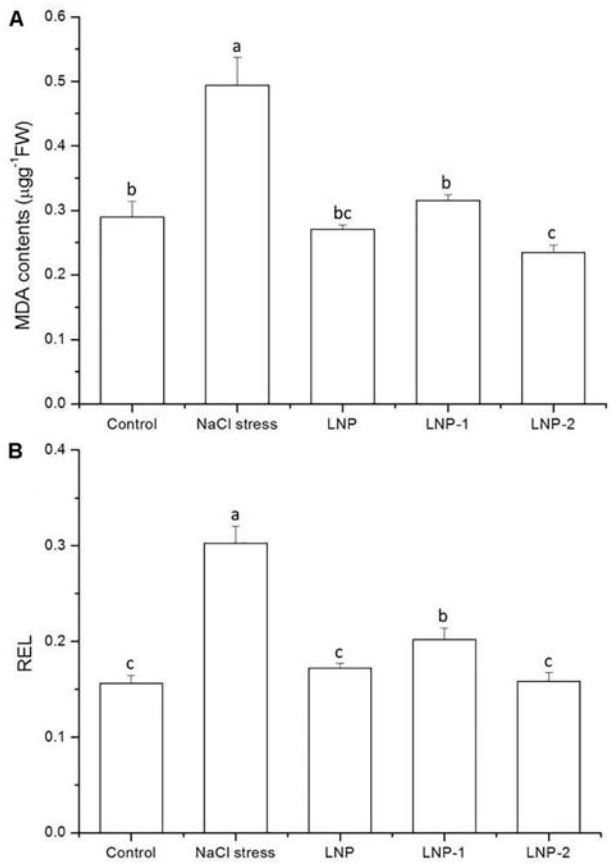


FIGURE 3 | Effect of LNP, LNP-1, and LNP-2 on MDA content **(A)** and REL **(B)** in leaves of wheat seedlings. Values are the mean ± SD of three replicates. Different letters indicate significant differences at $P < 0.05$. MDA, malondialdehyde; REL, REL.

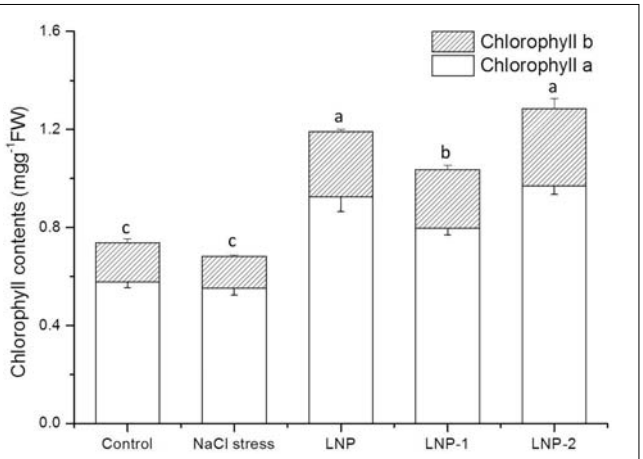


FIGURE 4 | Effect of LNP, LNP-1, and LNP-2 on chlorophyll content in wheat seedlings. Values are the mean ± SD of three replicates. Different letters indicate significant differences at $P < 0.05$.

by 94.2% due to NaCl stress (**Figure 3B**). LNP, LNP-1, and LNP-2 significantly reduced the REL to 43.1, 33.4, and 47.7% compared to the NaCl-treated group. Moreover, LNP-2 significantly reduced the MDA content and REL in the seedlings compared to NaCl-stressed plants more than the LNP-1.

Chlorophyll Content

When plants were exposed to stress, chlorophyll contents tended to decrease significantly. Therefore, chlorophyll content is widely used as an index of abiotic stress tolerance in plants. In the present study, Chl-a and Chl-b content decreased under NaCl stress (**Figure 4**). LNPs alleviated chlorophyll decline in wheat seedlings under salt stress. The Chl-a content of plants treated with LNP, LNP-1, and LNP-2 was significantly greater at 67.9, 44.7, and 75.9%, respectively than Chl-a of salt-stressed plants (**Figure 4**). Similarly, the Chl-b content of LNP, LNP-1, and LNP-2 was markedly higher (103.7, 81.9, and 141.1%, respectively), than in salt-stressed seedlings. Moreover, crude LNP and LNP-2 increased chlorophyll content much more than LNP-1 compared to NaCl stressed plants.

Soluble Sugar and Proline Content

In the present study, soluble sugar content increased significantly in wheat seedlings under salt stress compared to the control (**Figure 5A**). In wheat seedlings treated with LNP, LNP-1, and LNP-2, soluble sugar content increased by 25.2, 17.7, and 35.3%, respectively, compared with salt-stressed plants. Moreover, LNP-2 induced soluble sugar production more strongly than the other treatments. The fact that the soluble sugar content increased in wheat seedlings treated with different LNPs suggests that soluble sugars can help maintain osmotic balance and stabilize cell membranes in plants.

As shown in **Figure 5B**, in response to NaCl treatment, the proline content in wheat seedling leaves increased significantly by 22.0% relative to the control. There was a significant increase in the proline content of wheat seedlings treated with LNPs. In salt-stressed plants, LNP, LNP-1, and LNP-2

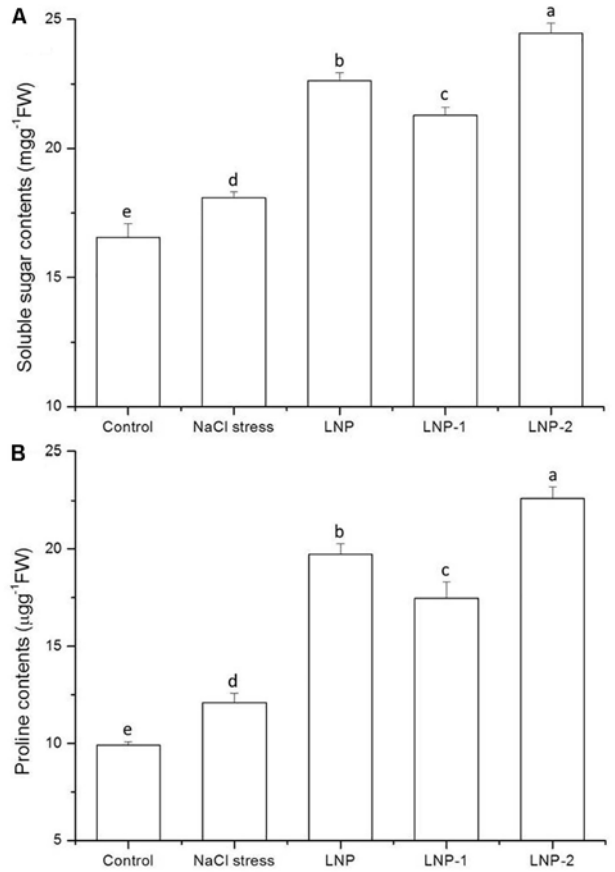


FIGURE 5 | Effect of LNP, LNP-1, and LNP-2 on soluble sugar **(A)** and proline content **(B)** in wheat seedlings. Values are the mean ± SD of three replicates. Different letters indicate significant differences at $P < 0.05$.

significantly increased the proline content by 63.4, 44.6, and 87.1%, respectively, compared with the NaCl-stressed group. The results showed that LNP-2 promoted proline production and accumulation more strongly than the other treatments.

Antioxidant Enzymes Activities

Soluble protein content significantly increased under salt stress (**Figure 6**). In wheat seedlings treated with LNP, LNP-1, and LNP-2, the soluble protein content increased by 9.7, 5.0, and 9.9%, respectively, compared to the negative control (**Figure 6A**). However, no significant difference in soluble protein content was observed between the NaCl-stressed group and the LNP-1 treatment group. In the present study, treatment with the various LNPs increased SOD activity. LNP, LNP-1, and LNP-2 significantly increased the SOD activities by 84.2, 67.9, and 96.0%, respectively, compared to the salt-stressed plants (**Figure 6B**). Similarly, LNP and LNP-2 increased the POD activities by 24.0 and 43.9%, respectively, relative to the NaCl-stressed plants (**Figure 6C**). However, there was no significant difference between the NaCl-stressed and the LNP-1-treated group. The CAT activity was only improved by treatment with LNP-2 (**Figure 6D**). This result suggested that antioxidant enzyme activities in the LNP-2 treatment were significantly higher than those in the other groups.

Na^+ and K^+ Accumulation in Different Tissues of Wheat Seedlings

The results showed that Na^+ content in different tissues of wheat seedlings increased significantly under salt stress. In the root, sheath, and leaf, the increase of Na^+ content in salt-stressed plants was 48.9, 105.6, and 49.7-times of that of the control (**Figure 7A**), respectively. The K^+ content in the root and leaf of salt-stressed plants increased by 146.3 and 37.4%, respectively. However, there was a reverse trend in K^+ content in the sheath of wheat seedlings (**Figure 7B**). The K^+ content in the salt-stressed group was slightly lower than that in the control group, but no significant difference was observed. In wheat seedlings treated with LNPs, Na^+ content was lower than that of salt-stressed plants, but was still higher than that of the control group. The results showed that Na^+ accumulated in different tissues under salt stress, especially in roots. In wheat seedlings treated with LNPs, Na^+ content decreased by 21.6, 19.4, and 25.6% in roots; 40.8, 38.2, and 50.0% in sheaths; and 51.3, 39.6, and 59.1% in leaves, respectively. However, there was no significant difference in the Na^+ content among the LNP groups. In contrast, LNP application increased the K^+ content in roots, sheaths, and leaves compared with salt-stressed plants. Consequently, a lower Na^+/K^+ ratio was observed in the roots, sheaths, and leaves of LNP-treated plants (**Figure 7C**).

Expression of Genes Encoding the Na^+/K^+ Transporter

Compared to the control, salt stress induced higher transcript levels of *TaHKT2;1*. Treatment with LNPs significantly down-regulated the expression of *TaHKT2;1* in the roots, sheaths, and leaves (**Figure 8A**). In contrast, the *TaNHX2* expression was obviously down-regulated in roots of the salt-stressed plants, but there was no significant difference in sheaths and leaves (**Figure 8B**). After LNP application, the *TaNHX2* expression was down-regulated in roots, but up-regulated in sheaths and leaves. Gene expression was 3.1-, 2.9-, and 4.1-fold in sheaths and 4.2-, 3.4-, and 4.8-fold in leaves in the LNP-, LNP-1-, and LNP-2-treated plants, respectively, compared to the salt-stressed plants. In plants treated with LNP, LNP-1, and LNP-2, the expression of *TaSOS1* was up-regulated by 31.1, 29.6, and 64.6% in roots; 66.4, 40.8, and 138.2% in sheaths; and 49.7, 31.4, and 152.7% in leaves, respectively. Moreover, the transcript levels of *TaSOS1* in plants treated with LNP-2 were significantly higher than those in the other groups (**Figure 8C**).

DISCUSSION

Salt stress restricts the growth and development of plants by influencing physiological and biochemical processes, such as osmotic pressure, superoxide ion homeostasis, and antioxidant responses. The seaweed extracts are used in agriculture due to their ability to regulate plant resistance responses to different environmental stresses. Our study demonstrated that exogenous

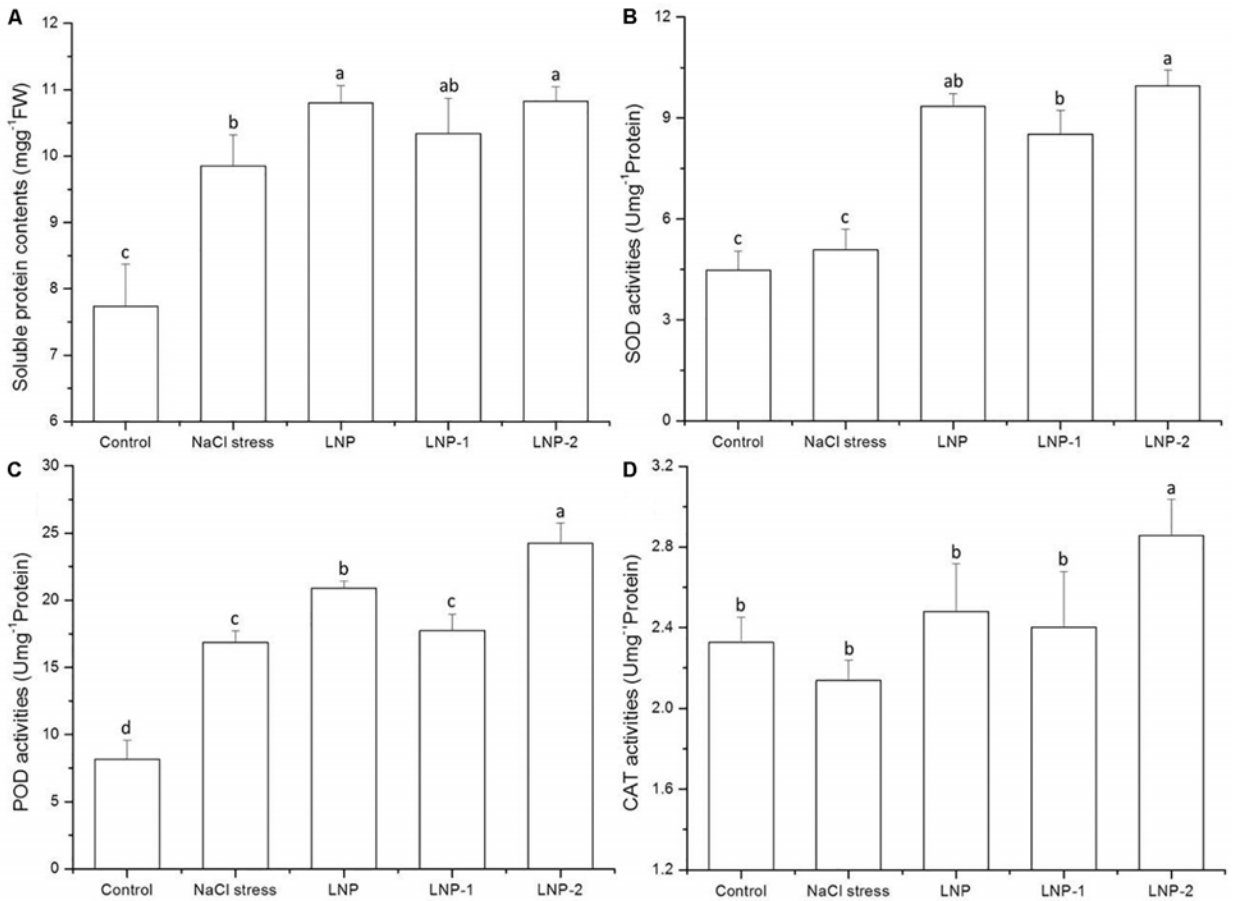


FIGURE 6 | Effect of LNP, LNP-1, and LNP-2 on soluble protein contents **(A)**, SOD **(B)**, POD **(C)**, and CAT **(D)** activities in wheat seedlings. Values are the mean ± SD of three replicates. Different letters indicate significant differences at $P < 0.05$. SOD, superoxide dismutase; POD, peroxidase activity; CAT, catalase.

supply of polysaccharides from *L. nigrescens* could alleviate the adverse effects of salt stress on the growth of wheat seedlings through improving antioxidant activities and regulating the efflux and compartmentation of intracellular ions.

The observed significant reductions in fresh and DW indicated that salt stress inhibited growth and caused damage to wheat seedlings. Salinity reduced root length, fresh and DW in agreement with earlier studies (Annunziata et al., 2017). Reduction in root extension rates might come from the marked lowering of root turgor and water potential (Rodriguez et al., 1997). The application of the LNPs could improve the wheat seedling growth parameters (**Table 2**). However, its mechanism is highly complex and needs to be further discussed.

Malondialdehyde, a marker of lipid peroxidation and cell damage, was significantly increased under salt stress treatments, but low levels of MDA were found in LNPs treated plants (**Figure 3A**). The results of the present study are similar to the previous findings that exogenous polysaccharides from *Pyropia yezoensis* could scavenge free radicals and prevent salt-stress-related lipid peroxidation (Bose et al., 2014).

Abiotic stresses seriously affect photosynthesis in plants, such as reduction in chlorophyll content, disintegration of chloroplast membranes, and disruption of photosystem biochemical reactions. The present study demonstrated that NaCl stress significantly decreased chlorophyll content in wheat seedlings (**Figure 4**). Degradation of chlorophyll under abiotic stress is usually related to the accumulation of ROS, which leads to lipid peroxidation of chloroplast membranes. In the current study, salt stress increased the MDA content of wheat seedlings, indicating that membrane lipids were damaged by ROS generation caused by salt stress. However, the MDA levels in LNPs-treated wheat seedling leaves declined compared to those of the NaCl-stressed group. In the present study, improvement in the growth of plants exposed to NaCl stress was determined by the increases in chlorophyll content. It was reported that the seaweed extract enhanced plant chlorophyll content by inducing its synthesis (Khan et al., 2009). The LNPs significantly increased chlorophyll levels in plants under NaCl stress. The results indicated that LNP treatment reduced lipid peroxidation and mitigated the salt-induced decline in chlorophyll content.

Proteins and other macromolecules rapidly decompose due to abiotic stresses. In the process, plant cell membranes are damaged and imbalances in osmotic pressure occur. Sugars act as an osmotic regulator and reduce membrane permeability,

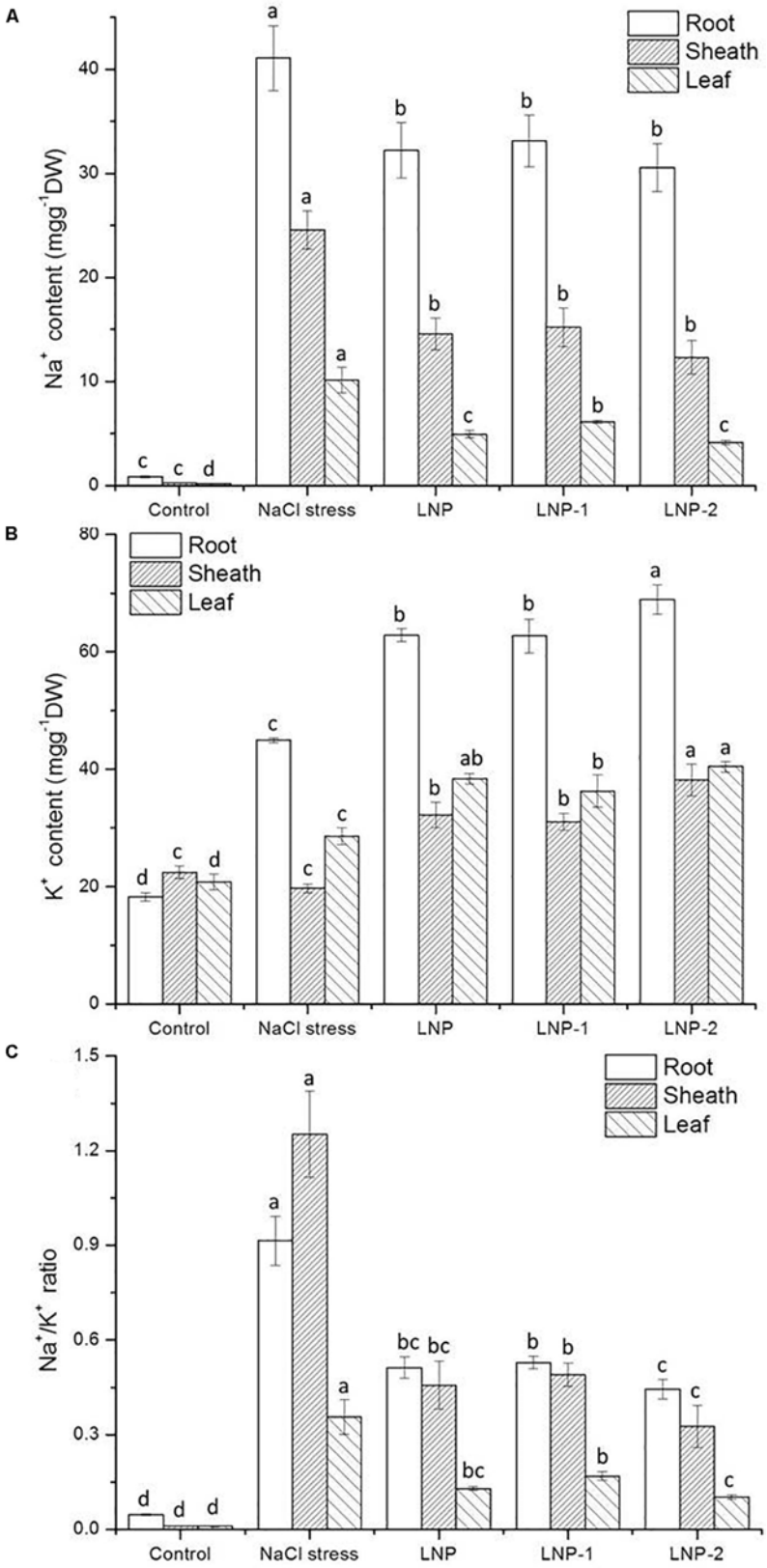


FIGURE 7 | Effect of LNP, LNP-1, and LNP-2 on Na^+ **(A)**, K^+ contents **(B)**, and Na^+/K^+ ratio **(C)** of root, sheath, and leaf in wheat seedlings. Values are the mean ± SD of three replicates. Different letters indicate significant differences at $P < 0.05$.

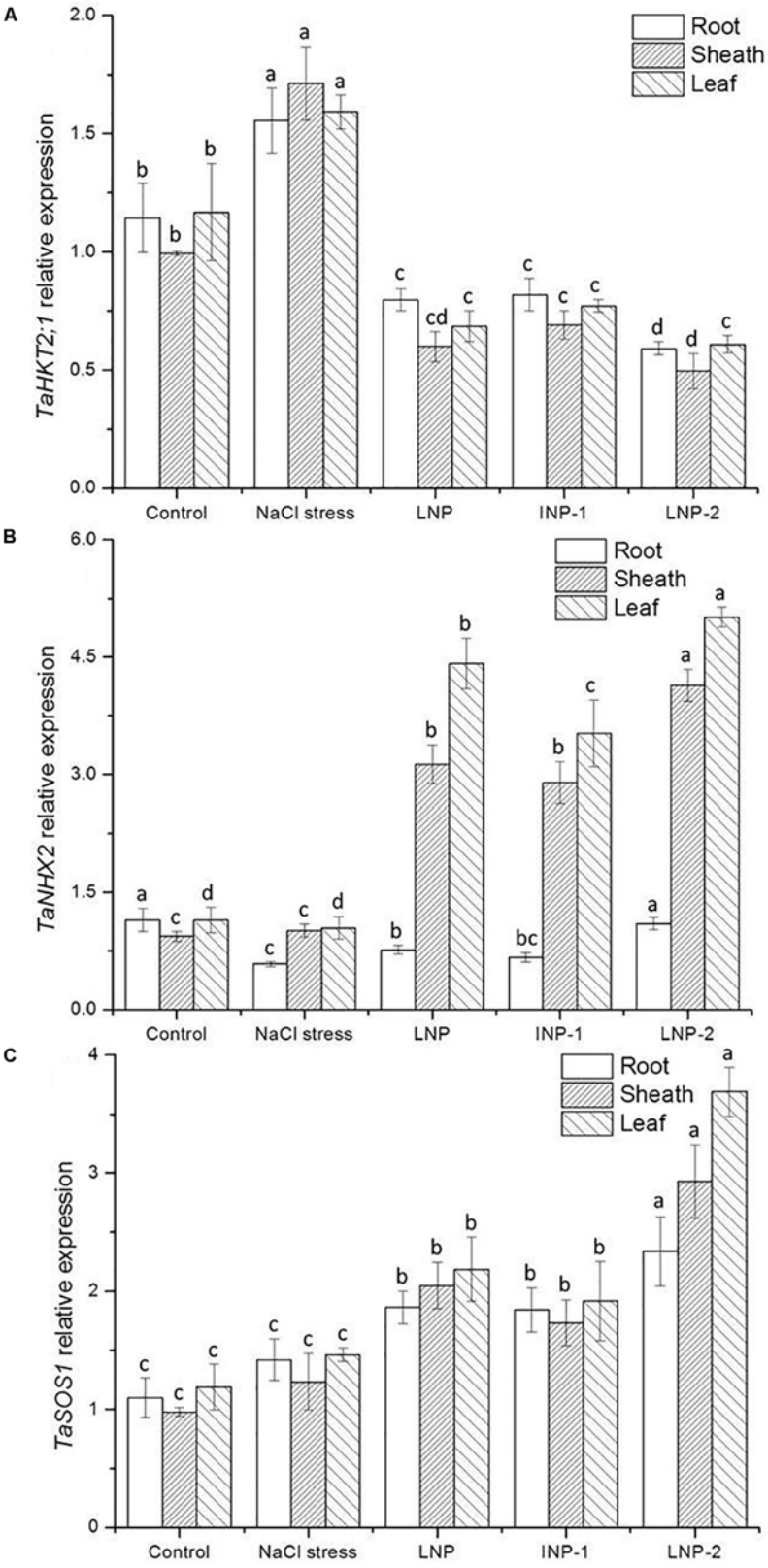


FIGURE 8 | Effect of LNP, LNP-1, and LNP-2 on *TaHKT2;1* **(A)**, *TaNHX2* **(B)**, and *TaSOS1* **(C)** expression of root, sheath, and leaf in wheat seedlings. Values are the mean ± SD of three replicates. Different letters indicate significant differences at $P < 0.05$.

specifically by reducing water potential in cells and stabilizing cell membranes. Under abiotic stress, the contents of compatible osmolytes such as proline, betaine, and free amino acids increased in the cytoplasm to prevent cytoplasm dehydration caused by salt stress. Proline not only acts as an osmotic protector under abiotic stress but also regulates osmotic potential, stabilizes cell structure, and reduces damage to the photosynthetic apparatus. Zhang et al. (2017) pinpoint that the proline metabolic pathway showed significant differences in response to salt stress. Proline induces the expression of salt stress-responsive genes, which promotes the adaptation of plants to salt stress. Proline may have a protective effect as it can induce antioxidant enzymes to scavenge ROS. Indeed, application of exogenous proline improved antioxidant activities, reduced the oxidative damage, and lipid peroxidation levels (Hasanuzzaman et al., 2014).

In this study, high proline content indicated that salt stress-induced proline biosynthesis in plants helped to ameliorate the osmotic imbalance. LNPs further increased proline content in plants under salt stress (**Figure 5B**). The proline content of the LNP-2 treatment plants was considerably higher than that of the other groups, indicating stronger osmotic regulation ability in the LNP-2-treated plants.

Salt stress leads to crop yield loss because of imbalances in mineral nutrient concentrations and osmotic effects, triggering excess ROS production (Mittler et al., 2004). Under normal physiological conditions, ROS are constantly produced by aerobic metabolism in chloroplasts, mitochondria, and peroxisomes. However, ROS overproduction as a consequence of stress exposure can lead to oxidative damage to lipids, proteins, nucleic acids, and cause membrane dysfunction and cell death (Mittler et al., 2004).

It has been reported that regulation of ROS generation in plants may effectively protect against oxidative damage and increase stress tolerance (Bose et al., 2014). An antioxidant system consisting of a variety of enzymes and small molecules protects plants from oxidative damage. Antioxidant enzymatic activities and accumulation of antioxidants play an important role in the inhibition of membrane protein and lipid peroxidation. Many studies have shown that SOD, POD, and CAT are closely related to salt resistance in plants (Qiu et al., 2014). SOD detoxifies $O_2\bullet$ radicals by forming H_2O_2, which is also toxic and must be eliminated by the concerted actions of CAT and POD.

In wheat, several studies showed that wheat plants alter the activity of antioxidant enzymes such as SOD, CAT, APX, POX, and GR under different abiotic stresses attempt to defend against oxidative damage (Caverzan et al., 2016). Most antioxidant enzymes increased their activity in response to the major abiotic stresses faced by wheat plants. For example, in wheat, an increase in the SOD transcript was observed under different heat shock treatment (Kumar et al., 2013). This is consistent with the results of the present study, since SOD and POD activities were significantly higher in salt-stressed plants than in the control. Plants treated with LNPs under NaCl stress also showed relative increases in SOD, POD, and CAT activities. The activation of these enzymes leads to wheat seedlings protection against oxidative damage. These results indicate that LNPs effectively induce ROS scavenging in wheat seedlings by modulating their antioxidant enzyme activities. Therefore, LNPs may enhance defense responses in plants under salt stress. In general, LNP-2 was significantly more effective at inducing antioxidant activity and ROS scavenging than the other groups.

In the present study, increased proline content and decreased MDA in plants after LNP pre-treatment was consistent with the increase of the antioxidant enzyme indicating that the pre-treatment with LNP can induce proline, regulates the enzymes, and decreases lipid peroxidation, thus reducing ROS directly and protects plants from salt stress.

The Na^+ content in all wheat tissues of control group was negligible, and treatment with 150 mM NaCl for 10 days resulted in a large amount of Na^+ accumulated in roots. Under salt stress, excess Na^+ can lead to an imbalance in cellular Na^+ and K^+ homeostasis, which play an important role in the growth and development of higher plants (Zhang et al., 2018). Sodium within the plant has a devastating effect on the metabolism of cytoplasm and organelles, because it tends to replace potassium in key enzymatic reactions. Restricting the transport and accumulation of Na^+ in leaves is the most important adaptation of plant to salt stress (Mekawy et al., 2015). Wheat is a classical "salt excluder," characterized by low rates of Na^+ transport to the shoot, thus keeping mesophyll cells as Na^+-free as possible (Munns and James, 2003; Colmer et al., 2005; James et al., 2006). In this study, the Na^+ content was significantly lower than that in roots, indicating that plants restricted the transport of Na^+ from roots to leaves. Moreover, the Na^+ content of leaves treated with exogenous LNPs was significantly lower compared to that of salt-stressed plants. Wheat seedlings treated with LNPs selectively excluded Na^+ from leaves, which are the basis tissue of photosynthesis (Lekshmy et al., 2015). Wheat seedlings treated with LNP and LNP-2 exhibited lower leaf Na^+ content and thus should be better adapted to salt stress.

However, Na^+ exclusion is not always sufficient to improve plant salt tolerance. In addition to low Na^+ transport rates to the shoots, a high selectivity for K^+ also plays an important role in salt tolerance in wheat. K^+ is one of the most abundant macronutrients in plants and is essential for maintaining membrane potential and swelling pressure, activating enzymes, regulating osmotic pressure, stomatal movement, and orientation (Laurie et al., 2002). Maintenance of a high cytosolic K^+:Na^+ ratio is a key feature of plant salt tolerance (Cuin et al., 2008). Under salt stress conditions, plants are affected by K^+ accumulation due to the competitive inhibition of Na^+ uptake, which often leads to a high Na^+/K^+ ratio that disrupts the intracellular balance (Mekawy et al., 2015). In this study, the cytosolic Na^+:K^+ ratio rose dramatically under salt stress due to excessive Na^+ accumulation in the cytosol. However, K^+ accumulation in LNP-treated plants was accompanied by a lower Na^+/K^+ ratio, thus increased salt tolerance. Moreover, the Na^+/K^+ ratio in LNP-2-treated plants was lower than that of other groups but without obvious difference with the LNP group. The transmembrane transport of Na^+ and K^+ in plants is mediated by several types of transporters and/or channels (Yao et al., 2010), some of which are closely related to

Na^+ exclusion in leaves, including high-affinity K^+ transporters (HKTs). In bread wheat, *TaHKT2;1* has been confirmed and is assumed to function in Na^+ uptake from the soil (Ariyarathna et al., 2014). Kumar et al. (2017) previously studied the expression of *TaHKT2;1* in contrasting wheat genotypes. The results showed that the transcript levels of *TaHKT2;1* were up-regulated in the shoots of the salt-sensitive genotype, but down-regulated in the salt-tolerant genotype. Furthermore, under salt stress an increase in cytosine methylation down-regulated *TaHKT 2;1* and *TaHKT2;3* expression in salt-tolerant wheat, thereby improving salt tolerance. This indicated that its inhibition of gene expression was associated with salt tolerance. In the present study, salt stress up-regulated the expression of the *TaHKT2;1* gene, while LNPs significantly down-regulated the expression of *TaHKT2;1* in roots. These results suggest that *TaHKT2;1* inhibits the absorption of Na^+ from the soil and thus decreases Na^+ content and Na^+/K^+ ratio in leaves.

In addition to limiting Na^+ entry into cells, salt tolerance mechanisms of plants also include Na^+ exclusion and compartmentalization of Na^+ into vacuoles to prevent Na^+ accumulation (Yamaguchi et al., 2013). The Salt Overly Sensitive 1 (*SOS1*) antiporter is a plasma membrane Na^+/K^+ antiporter that mediates the efflux of cytosolic Na^+ in roots and regulates the transportation of Na^+ from root to shoot to maintain appropriate K^+/Na^+ ratio in leaves (Qiu et al., 2002). NHX, an Na^+/K^+ antiporter localized in vacuolar membranes, can mediate Na^+ regionalization into vacuoles (Gaxiola et al., 2001). Under 200 mM NaCl concentration, *SOS1* and *NHX1* expression in salt-tolerant wheat genotypes increased significantly, thereby improving Na^+ exclusion and lowering the Na^+/K^+ ratio (Lekshmy et al., 2015). In this study, *TaSOS1* and *TaNHX2* overexpression were significantly up-regulated in wheat seedlings treated with LNPs. These seedlings showed a considerably higher resistance to high NaCl concentrations. In general, *TaSOS1* and *TaNHX2* transcripts in plants treated with LNP-2 were significantly higher than those of the other groups. These results indicated that LNP-2 effectively supported the efflux and regionalization of Na^+, thus alleviating salt stress damage.

Although there have been many reports that algae extracts contain biological stimulants and protective agents, due to the diversity and complexity of these extracts, it is difficult to determine the active compounds. The effects of biostimulants have generally been attributed to the presence of phytohormones, organic molecules, phenolic compounds, amino acids, and bioactive secondary metabolites (Stasio et al., 2018). Apart from these compounds, polysaccharides may also contribute to the observed beneficial effects. The present study proved that polysaccharides derived from *L. nigrescens* and its purified fractions can improve the resistance of plants to salt stress.

We found that polysaccharide activity mainly depends on MW and sulfate content. This conclusion is in line with the results of previous studies showing that both properties determine the biological activities of fucoidan from the brown seaweed *Adenocystis utricularis* (Ponce et al., 2003). However, Zha et al. (2016) stated that a low-MW polysaccharide isolated from *Laminaria japonica* has effective scavenging activities on ROS *in vitro*. This is in agreement with the results of Qi et al. (2006), who demonstrated that the low-MW polysaccharide fraction prepared from *Ulva pertusa* had stronger reducing power due to the higher number of reducing and non-reducing ends. Sangha et al. (2010) stated that the efficiency of inductive activity depended on the species and its sulfate degree of carrageenan. The pre-treatment of λ-carrageenan with high sulfation induced resistance to *S. sclerotiorum* resulting in less foliar damage while the ι-carrageenan with low sulfation increased the severity of the disease. In the current study, LNP-2 (40.2 kDa) treatment was more effective at inducing salt resistance than the LNP (45.4 kDa) and LNP-1 (63.9 kDa) treatments. This result may suggest a relationship between salt resistance and polysaccharide MW and the amount of sulfates. Specifically, salt resistance capacity seemed to be higher with lower MW and higher sulfate content. The presence of LNP-2 in LNP may explain the higher salt-resistance in the LNP treatment than in the LNP-1 treatment.

CONCLUSION

In the present study, polysaccharides extracted from *L. nigrescens* (LNP) have similar characteristics to species of the genus *Lessonia*. Crude polysaccharides were further separated and fractionated and two acidic polysaccharides (LNP-1 and LNP-2) from crude polysaccharide LNP were obtained. Moreover, the salt-resistance activity of plants induced by the three polysaccharides was studied. The results showed that all of the three polysaccharides could induce plant's resistance to salt stress, and LNP-2 showed more effective plant salt-resistance activity than the other groups. It can be concluded from this work that both the MW and the sulfate degree contribute to the salt-resistance activity of polysaccharides from *L. nigrescens*. The results of this study can make a positive contribution to the cultivation and promotion of crop and utilization of algae resources.

AUTHOR CONTRIBUTIONS

PZ conceived the study, did most of the experimental work, and wrote the manuscript. LM and HZ preparation of polysaccharide. YY chemical analysis of polysaccharide. XL plant growth analyses and study the effect of polysaccharides on wheat seedlings under salt stress. CZ and YL reviewed and edited the manuscript.

FUNDING

This work was supported by the Agricultural Science and Technology Innovation Program (ASTIP-TRIC07), Science Foundation for Young Scholars of Tobacco Research Institute of Chinese Academy of Agricultural Sciences (2016A01), Fundamental Research Funds for Central Non-profit Scientific

Institution (1610232016003), and Funded by Open Foundation of the State Key Laboratory of Bioactive Seaweed Substances (SKL-BASS1715).

ACKNOWLEDGMENTS

We would like to thank Editage (www.editage.cn) for English language editing.

SUPPLEMENTARY MATERIAL

The Supplementary Material for this article can be found online at: https://www.frontiersin.org/articles/10.3389/fpls.2019.00048/full#supplementary-material

FIGURE S1 | The MW of LNP, LNP-1, and LNP-2 measured by HPLC with dRI detector.

FIGURE S2 | Effects of LNP, LNP-1, and LNP-2 on growth of wheat seedlings.

REFERENCES

Annunziata, M. G., Ciarmiello, L. F., Woodrow, P., Maximova, E., Fuggi, A., and Carillo, P. (2017). Durum wheat roots adapt to salinity remodeling the cellular content of nitrogen metabolites and sucrose. *Front. Plant Sci.* 7:2035. doi: 10.3389/fpls.2016.02035

Ariyarathna, H. A., Ul-Haq, T., Colmer, T. D., and Francki, M. G. (2014). Characterization of the multigene family TaHKT 2;1 in bread wheat and the role of gene members in plant Na(+) and K(+) status. *BMC Plant Biol.* 14:159. doi: 10.1186/1471-2229-14-159

Aziz, A., Poinssot, B., Daire, X., Adrian, M., Bézier, A., Lambert, B., et al. (2003). Laminarin elicits defense responses in grapevine and induces protection against *Botrytis cinerea* and *Plasmopara viticola*. *Mol. Plant Microbe Interact.* 16, 1118–1128. doi: 10.1094/MPMI.2003.16.12.1118

Bose, J., Rodrigo-Moreno, A., and Shabala, S. (2014). ROS homeostasis in halophytes in the context of salinity stress tolerance. *J. Exp. Bot.* 65, 1241–1257. doi: 10.1093/jxb/ert430

Bradford, M. M., and Williams, W. L. (1976). New, rapid, sensitive method for protein determination. *Fed. Proc.* 35:274.

Buono, D. D., Ioli, G., Nasini, L., and Proietti, P. (2011). A comparative study on the interference of two herbicides in wheat and italian ryegrass and on their antioxidant activities and detoxification rates. *J. Agric. Food Chem.* 59, 12109–12115. doi: 10.1021/jf2026555

Castro, J., Vera, J., González, A., and Moenne, A. (2011). Oligo-carrageenans stimulate growth by enhancing photosynthesis, basal metabolism, and cell cycle in tobacco plants (var. Burley). *J. Plant Growth Regul.* 31, 173–185. doi: 10.1007/s00344-011-9229-5

Caverzan, A., Casassola, A., and Brammer, S. P. (2016). Antioxidant responses of wheat plants under stress. *Genet. Mol. Biol.* 39, 1–6. doi: 10.1590/1678-4685-GMB-2015-0109

Chen, C. F., Su, C. H., Lai, M. N., and Ng, L. T. (2018). Differences in water soluble non-digestible polysaccharides and anti-inflammatory activities of fruiting bodies from two cultivated *Xylaria nigripes* strains. *Int. J. Biol. Macromol.* 116, 728–734. doi: 10.1016/j.ijbiomac.2018.05.047

Chen, G., Wang, M., Xie, M., Wan, P., Chen, D., Hu, B., et al. (2018). Evaluation of chemical property, cytotoxicity and antioxidant activity *in vitro* and in vivo of polysaccharides from Fuzhuan brick teas. *Int. J. Biol. Macromol.* 116, 120–127. doi: 10.1016/j.ijbiomac.2018.04.184

Chernane, H., Latique, S., Mansori, M., and Kaoua, M. (2015). Salt stress tolerance and antioxidative mechanisms in wheat plants (*Triticum durum* L.) by seaweed extracts application. *IOSRJ Agric. Vet. Sci.* 8, 36–44. doi: 10.9790/2380-08313644

Colmer, T. D., Munns, R., and Flowers, T. J. (2005). Improving salt tolerance of wheat and barley: future prospects. *Aust. J. Exp. Agric.* 45, 1425–1443. doi: 10.1071/EA04162

Cuin, T. A., Betts, S. A., Chalmandrier, R., and Shabala, S. (2008). A root's ability to retain K+ correlates with salt tolerance in wheat. *J. Exp. Bot.* 59, 2697–2706. doi: 10.1093/jxb/ern128

Foley, S. A., Szegezdi, E., Mulloy, B., Samali, A., and Tuohy, M. G. (2011). An unfractionated fucoidan from *Ascophyllum nodosum*: extraction, characterization, and apoptotic effects in vitro. *J. Nat. Prod.* 74, 1851–1861. doi: 10.1021/np200124m

Gaxiola, R. A., Li, J., Undurraga, S., Dang, L. M., Allen, G. J., Alper, S. L., et al. (2001). Drought- and salt-tolerant plants result from overexpression of the AVP1 H+-pump. *Proc. Natl. Acad. Sci. U.S.A.* 98, 11444–11449. doi: 10.1073/pnas.191389398

Gonzalez, A., Beltran, J., Hiriart-Bertrand, L., Flores, V., De Reviers, B., Correa, J. A., et al. (2012). Identification Of cryptic species in the lessonia nigrescens complex (Phaeophyceae. Laminariales)(1). *J. Phycol.* 48, 1153–1165. doi: 10.1111/j.1529-8817.2012.01200.x

Hasanuzzaman, M., Alam, M. M., Rahman, A., Hasanuzzaman, M., Nahar, K., and Fujita, M. (2014). Exogenous proline and glycine betaine mediated upregulation of antioxidant defense and glyoxalase systems provides better protection against salt-induced oxidative stress in two rice (*Oryza sativa* L.) varieties. *Biomed. Res. Int.* 2014:757219. doi: 10.1155/2014/757219

Ibrahim, W. M., Ali, R. M., Hemida, K. A., and Sayed, M. A. (2014). Role of *Ulva lactuca* extract in alleviation of salinity stress on wheat seedlings. *ScientificWorldJournal* 2014:847290. doi: 10.1155/2014/847290

James, R. A., Munns, R., Von Caemmerer, S., Trejo, C., Miller, C., and Condon, T. (2006). Photosynthetic capacity is related to the cellular and subcellular partitioning of Na^+, K^+ and Cl^- in salt-affected barley and durum wheat. *Plant Cell Environ.* 29, 2185–2197. doi: 10.1111/j.1365-3040.2006.01592.x

Jardin, P. D. (2015). Plant biostimulants definition concept main categories and regulation. *Sci. Hortic.* 196, 3–14. doi: 10.1016/j.scienta.2015.09.021

Khan, W., Rayirath, U. P., Subramanian, S., Jithesh, M. N., Rayorath, P., Hodges, D. M., et al. (2009). Seaweed extracts as biostimulants of plant growth and development. *J. Plant Growth Regul.* 28, 386–399. doi: 10.1007/s00344-009-9103-x

Klarzynski, O., Bertrand, P., Joubert, J. M., Yvin, J. C., Kopp, M., Kloareg, B., et al. (2000). Linear beta-1,3 glucans are elicitors of defense responses in tobacco. *Plant Physiol.* 124, 1027–1037. doi: 10.1104/pp.124.3.1027

Kumar, R. R., Sharma, S. K., Goswami, S., Singh, K., Gadpayle, K. A., Singh, G. P., et al. (2013). Transcript profiling and biochemical characterization of mitochondrial superoxide dismutase (mtSOD) in wheat (*Triticum aestivum*) under different exogenous stresses. *Aust. J. Crop Sci.* 7, 414–424.

Kumar, S., Beena, A. S., Awana, M., and Singh, A. (2017). Salt-induced tissue-specific cytosine methylation downregulates expression of HKT genes in contrasting wheat (*Triticum aestivum* L.) genotypes. *DNA Cell Biol.* 36, 283–294. doi: 10.1089/dna.2016.3505

Latique, S., Aymen, E. M., Halima, C., Chérif, H., and Mimoun, K. (2017). Alleviation of salt stress in durum wheat (*Triticum durum* L.) seedlings through the application of liquid seaweed extracts of *Fucus spiralis*. *Commun. Soil Sci. Plant Anal.* 48, 2582–2593. doi: 10.1080/00103624.2017.1416136

Laurie, S., Feeney, K. A., Maathuis, F. J. M., Heard, P. J., Brown, S. J., and Leigh, R. A. (2002). A role for HKT1 in sodium uptake by wheat roots. *Plant J.* 32, 139–149. doi: 10.1046/j.1365-313X.2002.01410.x

Leal, D., Mansilla, A., Matsuhiro, B., Moncada-Basualto, M., Lapier, M., Maya, I. D., et al. (2018). Chemical structure and biological properties of sulfated fucan from the sequential extraction of subAntarctic *Lessonia* sp (Phaeophyceae). *Carbohydr. Polym.* 199, 304–313. doi: 10.1016/j.carbpol.2018.07.012

Lekshmy, S., Sairam, R. K., Chinnusamy, V., and Jha, S. K. (2015). Differential transcript abundance of salt overly sensitive (SOS) pathway genes is a determinant of salinity stress tolerance of wheat. *Acta Physiol. Plant.* 37, 169–179. doi: 10.1007/s11738-015-1910-z

Li, H., Chang, J., Chen, H., Wang, Z., Gu, X., Wei, C., et al. (2017). Exogenous melatonin confers salt stress tolerance to watermelon by improving photosynthesis and redox homeostasis. *Front. Plant Sci.* 8:295. doi: 10.3389/fpls.2017.00295

Ma, X., Zheng, J., Zhang, X., Hu, Q., and Qian, R. (2017). Salicylic acid alleviates the adverse effects of salt stress on *Dianthus superbus* (Caryophyllaceae) by activating photosynthesis, protecting morphological structure, and enhancing the antioxidant system. *Front. Plant Sci.* 8:600. doi: 10.3389/fpls.2017.00600

Marta, B., Szafranska, K., and Posmyk, M. M. (2016). Exogenous melatonin improves antioxidant defense in cucumber seeds (*Cucumis sativus* L.) Germinated under Chilling Stress. *Front. Plant Sci.* 7:575. doi: 10.3389/fpls.2016.00575

Mekawy, A. M., Assaha, D. V., Yahagi, H., Tada, Y., Ueda, A., and Saneoka, H. (2015). Growth, physiological adaptation, and gene expression analysis of two Egyptian rice cultivars under salt stress. *Plant Physiol. Biochem.* 87, 17–25. doi: 10.1016/j.plaphy.2014.12.007

Misra, N., and Saxena, P. (2009). Effect of salicylic acid on proline metabolism in lentil grown under salinity stress. *Plant Sci.* 177, 181–189. doi: 10.1016/j.plantsci.2009.05.007

Mittler, R., Vanderauwera, S., Gollery, M., and Van Breusegem, F. (2004). Reactive oxygen gene network of plants. *Trends Plant Sci.* 9, 490–498. doi: 10.1016/j.tplants.2004.08.009

Munns, R., and James, R. A. (2003). Screening methods for salinity tolerance: a case study with tetraploid wheat. *Plant Soil* 253, 201–218. doi: 10.1023/a:1024553303144

Munns, R., and Tester, M. (2008). Mechanisms of salinity tolerance. *Annu. Rev. Plant Biol.* 59, 651–681. doi: 10.1146/annurev.arplant.59.032607.092911

Nancy, P. C., Betty, M., Enrique, M., and Alejandra, M. (2004). Alginic acids in Lessonia vadosa: partial hydrolysis and elicitor properties of the polymannuronic acid fraction. *J. Appl. Phycol.* 16, 127–133. doi: 10.1023/B:JAPH.0000044778.44193.a8

Nancy, P. C., Betty, M., Enrique, M., and Vásquez, A. E. (2001). Alginic acids in Lessonia trabeculata- characterization by formic acid hydrolysis and FT-IR spectroscopy. *Carbohydr. Polym.* 46, 81–87. doi: 10.1016/S0144-8617(00)00286-1

Palanisamy, S., Vinosha, M., Manikandakrishnan, M., Anjali, R., Rajasekar, P., Marudhupandi, T., et al. (2018). Investigation of antioxidant and anticancer potential of fucoidan from *Sargassum polycystum*. *Int. J. Biol. Macromol.* 116, 151–161. doi: 10.1016/j.ijbiomac.2018.04.163

Ponce, N. M. A., Pujol, C. A., Damonte, E. B., Flores, M. L., and Stortza, C. A. (2003). Fucoidans from the brown seaweed *Adenocystis utricularis*: extraction methods, antiviral activity and structural studies. *Carbohydr. Res.* 338, 153–165. doi: 10.1016/S0008-6215(02)00403-2

Qi, H., Zhao, T., Zhang, Q., Li, Z., Zhao, Z., and Xing, R. (2006). Antioxidant activity of different molecular weight sulfated polysaccharides from *Ulva pertusa* Kjellm (Chlorophyta). *J. Appl. Phycol.* 17, 527–534. doi: 10.1007/s10811-005-9003-9

Qiu, Q. S., Guo, Y., Dietrich, M. A., Schumaker, K. S., and Zhu, J. K. (2002). Regulation of SOS1, a plasma membrane Na+/H+ exchanger in *Arabidopsis thaliana*, by SOS2 and SOS3. *Proc. Natl. Acad. Sci. U.S.A.* 99, 8436–8441. doi: 10.1073/pnas.122224699

Qiu, Z., Guo, J., Zhu, A., Zhang, L., and Zhang, M. (2014). Exogenous jasmonic acid can enhance tolerance of wheat seedlings to salt stress. *Ecotoxicol. Environ. Saf.* 104, 202–208. doi: 10.1016/j.ecoenv.2014.03.014

Rivero, R. M., Mestre, T. C., Mittler, R., Rubio, F., Garcia-Sanchez, F., and Martinez, V. (2014). The combined effect of salinity and heat reveals a specific physiological, biochemical and molecular response in tomato plants. *Plant Cell Environ.* 37, 1059–1073. doi: 10.1111/pce.12199

Rodriguez, H. G., Roberts, J., Jordan, W. R., and Drew, M. C. (1997). Growth, water relations, and accumulation of organic and inorganic solutes in roots of maize seedlings during salt stress. *Plant Physiol.* 113, 881–893. doi: 10.1104/pp.113.3.881

Sangha, J. S., Ravichandran, S., Prithiviraj, K., Critchley, A. T., and Prithiviraj, B. (2010). Sulfated macroalgal polysaccharides λ-carrageenan and ι-carrageenan differentially alter *Arabidopsis thaliana* resistance to *Sclerotinia sclerotiorum*. *Physiol. Mol. Plant Pathol.* 75, 38–45. doi: 10.1016/j.pmpp.2010.08.003

Stasio, E. D., Oosten, M. J. V., Silletti, S., Raimondi, G., Aversana, E. D., Carillo, P., et al. (2018). *Ascophyllum nodosum*-based algal extracts act as enhancers of growth, fruit quality, and adaptation to stress in salinized tomato plants. *J. Appl. Phycol.* 30, 2675–2686. doi: 10.1007/s10811-018-1439-9

Van Oosten, M. J., Silletti, S., Guida, G., Cirillo, V., Di Stasio, E., Carillo, P., et al. (2017). A benzimidazole proton pump inhibitor increases growth and tolerance to salt stress in tomato. *Front. Plant Sci.* 8:1220. doi: 10.3389/fpls.2017.01220

Yamaguchi, T., Hamamoto, S., and Uozumi, N. (2013). Sodium transport system in plant cells. *Front. Plant Sci.* 4:410. doi: 10.3389/fpls.2013.00410

Yao, X., Horie, T., Xue, S., Leung, H. Y., Katsuhara, M., Brodsky, Q. E., et al. (2010). Differential sodium and potassium transport selectivities of the rice OsHKT2;1 and OsHKT2;2 transporters in plant cells. *Plant Physiol.* 152, 341–355. doi: 10.1104/pp.109.145722

Ye, Z., Wang, W., Yuan, Q., Ye, H., Sun, Y., Zhang, H., et al. (2016). Box-Behnken design for extraction optimization, characterization and *in vitro* antioxidant activity of *Cicer arietinum* L. hull polysaccharides. *Carbohydr. Polym.* 147, 354–364. doi: 10.1016/j.carbpol.2016.03.092

Yuan, Y., and Macquarrie, D. (2015). Microwave assisted extraction of sulfated polysaccharides (fucoidan) from *Ascophyllum nodosum* and its antioxidant activity. *Carbohydr. Polym.* 129, 101–107. doi: 10.1016/j.carbpol.2015.04.057

Zha, S., Zhao, Q., Zhao, B., Ouyang, J., Mo, J., Chen, J., et al. (2016). Molecular weight controllable degradation of *Laminaria japonica* polysaccharides and its antioxidant properties. *J. Ocean Univ. China* 15, 637–642. doi: 10.1007/s11802-016-2943-7

Zhang, M., Cao, Y., Wang, Z., Wang, Z. Q., Shi, J., Liang, X., et al. (2018). A retrotransposon in an HKT1 family sodium transporter causes variation of leaf Na(+) exclusion and salt tolerance in maize. *New Phytol.* 217, 1161–1176. doi: 10.1111/nph.14882

Zhang, S., Gan, Y., and Xu, B. (2016). Application of plant-growth-promoting fungi *Trichoderma longibrachiatum* T6 Enhances Tolerance of Wheat to Salt Stress through Improvement of Antioxidative Defense System and Gene Expression. *Front Plant Sci* 7:1405. doi: 10.3389/fpls.2016.01405

Zhang, Z., Mao, C., Shi, Z., and Kou, X. (2017). The amino acid metabolic and carbohydrate metabolic pathway play important roles during salt-stress response in tomato. *Front. Plant Sci.* 8:1231. doi: 10.3389/fpls.2017.01231

Zou, P., Lu, X., Jing, C., Yuan, Y., Lu, Y., Zhang, C., et al. (2018). Low-molecular-weightt polysaccharides from *Pyropia yezoensis* enhance tolerance of wheat seedlings (*Triticum aestivum* L.) to salt stress. *Front. Plant Sci.* 9:427. doi: 10.3389/fpls.2018.00427

Conflict of Interest Statement: The authors declare that the research was conducted in the absence of any commercial or financial relationships that could be construed as a potential conflict of interest.

frontiers in Plant Science

ORIGINAL RESEARCH
published: 01 February 2019
doi: 10.3389/fpls.2018.01959

Exposure *in vitro* to an Environmentally Isolated Strain TC09 of *Cladosporium sphaerospermum* Triggers Plant Growth Promotion, Early Flowering, and Fruit Yield Increase

*Zhijian T. Li[1], Wojciech J. Janisiewicz[1], Zongrang Liu[1], Ann M. Callahan[1], Breyn E. Evans[1], Wayne M. Jurick II[2] and Chris Dardick[1]**

[1] Appalachian Fruit Research Station, United States Department of Agriculture – Agricultural Research Service, Kearneysville, WV, United States, [2] Food Quality Laboratory, Beltsville Agricultural Research Center, United States Department of Agriculture – Agricultural Research Service, Beltsville, MD, United States

OPEN ACCESS

Edited by:
Giuseppe Colla, Università degli Studi della Tuscia, Italy

Reviewed by:
Philipp Franken, Leibniz-Institut für Gemüse- und Zierpflanzenbau (IGZ), Germany
Paolo Bonini, Ngalab, Spain
Antonio Ferrante, University of Milan, Italy

***Correspondence:**
Chris Dardick
chris.dardick@ars.usda.gov

Specialty section:
This article was submitted to Plant Microbe Interactions, a section of the journal Frontiers in Plant Science

Received: *26 September 2018*
Accepted: *17 December 2018*
Published: *01 February 2019*

Citation:
Li ZT, Janisiewicz WJ, Liu Z, Callahan AM, Evans BE, Jurick WM II and Dardick C (2019) Exposure in vitro to an Environmentally Isolated Strain TC09 of Cladosporium sphaerospermum Triggers Plant Growth Promotion, Early Flowering, and Fruit Yield Increase. Front. Plant Sci. 9:1959. doi: 10.3389/fpls.2018.01959

A growing number of bacteria and fungi have been found to promote plant growth through mutualistic interactions involving elements such as volatile organic compounds (VOCs). Here, we report the identification of an environmentally isolated strain of *Cladosporium sphaerospermum* (herein named TC09), that substantially enhances plant growth after exposure *in vitro* beyond what has previously been reported. When cultured on Murashige and Skoog (MS) medium under *in vitro* conditions, tobacco seedlings (*Nicotiana tabacum*) exposed to TC09 cultures for 20 days increased stem height and whole plant biomass up to 25- and 15-fold, respectively, over controls without exposure. TC09-mediated growth promotion required >5 g/L sucrose in the plant culture medium and was influenced by the duration of exposure ranging from one to 10 days, beyond which no differences were detected. When transplanted to soil under greenhouse conditions, TC09-exposed tobacco plants retained higher rates of growth. Comparative transcriptome analyses using tobacco seedlings exposed to TC09 for 10 days uncovered differentially expressed genes (DEGs) associated with diverse biological processes including cell expansion and cell cycle, photosynthesis, phytohormone homeostasis and defense responses. To test the potential efficacy of TC09-mediated growth promotion on agricultural productivity, pepper plants (*Capsicum annuum* L.) of two different varieties, Cayenne and Minisweet, were pre-exposed to TC09 and planted in the greenhouse to monitor growth, flowering, and fruit production. Results showed that treated pepper plants flowered 20 days earlier and yielded up to 213% more fruit than untreated controls. Altogether the data suggest that exposure of young plants to *C. sphaerospermum* produced VOCs may provide a useful tool to improve crop productivity.

Keywords: microbial volatile organic compounds, *Cladosporium sphaerospermum*, plant growth promotion, biostimulant, increased productivity, *Nicotiana tabacum* L., *Capsicum annuum* L., expression profiling

INTRODUCTION

Innumerable bacterial and fungal microorganisms colonize various habitats and can influence the survival of plants. Many such microorganisms promote plant growth through mutualistic interactions which have been the subject of intensive studies for more than 150 years (Whipps, 2001; Berg, 2009). In general, beneficial microorganisms are categorized into a number of groups depending on their habitats and functional roles. They include organic matter decomposers, nitrogen fixing bacteria, mycorrhizal fungi, detoxifiers, mutualistic endophytes, and pathogen-antagonists (Barea et al., 2005). These microbes produce and release unique proteins, enzymes, antibiotics, phenolics, lipids, carbohydrates, and phytohormones improving the availability of essential nutrients and plant growth. In addition, they are capable of activating plant defenses and protecting against biotic and abiotic stresses (Wardle et al., 2004; Barea et al., 2005; Saharan and Nehra, 2011; De-la-Peña and Loyola-Vargas, 2014). Over the past several decades, concerted efforts have focused on the study of plant–microbe symbiotic relationships and endophytic interactions to elucidate plant growth promoting (PGP) activities of beneficial microorganisms (Barea et al., 2005; Berg and Smalla, 2009; Lambers et al., 2009; Saharan and Nehra, 2011; Ahemad and Kibret, 2014; De-la-Peña and Loyola-Vargas, 2014). Consequently, a wide range of microbial inoculants and biocontrol products were developed and marketed as biopesticides, plant strengtheners, phytostimulators, and biofertilizers to improve soil conditions, increase crop productivity and control soil borne pests while reducing reliance on agrochemicals. These products have the potential to encourage environmentally friendly and sustainable cultivation practices and protect biodiversity (Thakore, 2006; Berg, 2009; Baez-Rogelio et al., 2016).

The utilization of current microbial products designed to be applied to the rhizosphere or as inoculants for PGP commonly suffer from unpredictability and inconsistency (Berg, 2009; Ahemad and Kibret, 2014). Changes in soil conditions due to tillage and erosion, extreme weather events and capricious mutualistic interactions amongst microorganisms outside or within host plants can influence the biochemical, physiological and metabolic activities of microbial community and the survival of beneficial microorganisms (De Souza et al., 2015; Baez-Rogelio et al., 2016). A number of studies revealed that some beneficial microorganisms under certain growth and environmental conditions can also produce phytotoxic substances such as hydrogen cyanide (HCN) and other nitrogen and sulfur compounds that can adversely affect plant growth (Kremer and Souissi, 2001; Weise et al., 2013; Nadeem et al., 2014). Clearly, a better understanding of the responses of beneficial microorganisms to their environments will help the development of sustainable strategies that can maximize the effectiveness of microbial products and minimize the deleterious effects on plant growth.

Recently, research activities in the utilization of microorganisms that enhance plant growth via microbial volatile organic compounds (MVOCs) have been intensified based on the premise that they might provide an eco-friendly, cost-effective and sustainable strategy to benefit agriculture (Kanchiswamy et al., 2015a,b; Li et al., 2016). MVOCs are small (<300 Da) and vaporous/gaseous semiochemicals with relatively high vapor pressures, low boiling points and high levels of lipophilicity (Schulz and Dickschat, 2007; Lemfack et al., 2014). These compounds are mostly organic/hydrocarbon in nature (Audrain et al., 2015). Microorganisms from many bacterial and fungal species and genera are able to synthesize a plethora of MVOCs with various biological functions. According to a recent tally, 400 out of 10,000 microorganisms have been described for their ability to produce up to 1,000 MVOCs (Schulz and Dickschat, 2007; Lemfack et al., 2014; Peñuelas et al., 2014; Kanchiswamy et al., 2015b; Li et al., 2016). However, among the complex blends of MVOCs, only a handful have been demonstrated to function in PGP. For instance, 141 MVOCs were detected from 20 strains and 11 species of *Trichoderma* examined. Yet, only 18 compounds or 11% of the detectable pool were able to promote plant growth, whereas the remaining majority compounds were either neutral or inhibitory to plant growth (Lee et al., 2016). In some cases, phytopathogenic microorganisms are also capable of emitting PGP MVOCs but their positive activities are often subdued by strong phytotoxic substances and parasitic or pathogenic behaviors (Sánchez-López et al., 2016b; Cordovez et al., 2017). While specific correlation between microbe-derived volatiles and plant responses requires further examination and the mechanisms of action remain unknown in most cases, a growing number of studies have linked PGP MVOCs to diverse mechanisms. These include regulation of photosynthesis, starch accumulation, phytohormone homeostasis/signaling and cell expansion, activation of innate immunity and abiotic stress responses, reprogramming of developmental controls for promoted reproductive growth, and even altering the behaviors of other microorganisms through inter- and intra- kingdom microbial interactions (Ryu et al., 2003, 2004; Zhang et al., 2007; Minerdi et al., 2011; Pieterse et al., 2014; Bitas et al., 2015; Sánchez-López et al., 2016a,b; Cordovez et al., 2017; Farag et al., 2017; Tahir et al., 2017).

Despite these studies, challenges remain in applying MVOC-mediated PGP technology to crop production. More research is needed to identify and fully exploit novel MVOC-emitting microorganisms and develop effective deployment strategies (Ahmad et al., 2008; Berg, 2009; Kanchiswamy et al., 2015a; Turner and Meadows-Smith, 2016). In this study, we report on the discovery of a non-pathogenic airborne fungus *Cladosporium sphaerospermum*, strain TC09 that strongly stimulates plant growth through exposure *in vitro*. By using tobacco (*Nicotiana tabacum*) and pepper (*Capsicum annuum*) as models, conditions for fungal exposure and fungal cultivation to achieve optimal plant growth stimulation were investigated. We demonstrated growth stimulation, early flowering and fruit yield increases following a relatively short duration of *in vitro* MVOC exposure at seedling stage. Comparative transcriptome analysis was conducted to reveal differential gene expression associated with promoted plant growth and development. PGP activities following *in vitro* exposure using related fungal species and isolates were also compared.

MATERIALS AND METHODS

Culture Media

Premixed medium powder containing basal salts and vitamins of MS medium (Murashige and Skoog, 1962) were purchased from PhytoTechnology Laboratories (Cat No. M519, Overland Park, KS, United States). For *in vitro* culture of plants, full strength MS medium supplemented with 30 g/L sucrose (Sigma-Aldrich, St. Louis, MO, United States, S5391) was prepared with pH adjusted to 5.8 using 1N KOH prior to addition of 7 g/L gelling agar (Sigma, A7921). The medium was then autoclaved at 121°C for 20 min and cooled down to 45°C prior to dispensing to Magenta GA7 vessels (100 ml per vessel) and 15 × 100 mm Petri dishes (30 ml/Plate).

Species Identification of TC09

TC09 was an environmentally isolated strain of unknown fungus and was subsequently deposited at the Agricultural Research Service Culture Collection (NRRL) with an assigned #: NRRL 67603. To determine the species identity, single spore cultures were transferred to MS medium in Petri plates and incubated at 25°C. Fungal spores were collected from the cultures and kept in a 1.5 ml microcentrifuge tube at −20°C. Genomic DNA was isolated using the DNEasy Plant Mini Kit (Qiagen, Hilden, Germany). Briefly, conidia were removed from the freezer and liquid nitrogen was added to the microcentrifuge tube. The conidia were ground to a fine powder using a motorized pestle mixer (VWR Pellet Mixer, VWR, Intl., Radnor, PA, United States). The DNA was isolated following the manufacturer's protocol with one exception; in the final step, DNA was eluted from the spin column using 100 μL hot (65°C) nuclease-free water. DNA concentration was determined using a Qubit® 2.0 fluorometer and the dsDNA HS Assay Kit (Life Technologies, Carlsbad, CA, United States). Polymerase chain reaction (PCR) was performed with the genomic DNA as a template. The internal transcribed spacer 1 (ITS1) and ITS2 regions associated with the 5.8S ribosomal RNA (rRNA) gene in fungal organisms were targeted for species identification. Two reactions using primer pairs ITS1/2 and ITS3/4, respectively, were conducted in a Bio-Rad thermocycler using 30 reaction cycles each consisting of 94°C for 1 min, 60°C for 1 min, and 72°C for 3 min. Sequences of the primer pairs are as described by White et al. (1990) (**Table 1**). Both amplicons were visualized on a 0.8% agarose gel stained with ethidium bromide (EtBr) following electrophoresis. DNA products in single bands were purified from isolated gel blocks using Qiagen PCR Clean Up kit and DNA concentrations were quantified using a Nanodrop spectrophotometer. Purified DNA Products were subject to Sanger sequencing by Eurofins MWG Operon (KY, United States) and sequence data were analyzed using Geneious software (Biomatters Inc., Newark, NJ, United States).

To determine phylogenetic relationships, DNA sequences of 145 related fungal species/isolates with over 95% homology to the consensus sequence of the ITS3/4 primer pair-amplified PCR products were retrieved via a BLAST search of GenBank database. The sequences were aligned and analyzed using default settings of the Phylogeny Module of CLC Genomics Workbench software program (Version 10, Qiagen, Redwood City, CA, United States) with Neighbor-Joining method with 1,000 bootstrap replicates.

For microscopic examination, TC09 was grown on PDA (Potato dextrose agar) or MS media for 7–10 days at 22°C under continuous light. Squares of transparent adhesive tape (Scotch Magic tape, 3M, St. Paul, MN, United States) were gently placed along the edge of the colony with forceps. Tape squares were removed from the colony margin and stained for 20 min with 1% aqueous Calcofluor white M2R (Fluorescent brightener 28, Sigma, St. Louis, MO, United States). Tape squares were gently rinsed in sterile distilled water and mounted between drops of 50% glycerol under a glass cover slip. The cover slip was affixed in

TABLE 1 | Oligonucleotide primers and amplicons targeting the internal transcribed spacer 1 (ITS1) and ITS2 regions associated with the 5.8S ribosomal RNA (rRNA) gene in fungi.

Sequence type	Sequence name	Sequence 5′ to 3′	Nucleotide
Forward primer	ITS1	TCCGTAGGTGAACCTGCGG	19
Reverse primer	ITS2	GCTGCGTTCTTCATCGATGC	20
Forward primer	ITS3	GCATCGATGAAGAACGCAGC	20
Reverse primer	ITS4	GGAAGTAAAAGTCGTAACAAGG	24
Amplicon	ITS1/2	GGCCGGGGATGTTCATAACCCTTTGTTGTC CGACTCTGTTGCCTCCGGGGCGACCCTGCC TTTTCACGGGCGGGGGCCCCGGGTGGACA CATCAAAACTCTTGCGTAACTTTGCAGTCT GAGTAAATTTAATTAATAA	138
Amplicon	ITS3/4	TTCAGTGAATCATCGAATCTTTGAACGCAC ATTGCGCCCCCTGGTATTCCGGGGGGCAT GCCTGTTCGAGCGTCATTTCACCACTCAAG CCTCGCTTGGTATTGGGCGACGCGGTCCG CCGCGCGCCTCAAATCGACCGGCTGGGTC TTCTGTCCCCTCAGCGTTGTGGAAACTATT CGCTAAAGGGTGCCACGGGAGGCCACGCC GAAAAACAAACCCATTTCTAAGGTTGACC TCGGATCAGGTAGG	249

place using clear nail polish. Mounted specimens were visualized through confocal microscopy (Zeiss LSM-800, Carl Zeiss AG, Oberkochen, Germany) and images were captured using the manufacturer software.

Assay of TC09 Promotion Activities on Tobacco Plant Growth

Tobacco seeds (*Nicotiana tabacum* cv. "Samsun") were surface-sterilized by rinsing briefly in 95% ethanol and then immersed in 20% (v/v) bleach containing 8.25% w/v sodium hypochlorite with constant agitation for 10 min. After 3× rinse with sterile water, seeds were then spread evenly on the surface of MS medium in Petri plates. The plates were sealed with plastic wrap and maintained at 25°C under 16-h light (50 μmol m^2 s^1) and 8-h dark photoperiods for 6 days. Seedlings with fully expanded cotyledons were selected based on a uniform plant size and an appearance free of abnormality (∼80–90% of sowed seeds).

TC09 inoculant in aqueous conidial suspension was prepared by first culturing the fungal conidia on MS plate for 1 week followed by collecting conidia in sterile 0.01% Triton X-100/water solution and adjusting density to 10^5 conidia per ml prior to use or storage at −80°C. For initial experiments, fungal cultures grown in Eppendorf tubes were placed inside the plant tissue culture vessel but physically separated using sealed biological filters that restrict the movement of conidia but not volatiles. First, aliquots of 300 μl warm MS medium were poured into 1.5 ml Eppendorf microcentrifuge tubes. Tubes were positioned diagonally to form a slant surface while cooling in laminar flow hood. Ten microliters of an aqueous conidial suspension of TC09 was transferred onto the surface of the medium in each tube and thus inoculated tubes were plugged with a sterile aerosol substance- and liquid-resistant filter (Rainin #17001945, Mettler Toledo, Oakland, CA, United States). Two tubes were directly inserted at separate corners of a Magenta GA7 vessel containing three tobacco seedlings each. The vessels were then sealed with plastic wrap and maintained under light conditions with a 16-h photoperiod at 25°C. Plant growth was monitored and compared to controls grown without fungal cultures.

In subsequent experiments open-end culture tube closures (Sigma C5791) were used to reduce premature build-up of condensation water on the surface of the fungal mycelium as found in above microcentrifuge tube setting. Each sterile closure contained 5 ml semi-solid MS medium followed by addition of 10 μl of TC09 suspension as inoculant. One inoculated closure was then placed in each Magenta GA7 vessel that contained three tobacco seedlings for fungal exposure treatment. For controls, a MS-containing closure without fungal inoculant was added. Vessels were placed under above-mentioned light conditions at 25°C. To measure plant growth promotion under *in vitro* conditions, a 20-day exposure duration was employed which was comparable to previous studies using tobacco (Paul and Park, 2013; Lee et al., 2016).

Three plants were grown in each replicate vessel and three vessels were used per treatment. The experiment was conducted three times unless otherwise stated. Plant growth was determined by measurement of major growth parameters including total plant height, total plant fresh weight, stem length, number of traceable leaves, root system length, and length of largest leaf. Data were analyzed by using SAS statistics program with standard statistical approaches including standard error, *t*-test (two means), ANOVA and Tukey's HSD mean separation test (multiple means) with a statistical significance level $P < 0.05$.

Effect of TC09 Exposure Duration and Sugar Concentration

For experiments to test the effect of exposure time under *in vitro* conditions, control tube closures with medium-only (CK) and those with fungal cultures were placed in vessels containing tobacco seedlings. Fungal cultures were then removed after an incrementally extended exposure duration from 1, 4, 10, or 20 days. Vessels containing medium only were treated as 0 day and served as a control. All plants were allowed to grow continuously in the vessels until 20 days after introduction of fungal cultures when growth was measured. The experiment was conducted two times.

A total of six sucrose concentrations including 0, 5, 10, 20, 30, and 40 g/L were incorporated in MS medium and used to culture tobacco seedlings. Introduction of TC09 cultures in tube closures to Magenta GA7 vessels was carried out as aforementioned. After a 20-day fungal exposure, plants were collected, measured and analyzed as described above. Experiments were conducted two times.

Plant Growth Under *ex vitro*/Soil Conditions in the Greenhouse

In vitro grown plants following fungal exposure were transplanted to potting soil mix (Metro Mix 360, Sun Gro Horticulture, Canada) and maintained in a greenhouse to monitor growth. Soil mix was pre-sterilized by autoclaving at 121°C for 90 min prior to use. Plants were maintained in a greenhouse. Six to ten individual tobacco plants from each treatment and untreated controls were transplanted for each test and experiments were conducted three times. Plant growth and development were evaluated via periodic measurement of plant height and total leaf numbers over the course of 70 days beginning from the time the seeds were sown. Experimental data were analyzed using standard statistical approaches as mentioned above.

Effect of TC09 Growth Media on Plant Growth Promotion

A total of six media were examined for fungal cultivation including MS (Murashige and Skoog, 1962), PDA (potato dextrose agar), Czapek (CYA, Czapek-DOX Yeast agar), Malt (Malt extract agar), yeast (Yeast extract extract) and Hutner's medium. Formula and preparation procedures for the last five culture media were described previously (Sinclair and Dhingra, 1995). Organic potatoes were purchased locally and used to prepare potato infusion and PDA medium. For these experiments, tobacco and closure-mediated method for fungal exposure were employed. Final determination of plant

growth was made at the end of an exposure duration of 20 days. There were three plants per replicate vessel and three vessels per treatment. The experiment was repeated twice.

Effect of TC09-Related Fungal Species/Isolates on Plant Growth Promotion

Comparative assays were performed to determine the effectiveness of exposure *in vitro* using various species and isolates belonging to *Cladosporium* that were kindly provided by Dr. Frank M. Dugan of USDA (59 Johnson Hall, WSU, Pullman, WA, United States). These include *C. sphaerospermum* NRRL8131, *C. cladosporioides* 113d, *C. asperulatum* 208db, *C. subtilissimum* WF99-209, *C. cladosporioides* W99-175a, and *C. macrocarpum* Clad ex Phyl 8. Tobacco plants were used for growth comparison using MS medium for fungal culture. Three replicate vessels with three plants each were used for each treatment. The experiment was repeated twice.

Comparative Transcriptome Analysis of Tobacco Plants Following Exposure to TC09

Transcriptome analysis was carried out using total RNA isolated from tobacco seedlings grown under *in vitro* conditions with or without TC09 exposure for 10 days. For RNA isolation, shoot tips carrying the apical meristem and three to four terminal leaves were collected from each replicate plant, placed in 1.5 ml microcentrifuge tubes, and immediately immersed in liquid nitrogen. Samples were pulverized into a fine powder using Geno/Grinder 2010 (SPEX Sample Prep, Metuchen, NJ, United States). Total RNA was then extracted from 100 mg tissue powder using the RNeasy® Plant Mini Kit per manufacturer's instructions (Qiagen, Redwood City, CA, United States). There were four biological replicates from each treatment. RNA sequencing was performed by Genewiz via Illumina HiSeq platform (50 bp single-end reads) (South Plainfield, NJ, United States). Raw sequences were paired, trimmed and filtered to obtain quality reads. Reads were mapped to the *Solanum lycopersicum* reference genome with CLC Genomics Workbench (version 10, Qiagen, Redwood City, CA, United States) using default parameters (The Tomato Genome Consortium, 2012). Differentially expressed genes (DEGs) were identified using false discovery rate (FDR) $\leq$ 0.05. Genes were annotated per referenced tomato (*S. lycopersicum*) transcriptome dataset available on https://solgenomics.net and associated gene ontology (GO) terms. The RNAseq data was deposited to NCBI GEO under reference ID GSE120288.

Effect of TC09 Exposure *in vitro* on Pepper Plant Growth and Production

Two different varieties of pepper (*Capsicum annuum*), cayenne pepper 'Long Thin' and sweet bell pepper 'Minisweet Pepper Mixed,' were tested to determine growth response to TC09 exposure *in vitro*. Pepper seeds were acquired from commercial source (Plantation Product LLC., Norton, MA, United States) and sterilized by previously described method prior to *in vitro* cultivation or seeded directly in the soil as controls. Germinated pepper seedlings were exposed to closure-contained TC09 while culturing on MS medium in GA7 vessels for 20 days and then transplanted to soil in 8-inch pots. Plants were maintained in the greenhouse using standard management practice. Plant growth and fruit production were monitored continuously until fruit ripening. In these experiments each treatment consisted of four to five individual plants. Experiments were conducted two times each in a similar seasonal span from late winter to early spring in 2017 and 2018, respectively.

RESULTS

Isolation and Species Identification of TC09 Fungus

An airborne fungal contaminant on a tissue culture plate containing tobacco seedlings growing on MS medium was isolated and single-spore purified based on the preliminary visual observation that plants in the contaminated plate grew much larger than comparable plants on uncontaminated plates. To identify the species, DNA was extracted and the internal transcribed spacer was amplified by PCR and subsequently sequenced. The sequence of the two fragments generated from the primer pairs are specified in **Table 1**.

Upon comparison with existing sequences, the 138 bp ITS1/2 amplicon was 100% identical to a GenBank accession KU926349.1 belonging to *Cladosporium sphaerospermum* isolate UACH-124. The 249 bp ITS3/4 amplicon was 100% identical to a GenBank accession KX982238.1 of *Cladosporium sphaerospermum* strain 7. Phylogenetic analysis of 148 sequences homologous to the ITS3/4 amplicon that were available in GenBank showed that TC09 clusters with known isolates of *C. sphaerospermum* (**Supplementary Figure S1**).

To confirm the results of genetic identification, TC09 was cultured on PDA medium, which is commonly used as a standard condition for fungal species identification. TC09 colonies grew to 20 mm and 35 mm in diameter during 7 and 14 days, respectively, on PDA at 20°C.=. Colonies were olivaceous-gray in the center and olivaceous to iron-gray in the outer region (**Supplementary Figure S3A**). On the reverse they were iron-gray and turned almost blackish at 14 days. Conidiophores were not much differentiated, occasionally branched with conidia produced in branching chains with variable shapes with smaller size toward the apex (**Supplementary Figures S3B,C**). Intercalary conidia were 1.7–3.2 × 2.4–8.0 μm and terminal conidia 1.9–2.5 × 2.1–2.9 μm. Hyphae was 2.6–3.2 μm wide, sparsely to profusely branched at 45–90° angles, distinctly septate with cell length averaging 17.5 μm and ranging from 12.4 to 27.3 μm (**Supplementary Figure S3D**). Collectively, the morphological results were consistent with TC09 being an isolate

of *C. sphaerospermum* as described by Dugan et al. (2008) and Ababutain (2013).

Stimulation of Plant Growth Is due to *in vitro* Exposure to TC09

Since the fungal mycelium and tobacco plants appeared to be physically separated in the original contaminated culture plate, possible involvement of MVOCs in plant growth promotion was investigated. Tobacco plants were tested with and without the inclusion of biological filter-sealed Eppendorf tubes containing TC09 in Magenta culture vessels. In this manner, the plants were exposed only to volatiles produced by the fungus. Visual observation indicated that plants with fungal exposure for 10 days exhibited more vigorous growth and thicker stems, larger leaves, and a more robust root system relative to plants without fungal exposure (**Figure 1A**). No conidia from the filter-sealed Eppendorf tubes were visually detected in either the culture vessel headspace or plant culture medium.

Next, plant growth stimulation was quantified using a 20-day exposure duration and open-end culture tube closures to prevent excessive condensation in the fungal culture. Exposed plants grew larger and filled up the headspace of culture vessels, produced elongated stems with larger diameters and longer internodes as well as a larger root systems as compared to controls (**Figure 1B**). Plants were subsequently transplanted to soil and growth differences were maintained (**Figure 1C**).

As compared to controls, the individual plants following 20-day *in vitro* exposure treatment exhibited the following growth increase: 25-fold for stem length, 15-fold for shoot biomass, 15-fold for root biomass, 3-fold for base-to-top length of largest leaf, and 10-fold for largest leaf biomass (**Figure 2**). Results

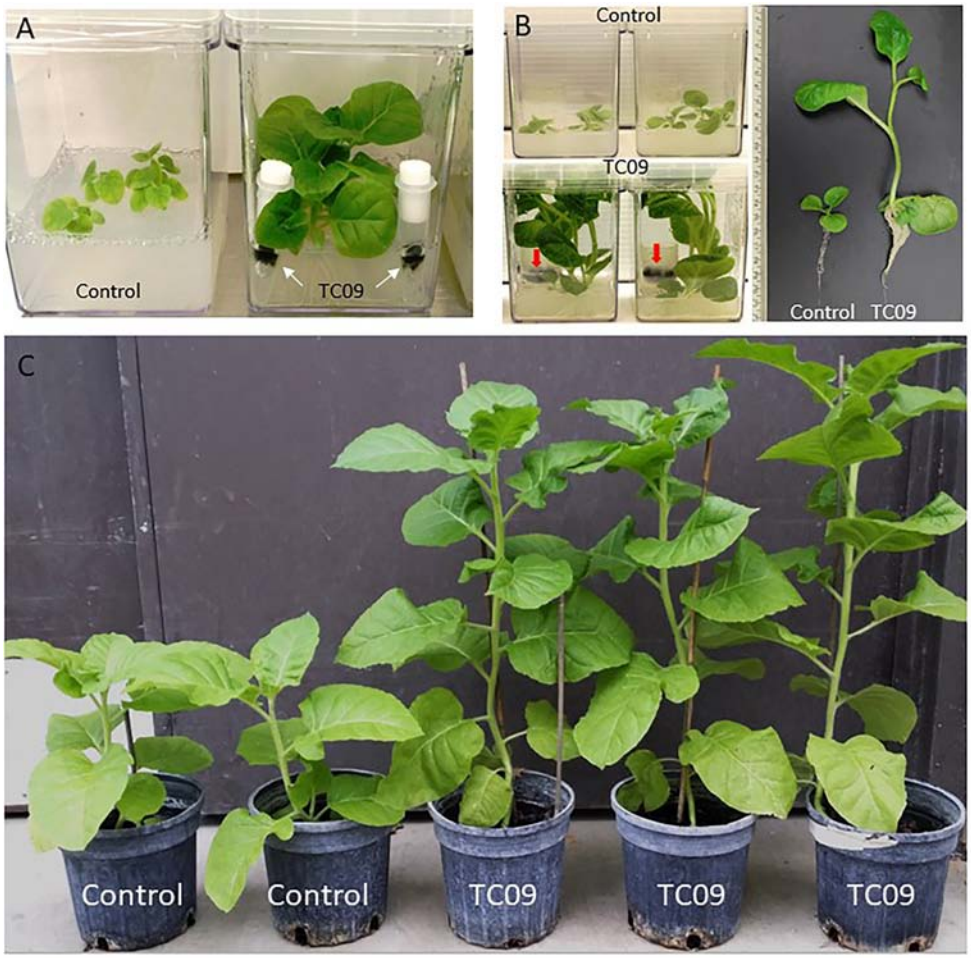


FIGURE 1 | Growth promotion of tobacco by exposure to TC09. **(A)** Control tobacco plants without fungal exposure (left) and plants exposed to physically separated TC09 contained in filter-sealed microcentrifuge tubes (right). Arrows indicated the fungus with dark-colored mycelium. Photograph was taken 10 days after fungal exposure. **(B)** Tobacco plants exposed to TC09 contained in open-end tube closures. Images were taken 20 days after introduction of fungal cultures. Left and upper row: control plants without TC09 exposure in triplicates per vessel, Left and lower row: plants with exposure to fungal cultures in triplicates per vessel. Arrows indicate TC09 mycelium in closure. Right panel: representative plants of control (left) and TC09-exposed treatment (right). **(C)** Evaluation of plant growth in greenhouse following *in vitro* seedling treatment with or without TC09 exposure. Plants with (right three) or without (left two) exposure to TC09 for 20 days were transplanted to soil and maintained in greenhouse using standard management practices. Photograph was taken 70 days after seed sowing.

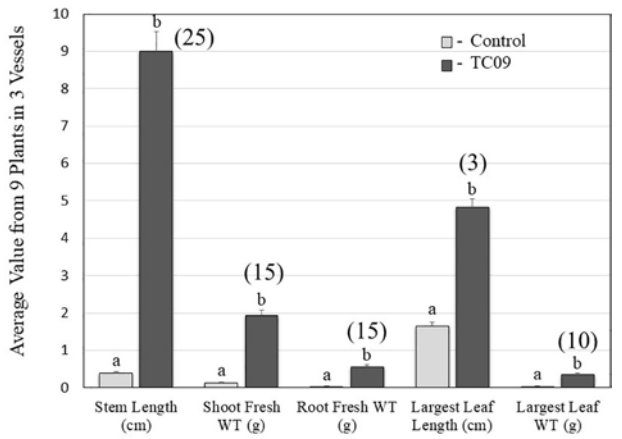


FIGURE 2 | Quantification of five growth parameters of control and TC09-exposed *in vitro* tobacco plants. Six-day-old tobacco seedlings following seed sowing were exposed to medium-containing closures (control) or fungal culture containing closures for 20 days and then subject to measurement to determine growth status. Values represent means obtained from triplicate vessels for each treatment. Each vessel contains three individual plants. Data in shaded bars from TC09-exposed plants were converted into fold increase over corresponding values from control plants and labeled in parentheses. Standard errors of the means were marked as vertical lines. Paired treatments sharing a letter on top of bars are not significantly different at $P > 0.05$ based on Tukey's test.

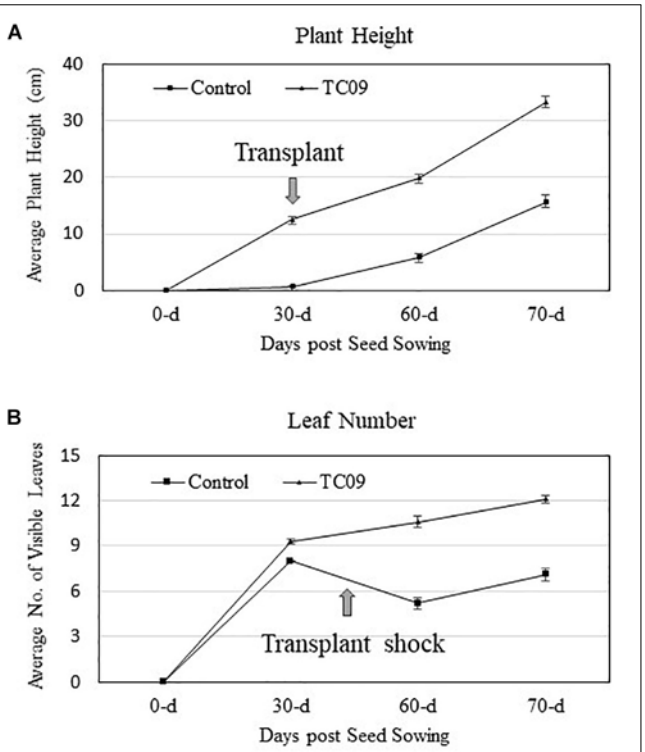


FIGURE 3 | Stem growth and leaf production of greenhouse-grown tobacco plants with or without *in vitro* exposure to TC09. Thirty-day-old plants with or without prior fungal exposure for 20 days were transplanted to soil and maintained in greenhouse using standard management practices. Plant height and the number of visible fully expanded leaves per plant were determined periodically. **(A,B)** Graphs denote changes in height and leaves, respectively, during the monitored growth period. Values represent average data from nine replicated plants per treatment and two repeated experiments.

from repeat experiments essentially gave rise to similar increased levels of plant growth with exposure to the fungal culture (data not shown). When planted in soil, the treated plants maintained a near twofold increase in height and in leaf number at 70 days post seed sowing relative to controls (**Figures 3A,B**).

Effect of TC09 Exposure Duration on Plant Growth

The potency of PGP activity of TC09 was ascertained using incrementally extended exposure durations. Visual inspection at the end of a 26-day culture period post seed sowing indicated that 1-day exposure of tobacco seedling to the freshly set-up TC09 culture was sufficient to double the plant size as compared to plants without exposure (0 day vs. 1 day) (**Figure 4A**). Plant growth was incrementally increased after 4-, 10-, and 20-day exposures with 10 days being maximal (**Figure 4A**). Based on measurements of major growth parameters, 10-day or 20-day exposure duration led to increase in stem length, stem caliper (diameter), and total plant biomass 16-, 3-, and 12-fold, respectively, over the control without TC09 exposure (**Figure 4B**). Repeated experiments yielded similar results (data not shown).

Effect of Sucrose Concentration and TC09 Exposure on Plant Growth

To investigate whether carbon source availability was a contributing factor for PGP activity, various concentrations of sucrose were tested in conjunction with TC09 exposure for 20 days. Without fungal exposure, tobacco plants did not show any measurable increase in growth rate in response to increasing concentrations of sucrose in the medium (**Figure 5**). When plants were exposed to TC09, only minimal plant growth promotion was observed in media containing 0 and 5 g/L sucrose whereas significant growth promotion was achieved when plants were maintained on a medium containing > 10 g/L sucrose (**Figure 5**). These results were also confirmed in a repeat experiment (data not shown).

Effect of Fungal Culture Media on PGP Activities of TC09

Initial PGP activity of TC09 was characterized using MS medium. Thus, to investigate the influence of culture conditions, several common fungal media were also tested. Variations in visual size and morphology of TC09 mycelium were discernable among various culture media used (**Figure 6**). Noticeable differences in the level of PGP activity of fungal cultures on various media were also observed (**Figure 6**). Twenty days post treatment, plants were measured to determine growth differences including total plant height, total plant biomass, stem length, and length of largest leaf. Results revealed that the order of growth stimulation level from highest to lowest among tested fungal culture media

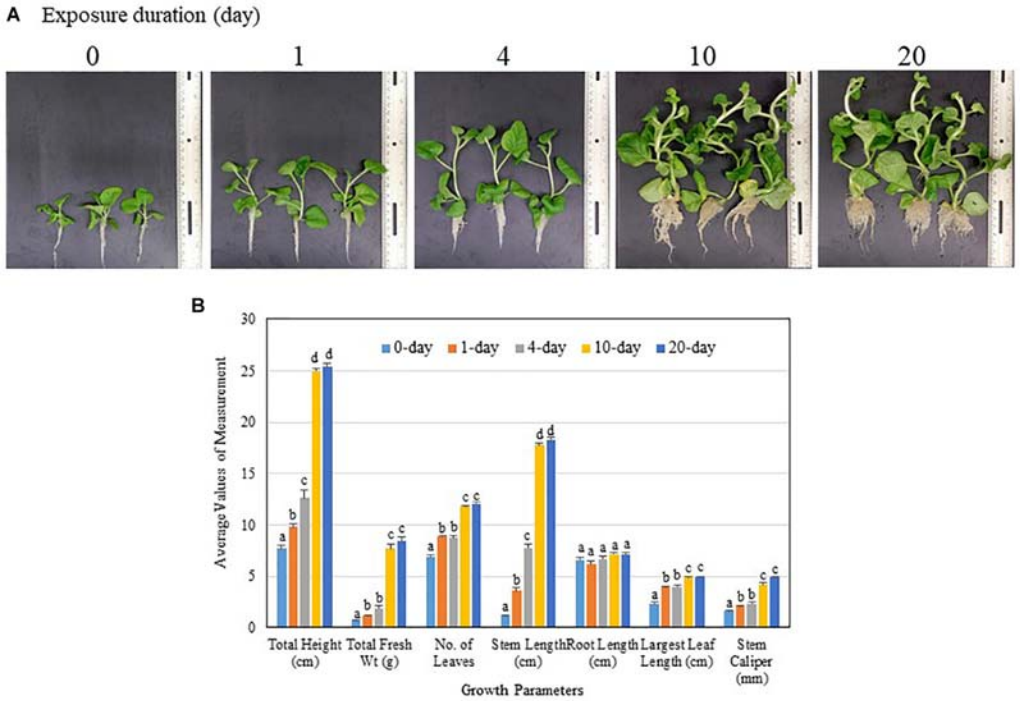


FIGURE 4 | Effect of MVOC exposure duration on tobacco growth under *in vitro* conditions. Seedlings 6 days after seed sowing were exposed to TC09 for specified durations from 1, 4, 10 to 20 days as TC09 exposure treatments or maintained without MVOC exposure (0 day as control). Plants were then allowed to grow continuously until the time for the last treatment was met, i.e., 20 days of exposure. **(A)** Representative plants from each treatment taken at the end of 26-day cultivation. Exposure durations were labeled on each image. **(B)** Values of seven growth parameters of control and TC09-exposed *in vitro* tobacco plants determined at the end of 26-day cultivation using various exposure duration. Bar values represent means from triplicate culture vessels for each treatment with each vessel containing three plants. Lines on top denote standard errors. Means sharing a letter in the group label are not significantly different at $P > 0.05$ according to Tukey's test.

was as follows: MS > PDA > Czapek > Yeast > Malt > Hutner's (**Figure 6B**). Hutner's medium contained rich vitamins and the same amount of sucrose as MS medium, but it showed the lowest levels of TC09 growth and PGP activity. On the other hand, Malt and Yeast media, both with reduced PGP activities as compared to MS, encouraged vigorous TC09 growth with comparable colony sizes to that of other media except for Hutner's medium. In addition, Malt medium seemed to increase hydrophilicity of the mycelium of TC09 and promote hyphal penetration into culture medium (**Figure 6A**). We conducted a repeat experiment and observed an identical trend of growth stimulation associated with various fungal media (data not shown).

Comparison of PGP by TC09 and Other Related Fungi

To investigate whether the observed PGP activity was unique to TC09-or common among related fungal species, we compared a total of seven *Cladosporium* species/isolates for their ability to promote *in vitro* tobacco growth. All strains exhibited PGP activity as compared to control without fungal exposure, although there were measurable differences among the species/isolates (**Figure 7A**). After 20 days of exposure, TC09 gave the highest level or PGP activity in five out of seven growth parameters measured (**Table 2**). Exposure to *C. sphaerospermum* NRRL8131 produced the longest stems among all compared fungi (**Table 2**). Colony morphology and growth pattern of various *Cladosporium* fungi when cultured on MS medium were also noticeably varied (**Figure 7B**). Four of the tested fungi including *C. sphaerospermum* TC09 (1), *C. sphaerospermum* NRRL8131 (2), *C. cladosporioides* 113 db (3) and *C. asperulatum* 208 db (4) produced mycelium in a similarly large diameter and dark greenish brown color. The remaining fungi including *C. subtilissimum* WF99-209 (5), *C. cladosporioides* W99-175a (6) and *C. macrocarpum* Clad ex Phyl 8 (7) formed smaller, black colonies. *C. cladosporioides* W99-175a (6) is the only fungus which produced dark brownish compounds that penetrated into the culture medium, whereas all other fungi demonstrated hydrophobicity of the mycelium on MS medium (**Figure 7B**). Subsequently, we conducted a repeat experiment that yielded similar results (data not shown).

RNAseq Analysis of Differential Gene Expression (DEG) Associated With TC09 Induced Growth Promotion

To assess host responses at the RNA level, tobacco seedlings treated with and without TC09 exposure for 10 days were subject to RNAseq analysis to identify DEGs. The sampling point was chosen based on the consistent levels of plant growth

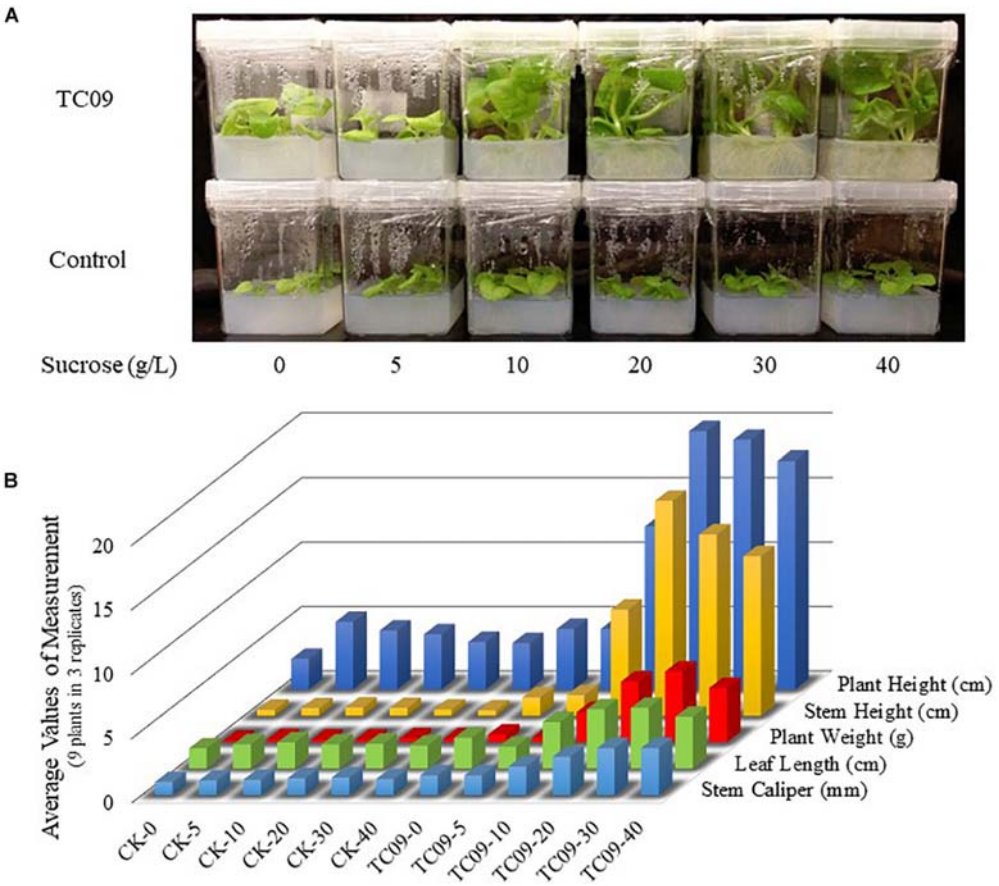


FIGURE 5 | Effect of sucrose concentration and TC09 exposure on tobacco growth under *in vitro* conditions. Seedlings 6 days post seed sowing were cultured on MS medium containing various concentrations of sucrose and exposed to freshly prepared TC09 culture in tube closure. **(A)** Plants were imaged 20 days after inset of fungal exposure. **(B)** Bars represent average values of growth parameters from three plants in each culture vessel with three replicate vessels per treatment.

stimulation achieved following 10 days of MOVC exposure (**Figure 4**). Experimental design included four replicate TC09 treated plants and four untreated control plants sampled after 10 days of exposure. Approximately 20 million quality reads per sample were obtained. A total of 3,562 DEGs were identified consisting of 1,594 up-regulated and 1,968 down-regulated genes (**Supplementary Table S1** and **Supplementary Figure S2**). Overall, the most highly repressed DEGs included reactive oxygen response genes such as those encoding catalases and superoxide dismutases involved in redox balance as well as photosystem functions including photosystem II D1 core genes along with constituents of the cytochrome and ATP synthase complexes. Among the over-represented DEGs were a number of genes involved in cell wall biosynthesis, cell expansion and multiplication. Numerous genes associated with the biosynthesis and response to phytohormones were differentially expressed with those related to auxin, gibberellin, and ethylene being the most abundant.

JA-inducible genes were notably up-regulated as the result of TC09 exposure. These included genes related to herbivore defense including proteinase inhibitors, Kunitz trypsin inhibitor, and *O*-methyltransferases – which catalyze multiple reactions in the biosynthesis of furanocoumarins (FCs) that act as insect repellants (**Table 3**).

Among cyclin-associated genes, cyclin A and B and two cyclin D genes increased twofold to threefold. Two cyclin-dependent kinases (CDKs) genes increased twofold to eightfold, respectively, in expression. In addition, one CDK regulator gene showed a 2.6-fold up-regulated expression. A number of genes encoding auxin regulators and sugar transporters were also up-regulated. Expression of several gylcosyltransferases functioning in glycosylation was reduced 2- to 10-fold (**Table 3**).

TC09 Exposure Increases Productivity in Peppers

Two varieties of pepper (*Capsicum annuum*) were used to investigate whether exposure to TC09 at the seedling stage could positively influence fruit production in a Solanaceous crop plant. Two independent experiments were carried out for each variety. The pepper seedlings were treated with and without TC09 for 20 days under *in vitro* conditions as described for tobacco. TC09–exposed cayenne and minisweet pepper plants responded to fungal stimulation in a growth pattern similar to tobacco

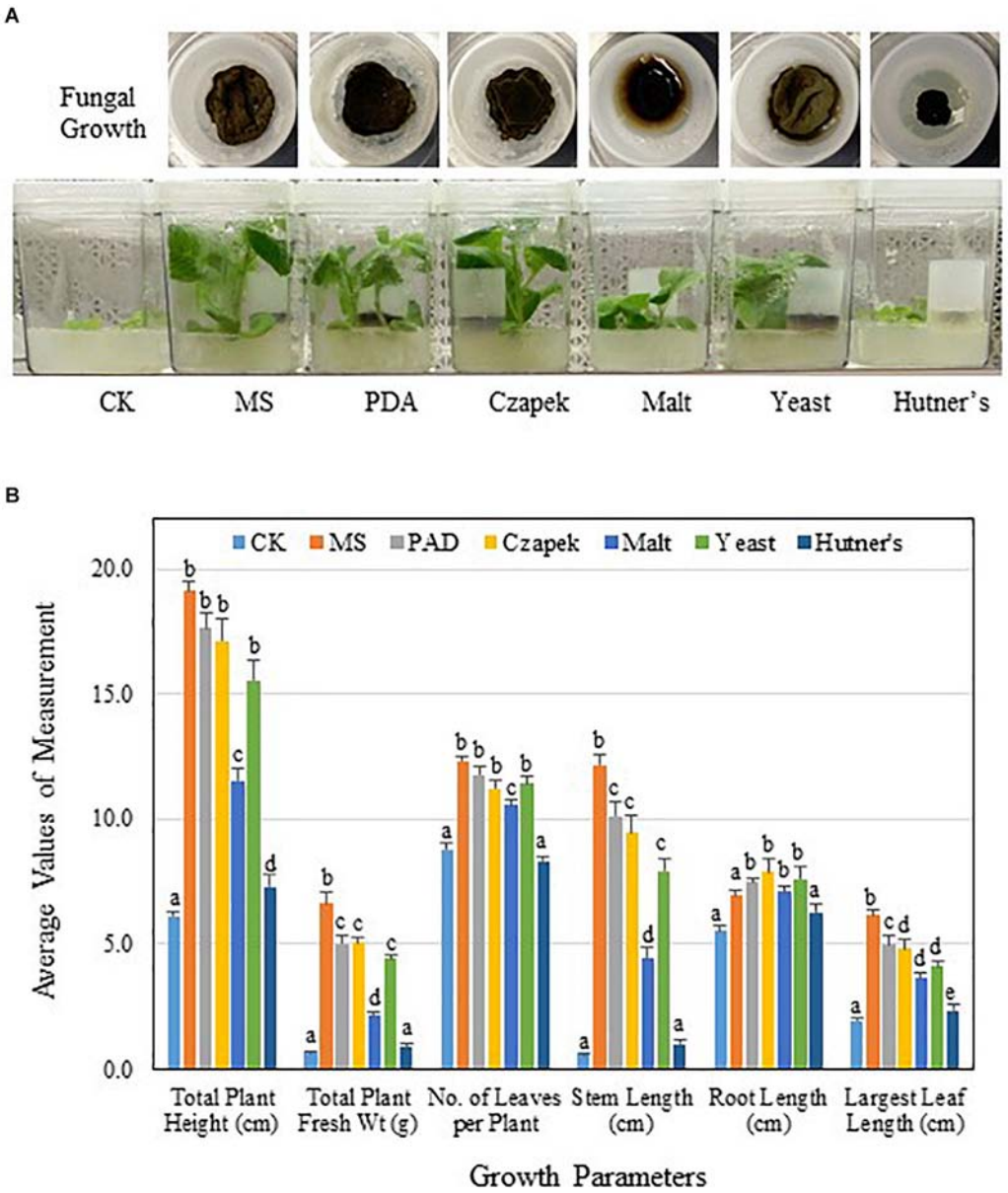


FIGURE 6 | PGP activity of TC09 affected by fungal culture media. **(A)** Fungal growth or hyphal development in various culture media and corresponding plant growth promotion. TC09 cultures were initiated in closures and used for exposure to 6-day-old tobacco seedlings according to described procedure. Images were taken 2 weeks after exposure initiation. Abbreviated names of fungal culture media are labeled. **(B)** Major growth parameters of control and TC09-exposed *in vitro* tobacco plants using various fungal culture media. Measurements were made 20 days after fungal exposure. Values represent means of three plants each of triplicate vessels per treatment. Standard errors of the means were labeled as vertical lines. Means sharing a letter in the group label are not significantly different ($P > 0.05$) according to Tukey's test.

and grew significantly larger than untreated controls that were either started in tissue culture or planted directly to soil. For cayenne pepper, following transplanting to soil, the first flowers among TC09-exposed plants were observed 20 days earlier than control plants along with a denser canopy and more branches (**Figure 8A**). By 116 days post seed sowing, visual inspection of the root system showed that TC09-exposed plants had more vigorous root growth than both direct soil seeding and tissue culture control plants without fungal exposure (**Figure 8B**).

Cayenne peppers longer than 1 cm in length were collected from all plants at 116 days post seed sowing. Results showed that TC09-exposed plants had the heaviest fruit load and the most vine-ripened fruits (reddish color) among all treatments (**Figure 8C**, left panel). Similar patterns of fruit production and development were also observed in minisweet pepper when all fruits were collected at 160 days post seed sowing (**Figure 8C**, right panel). For cayenne pepper, TC09-treated plants produced on average 49 fruit per plant whereas direct seeding and tissue

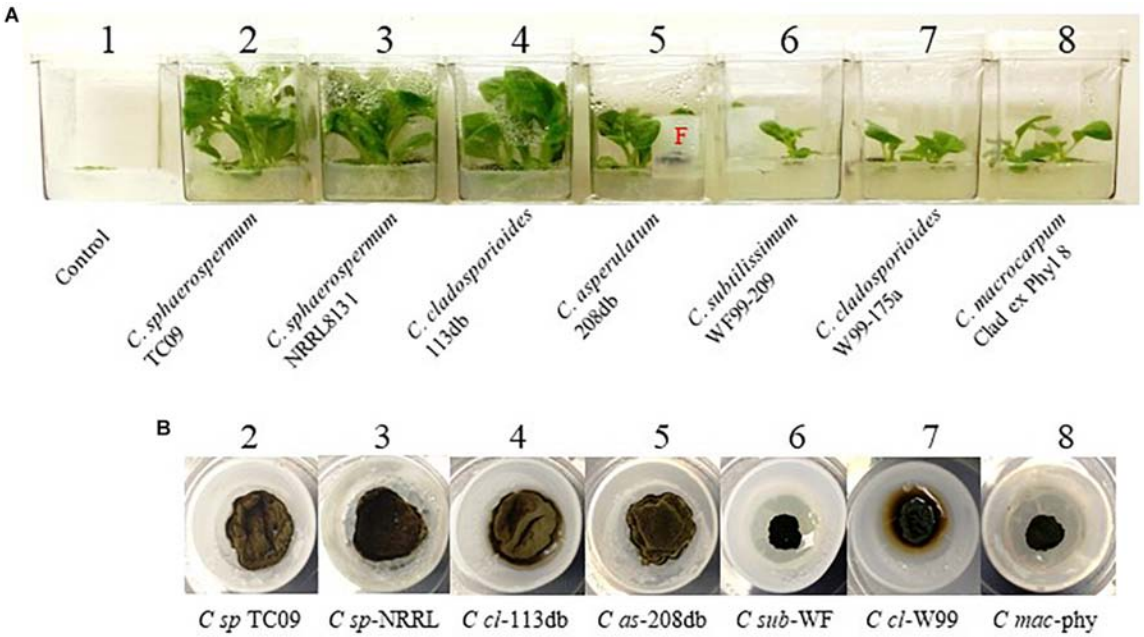


FIGURE 7 | Comparison of growth stimulation activity of various isolates/species of *Cladosporium*. **(A)** Tobacco plants exposed for 15 days to seven isolates/species of *Cladosporium* along with controls without exposure (#1). Names of species and isolates of *Cladosporium* are labeled. F denotes fungal culture in a closure. **(B)** Mycelium of various compared fungi in closures containing MS medium.

culture-treated plants only put out on average 10 and 18 fruit per plant, respectively. Likewise, for minisweet peppers, the number of fruits per plant from these three treatments were 28, 17, and 17, respectively (**Figure 9A**). Overall, the number of fruits per plant increased 174% and 62% for these two pepper varieties following exposure to TC09 as compared to tissue culture controls. When fruit weight was determined, cayenne pepper generated 173.1, 34.2, and 55.4 g of collected fruit per plant; and minisweet pepper produced 417.9, 247.8, and 272.0 g per plant for TC09, direct seeding and tissue culture treatments, respectively (**Figure 9B**). These results indicate that TC09 treatment gave rise to 213% and 54% yield increase over tissue culture control in cayenne and minisweet peppers, respectively. During the period from early 2017 to late 2018, we conducted a total of two experiments with minisweet pepper and three experiments with cayenne pepper. All results were highly consistent (data not shown).

DISCUSSION

In recent years there have been an increasing number of reports on MVOC-mediated PGP by beneficial and, in some cases, phytopathogenic microorganisms (Ryu et al., 2003; Zou et al., 2010; Minerdi et al., 2011; Paul and Park, 2013; Kai and Piechulla, 2014; Kanchiswamy et al., 2015a; Sánchez-López et al., 2016b; Cordovez et al., 2017; Tahir et al., 2017). Reported MVOCs are able to stimulate photosynthesis, cell expansion and starch accumulation, early flowering and fruit development and enhance host defense and stress tolerance (Ryu et al., 2004; Zhang et al., 2007; Ahemad and Kibret, 2014; Pieterse et al., 2014; Liu and Zhang, 2015; Chung et al., 2016; Sánchez-López et al., 2016a; Cordovez et al., 2017). This work has highlighted potential benefits and opportunities to utilize MVOC-mediated technology to improve agriculture in an environmentally friendly and sustainable fashion (Berg, 2009; Kanchiswamy et al., 2015a; Baez-Rogelio et al., 2016). However, with the relatively low levels of growth enhancement demonstrated thus far and the few plant species studied, the practical application involving direct use of MVOC emitting microorganisms has remained limited (Song and Ryu, 2013; Li et al., 2016; Cordovez et al., 2017; Fincheira and Quiroz, 2018). Furthermore, all effective MVOC species, their interactions and direct correlation between MVOC and plant response need to be determined. In this study, we demonstrated that short term exposure *in vitro* to an environmentally isolated fungal strain TC09 identified as *C. sphaerospermum* dramatically promoted tobacco plant growth via MVOCs. We showed in pilot scale experiments under greenhouse conditions that crop productivity of pepper plants could be significantly enhanced by exposure *in vitro* to TC09 at the seedling stage.

The effects of TC09 exposure on plant growth were largely correlated with the duration of exposure. The stem length of treated tobacco plants doubled after 20 days relative to controls with as little as a 24-h exposure. Exposure duration of 10 days gave maximal PGP as durations beyond 10 days had no significant additive effect. These observed dynamic changes in growth stimulation could potentially be explained by the loss of MVOC emission by TC09 over longer culturing times, saturated plant signaling receptors, or alternatively as a consequence of the depletion of nutrients in the media by rapidly growing plants. To test the latter hypothesis, we performed experiments to test

TABLE 2 | Fold increase over control of growth parameters from tobacco plants exposed to MVOCs emitted by various *Cladosporium fungi*.

Growth parameters	Item	Species/isolates of *Cladosporium*							
		CK	***C sp* TC09**	***C sp* NRRL**	***C cl* 113 db**	***C as* 208 db**	***C sub* WF**	***C cl* W99**	***C mac* phy**
Total plant	Average (SD)	4.63 (0.880)	16.92 (0.657)	17.44 (1.079)	17.07 (2.389)	16.66 (1.316)	10.71 (1.263)	12.19 (0.686)	8.78 (1.241)
height (cm)	Fold over CK[5]	0	2.7	**2.8**	2.7	2.6	1.3	1.6	0.9
	SD label[1]	a	b	b	b	b	c	c	d
Total plant	Average (SD)	0.05 (0.015)	6.22 (0.829)	4.21 (1.063)	4.76 (0.595)	3.65 (1.170)	1.18 (0.311)	1.50 (0.709)	1.18 (0.179)
fresh Wt (g)	Fold over CK	0	**123.4**	83.1	94.1	72.0	22.7	29.1	22.6
	SD label	a	b	c	c	c	d	d	d
No. of leaves	Average (SD)	5.7 (0.707)	10.4 (0.527)	8.9 (0.500)	8.6 (0.500)	9.7 (0.333)	8.7 (0.527)	8.4 (0.527)	7.3 (0.500)
per plant	Fold over CK	0	**0.8**	0.6	0.5	0.7	0.5	0.5	0.3
	SD label	a	b	c	c	b	c	c	d
Stem	Average (SD)	0.30 (0.050)	10.59 (0.764)	11.72 (1.045)	11.20 (1.997)	11.27 (1.054)	4.22 (1.032)	6.04 (0.704)	2.81 (0.562)
length (cm)[2]	Fold over CK	0	34.3	**38.1**	36.3	36.6	13.1	19.1	8.4
	SD label	a	b	b	b	b	c	c	c
Root	Average (SD)	4.33 (0.910)	6.33 (1.067)	5.72 (0.885)	5.89 (0.985)	5.39 (1.179)	6.49 (0.800)	6.14 (0.987)	5.97 (0.837)
length (cm)[3]	Fold over CK	0	**0.5**	0.3	0.4	0.2	0.5	0.4	0.4
	SD label	a	b	a	b	a	b	b	b
Largest leaf	Average (SD)	0.50 (0.087)	5.48 (0.576)	5.02 (0.733)	5.13 (0.572)	4.02 (1.017)	2.70 (0.695)	3.11 (0.622)	3.13 (0.180)
length (cm)[4]	Fold over CK	0	**10.0**	9.0	9.3	7.0	4.4	5.2	5.3
	SD label	a	b	b	b	c	d	cd	cd
Stem	Average (SD)	0.92 (0.136)	4.35 (0.235)	3.44 (0.575)	3.39 (0.317)	3.17 (0.521)	1.74 (0.345)	2.09 (0.416)	2.22 (0.417)
diameter (mm)	Fold over CK	0	**3.7**	2.7	2.7	2.4	0.9	1.3	1.4
	SD label	a	b	c	c	c	d	d	d

Names of species and isolates are abbreviated and described in the section "Materials and Methods." Average values were derived from triplicate vessels with three plants each for each treatment. Highest values of fold increase over control (CK) in each category were in bold letters. [1] Significant difference label generated by Tukey's mean separation test. Average values sharing a letter within a growth parameter are not significantly different at P = 0.05. Data were averaged from three plants in each replicate vessel and three vessels per treatment. [2] The length of stem segment measuring from the base of the plant to the top of shoot apex. [3] The length of root system measuring from the base of the plant to the tip of longest root. [4] The vertical length of the blade of a largest leaf of each plant excluding petiole. [5] Fold over CK reflects average value fold increase of treatment over control as follows:

$$\text{Fold over CK} = \frac{\text{Average value of treatment} - \text{Average value of control}}{\text{Average value of control}}$$

the requirement for sucrose in the tissue culture media and found that sugar concentrations of >5 g/L were necessary for the observed PGP activity but did not enhance PGP beyond 10g/L. These data suggest that endogenous sugar production in young plants is not sufficient to support robust PGP by TC09 and that sustained PGP requires exogenous carbon sources. This finding may also imply that TC09 MVOCs function by activating some signaling pathway(s) in the plant that allows it to better utilize carbon resources available directly through root uptake.

Another contributing factor to PGP by TC09 was the fungal cultivation medium. MS medium supported the highest level of PGP while some common fungal growth media such as Hutner's medium not only suppressed fungal growth but also reduced PGP activity. MVOC-mediated PGP has been previously shown to vary according to microbial culture conditions. By using rhizobacteria *Bacillus mojavensis* RRC101, Rath et al. (2018) demonstrated such variability in microbial metabolism in conjunction with PGP activity. When cultured on ½ strength MS medium, the bacteria produced known PGP compounds acetoin and 2,3-butanediol, thus leading to improved growth of Arabidopsis seedlings. However, the use of nutrient agar for bacterial cultivation resulted in the production of relatively high levels of acetone and 2-propanone and correlated with phytotoxicity. In addition, the microbial volatile compound, e.g., β-caryophyllene which is commonly produced by fungi was shown to switch its mode of action from stimulatory to inhibitory depending on concentrations (Wang et al., 2009). Other studies also established that both the diversity and absolute quantity of MVOCs emitted by many microbes are dictated by growth conditions and external stimuli (Sunesson

TABLE 3 | DEGs, associated major biological functions and expression changes from tobacco seedlings with exposure to TC09 for 10 days.

Annotation	Proposed function	Feature ID	Fold Incr
Auxin response factor 8B	Phytohormone synthesis and regulation	Solyc02g037530.3	2.3
Auxin-regulated IAA17	Phytohormone synthesis and regulation	Solyc06g008590.3	4.3
Auxin-regulated IAA19	Phytohormone synthesis and regulation	Solyc03g120380.3	2.6
Auxin-regulated IAA7	Phytohormone synthesis and regulation	Solyc06g053830.3	2.1
Auxin-regulated IAA16	Phytohormone synthesis and regulation	Solyc01g097290.3	2.1
GRAS family transcription factor	Phytohormone synthesis and regulation	Solyc08g014030.1	4.0
Cytokinin oxidase/dehydrogenase	Phytohormone synthesis and regulation	Solyc04g080820.2.1	4.3
Protease inhibitor/seed storage	Insect Resistance	Solyc01g066910.3	3.6
Kunitz trypsin inhibitor	Insect Resistance	Solyc06g072230.1	2.3
Major latex-like protein	Insect Resistance	Solyc05g046140.3	3.3
Major latex-like protein	Insect Resistance	Solyc05g046160.1	4.0
Cytochrome P450 family protein	Insect R, Coumarins biosynth	Solyc04g080100.3	3.4
Cytochrome P450	Insect R, Coumarins biosynth	Solyc04g083160.2	2.2
O-Methyltransferase 3	Insect R, Furanocoumarin biosynth	Solyc10g008120.2.1	1.9
O-Methyltransferase	Insect R, Furanocoumarin biosynth	Solyc01g111900.3	3.2
O-Methyltransferase-like protein	Insect R, Furanocoumarin biosynth	Solyc12g009110.1.1	3.0
O-Methyltransferase	Insect R, Furanocoumarin biosynth	Solyc06g064510.2.1	3.6
O-Methyltransferase	Insect R, Furanocoumarin biosynth	Solyc01g068550.2.1	4.0
O-Methyltransferase	Insect R, Furanocoumarin biosynth	Solyc10g047520.1.1	9.1
O-Methyltransferase	Insect R, Furanocoumarin biosynth	Solyc06g064500.2.1	14.6
Jasmonic acid 1 (JA-1)	ISR activation, JA signaling pathway	Solyc05g007180.3	2.3
Pathogenesis-related thaumatin	Defense	Solyc04g081560.3	4.4
Defensin	Defense	Solyc04g008470.3	4.0
Pathogenesis-related thaumatin	Defense	Solyc02g083790.3	2.3
Pathogenesis-related thaumatin	Defense	Solyc10g084840.2	2.3
Pathogenesis-related thaumatin	Defense	Solyc03g118780.3	2.3
Peroxidase	Defense	Solyc02g094180.3	2.3
Endoglucanase	Defense	Solyc12g055970.2	2.2
UDP-glucose glucosyltransferase	Glycosylation, hormone modulation	Solyc08g062220.2.1	−9.9
UDP-glucose glucosyltransferase	Glycosylation, hormone modulation	Solyc04g016220.2.1	−2.9
UDP-glucose glucosyltransferase	Glycosylation, hormone modulation	Solyc08g006330.2.1	−2.47
UDP-glucose glucosyltransferase	Glycosylation, hormone modulation	Solyc08g006350.2.1	−2.3
UDP-glucosyltransferase family 1 protein	Glycosylation, hormone modulation	Solyc12g042600.1.1	−2.2
UDP-glucose glucosyltransferase	Glycosylation, hormone modulation	Solyc08g006410.2.1	−2.2
Sucrose transporter-like protein	Alternative carbon assimilation	Solyc05g007190.2.1	1.5
Glucose-1-phosphate adenylyltransferase	Alternative carbon assimilation	Solyc01g109790.2.1	1.4
Glucose-6-phosphate translocator	Alternative carbon assimilation	Solyc07g064270.2.1	1.4
Glucose transporter 8	Alternative carbon assimilation	Solyc01g080680.2.1	1.8
UDP-glucose 6-dehydrogenase	Alternative carbon assimilation	Solyc02g088690.2.1	1.5
UDP-D-glucose dehydrogenase	Alternative carbon assimilation	Solyc02g067080.2.1	1.7
UDP-glucose dehydrogenase	Alternative carbon assimilation	Solyc03g115380.1.1	1.8
Cyclin A1	Cell cycle	Solyc11g005090.1.1	1.7
Cyclin-dependent kinase	Cell cycle	Solyc09g065200.2.1	1.8
Cyclin B	Cell cycle	Solyc12g094600.1.1	1.9
Cyclin D	Cell cycle	Solyc12g088650.1.1	2.1
Cyclin D3-1	Cell cycle	Solyc04g078470.2.1	1.8
Cyclin-like protein	Cell cycle	Solyc01g087450.2.1	2.3
Cyclin-dependent kinase regulator Pho80	Cell cycle	Solyc01g089850.2.1	2.6
Cyclin-dependent kinase	Cell cycle	Solyc01g090800.2.1	8.0
Extensin	Cell expansion	Solyc04g071070.2	4.4
Expansin-1	Cell expansion	Solyc06g076220.2.1	1.6
Expansin – pollen	Cell expansion	Solyc12g089380.1.1	1.6
Expansin	Cell expansion	Solyc02g081210.2.1	1.7

(Continued)

TABLE 3 | Continued

Annotation	Proposed function	Feature ID	Fold Incr
Expansin – pollen	Cell expansion	Solyc06g051800.2.1	2.1
Expansin – pollen	Cell expansion	Solyc09g010860.2.1	2.4
Expansin-1	Cell expansion	Solyc10g086520.1.1	2.5
Expansin – pollen	Cell expansion	Solyc00g017230.1.1	2.6
Expansin – pollen	Cell expansion	Solyc10g084780.1.1	2.9
Expansin B1 – pollen	Cell expansion	Solyc10g008440.2.1	3.0
Expansin	Cell expansion	Solyc10g084780.2	3.5
Expansin – pollen	Cell expansion	Solyc02g088100.2.1	4.7

Fold change in expression levels was calculated based on means of four biological replicates for both control and MVOC-treated plants. Genes were annotated using GO terms and tomato (Solanum lycopersicum) reference genes available on https://solgenomics.net.

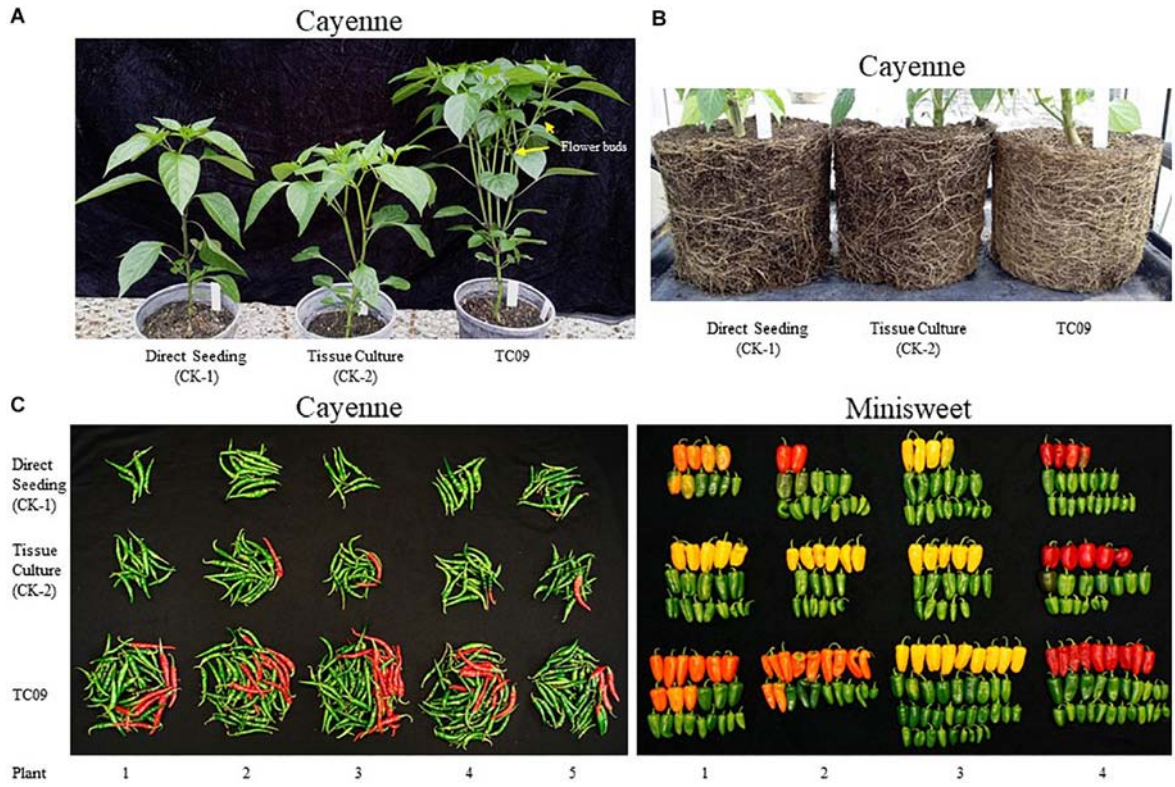


FIGURE 8 | Plant growth and fruit production of pepper (*Capsicum annuum*) varieties cayenne 'Long Thin' and sweet bell 'Minisweet Pepper Mixed' promoted by exposure *in vitro* to TC09. TC09 exposure for 20 days at seedling stage was identical to that used for tobacco. **(A)** Representative cayenne plants from direct seeding, tissue culture and TC09 treatments at 50 days post seed sowing. **(B)** Representative belowground image of cayenne pepper plants from various treatments at 116 days post seed sowing to depict the density and development of fibrous root mass. **(C)** Lineup of fruits collected from cayenne pepper (left panel, at 116 days post seed sowing) and minisweet pepper (right panel, at 160 days post seed sowing) from all treatments. Fruits in reddish color indicate vine-ripening.

et al., 1995; Weikl et al., 2015; Lee et al., 2016; Nieto-Jacobo et al., 2017). Our findings of altered PGP activities derived from the use of different fungal culture media suggest that culture conditions for TC09 and other PGP microbes can be further exploited and optimized to achieve more effective PGP.

The observed levels of PGP upon exposure of tobacco seedlings to TC09 greatly exceeded those previously reported using other microorganisms (Ryu et al., 2003; Minerdi et al., 2011; Paul and Park, 2013; Bitas et al., 2015; Lee et al., 2016; Sánchez-López et al., 2016b; Cordovez et al., 2017; Tahir et al., 2017). We found that varied levels of PGP activity were also exhibited by diverse species of *Cladosporium*, however, this activity was largely strain specific. Paul and Park (2013) previously reported an approximately 10-fold increase in fresh weight of tobacco plants exposed to *C. cladosporioides* CL-1 cultured on PDA for 28 days (4 weeks). The results reported here suggest that differences between various studies could be due to several factors including the specific strain or isolate used, the culture media used for both the plant and fungus,

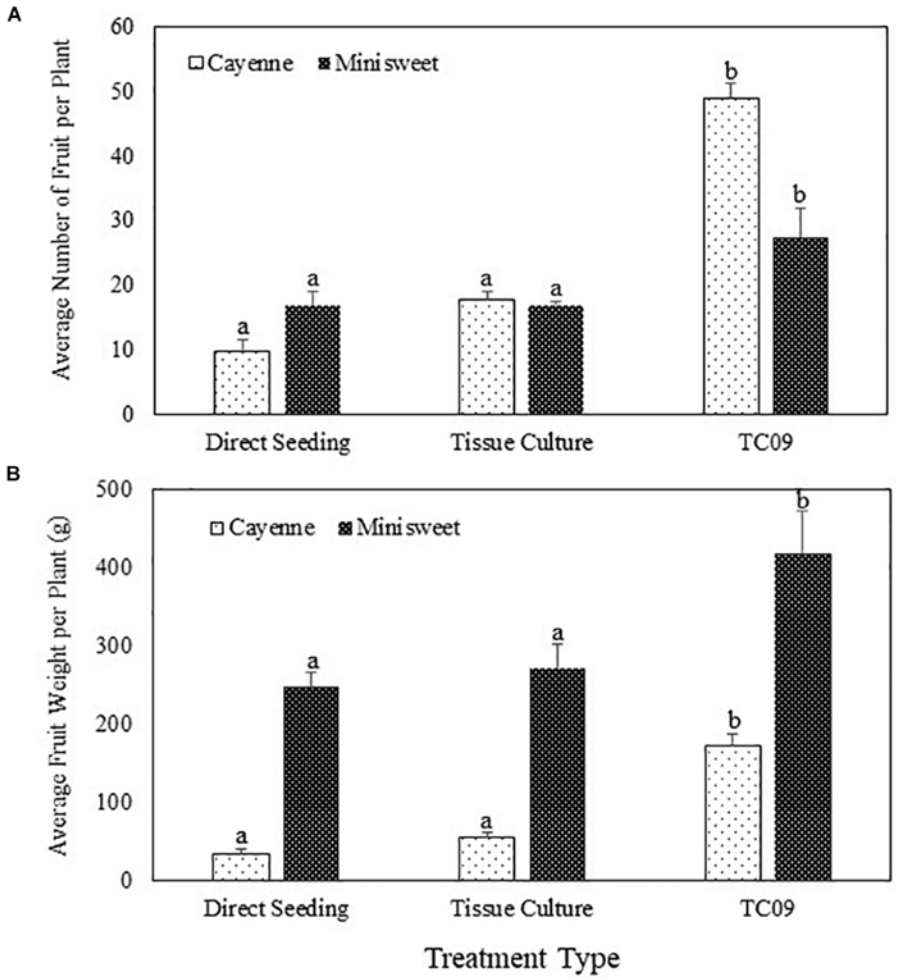


FIGURE 9 | Fruit yield of pepper (*Capsicum annuum*) varieties cayenne 'Long Thin' and sweet bell 'Minisweet Pepper Mixed' promoted by exposure *in vitro* to TC09. Fruits were collected at 116 days and 160 days post seed sowing for cayenne and minisweet peppers, respectively. Greenhouse tests with these two pepper varieties were carried out separately at different time periods of the year due to space limitations. **(A)** Average number of fruit per plant. **(B)** Average fresh weight of fruit per plant. Standard errors of the means were labeled as vertical lines on top of data bars. Means sharing a letter in the group label are not significantly different ($P > 0.05$) according to Tukey's test.

and the availability of sufficient carbon resources to support maximal plant growth rates. Nevertheless, the rapid response to growth stimulants should facilitate further investigation of the mechanisms underlying PGP activity as recently outlined by Fincheira and Quiroz (2018).

A potential limitation for the use of MVOC-mediated PGP is plant damage resulting from the use of phytopathogens or microbes with parasitic behaviors. Phytopathogenic microorganisms can cause adverse effects on target plants in the case of escape and contamination during fungal exposure. In this study, TC09 did not induce any discernable pathogenic responses in all test plants, consistent with its non-pathogenic nature. The non-pathogenic behavior of TC09 may be attributed to its environmental origin and non-adaptive relationship with plants. *C. sphaerospermum* has long been known as one of the most ubiquitous air-borne, saprobic fungal species and can be found in diverse environments, air and physical structures (Park et al., 2004; Zalar et al., 2007). In contrast, we observed leaf tissue necrosis in tobacco treated with *C. sphaerospermum* NRRL 8131 (data not shown). This particular isolate was originally referenced as *Cladosporium lignicolum* Corda based on its sylvan habitat and the ability to degrade and absorb nutrients from lignified woody materials (Dugan et al., 2008). Even though it promoted plant growth similar to TC09, its ability to induce phytotoxic reactions in target plants suggests pathogenic propensity. The ability of TC09 to significantly promote plant growth via MVOCs while lacking harmful phytotoxic effects makes it particularly attractive for further exploitation and the development of practical applications to improve crop productivity.

Transcriptome analyses previously revealed the correlation between differential gene expression and PGP as well as enhanced tolerance to biotic and abiotic stresses following treatment with

beneficial microorganisms and MVOCs (e.g., van Loon, 2007; Zhang et al., 2007; Sánchez-López et al., 2016a). Our RNAseq data from untreated and treated tobacco plants provided evidence that diverse metabolic and signaling pathways are altered as a result of exposure to TC09. The most striking observation was that TC09 treatment was accompanied by down-regulation of photosystem functions. This finding is somewhat inconsistent with several previous reports that MVOCs from various microbes caused substantial positive impacts on photosynthesis and chlorophyll production (see review by Fincheira and Quiroz, 2018). The transcriptome data also revealed a potential increase in cell wall metabolism including up-regulation of numerous cellulose synthase genes, endoglucanases, pectinesterases, fasciclins, and xyloglucan endotransglucosylases. A number of putative expansins were also induced which are known to induce slippage of cellulose microfibrils via a non-enzymatic cell wall loosening mechanism and are responsible for cell size increase (Sampedro and Cosgrove, 2005; Choi et al., 2008). In previous MVOC-related studies, *Fusarium oxysporum* reportedly was able to upregulate expression of an expansin A5 gene in lettuce (Minerdi et al., 2011). Volatiles from rhizobacteria *B. subtilis* (GB03) also reportedly increased expression of eight expansin genes ∼2-fold (Zhang et al., 2007). Our results indicate that up to 11 expansin genes in treated tobacco plants were up-regulated twofold to fivefold (five genes with more than threefold) over the control.

The rapid growth enhancement following MVOC or microbial exposure may also reflect promoted mitosis and cell cycle that give rise to increased cell numbers. We identified a number of cyclins and CDKs as DEGs in tobacco plants exposed to TC09. Mitotic-specific cyclins and CDKs are known to interact in partner-dependent fashion to form active complexes that function to modulate cell cycle during different phases of cell division (Mironov et al., 1999). This process is also regulated by mitogenic stimulation through protein docking, CDK phosphorylation, and proteolytic degradation of cell cycle proteins (Breyne et al., 2002). Homeostatic alterations in the expression of these proteins has been linked to either faster or slower cell multiplication (Komaki and Sugimoto, 2012). Noticeably, a previous study reported that ectopic over-expression of a cyclin B in Arabidopsis resulted in promoted cell proliferation in roots (Doerner et al., 1996). Our findings provide a direction for further study to address the influence of MVOCs on cell cycle regulation.

Phytohormones including cytokinin, GA3 and auxin can modulate plant growth and development and their activities are dictated by either biosynthesis or homeostasis of active forms. Interestingly, we observed a reduction (2- to 10-fold) in six UDP-glucose glycosyltransferases (UGGTs), which play an important role in the deactivation, homeostasis, storage and secretion of numerous macromolecules including phytohormones via glycosylation modification (Jones and Vogt, 2001; Ostrowski and Jakubowska, 2014). Kim et al. (2015) also reported the down-regulation of a glycosyltransferase in tobacco in response to MVOCs emitted by rhizobacteria *B. subtilis* although the functional involvement was not elucidated. We postulate that down-regulation of this class of genes may reduce UGGT activity. This consequently leads to minimized deactivation of phytohormones thus potentially increasing the quantity of active phytohormones galvanizing various aspects of plant growth. Early flowering induced by MVOCs was previously attributed to the enhancement of cytokinin action (Sánchez-López et al., 2016b).

Our transcriptome data indicated that the core transcriptional regulator JA-1 in jasmonic acid (JA) signaling pathway was up-regulated more than twofold in tobacco plants exposed to TC09. Also, many herbivore defense genes were up-regulated as well, including proteinase inhibitor II (21-fold), seed storage type protease inhibitor (3.6-fold), Kunitz trypsin inhibitor (2.3-fold) and three cytochrome P450 proteins with sequences homologous to a previously reported protein associated with wound healing and pest resistance (Noordermeer et al., 2001; Dunaevsky et al., 2005). Increased expression levels (threefold to fourfold) of eight latex-like proteins were identified which have been shown to have insecticidal activities against herbivore pests (Konno, 2011). Likewise, seven *O*-methyltransferases belonging to a class of catalytic enzymes involved in biosynthesis pathway of FCs also showed elevated expression (2- to 15-fold) (Bourgaud et al., 2006, 2014). FCs are secondary metabolites that function mainly as insect repellants (Nitao et al., 2003). These findings prompt future studies to evaluate whether TC09-exposed plants exhibit enhanced resistance to insect pests. Indeed, previous studies have likewise identified MVOC-induced expression of genes associated with JA signaling pathway, broad-spectrum induced systemic resistance (ISR) and herbivore resistance (Song et al., 2013; Naznin et al., 2014; Kanchiswamy et al., 2015a; Chung et al., 2016).

In this study, we demonstrated significant stimulation of plant growth, early reproduction and fruit yield increase in pepper by exposing seedlings to TC09 for a relatively short duration. Although results were achieved under greenhouse conditions, the high level of productivity increase without the additional input of other resources warrants further investigation as to whether such technology could be practically applied at a production scale. Many considerations have to be taken into account including microbe cultivation and conditions, MVOC deployment and dosage control, plant treatment stage and duration, post treatment process and transplanting methodology. In addition, future investigations are needed to study the genome, transcriptome and proteome of TC09 to identify genes, pathways and regulatory mechanisms associated with VOC production and PGP activity. The TC09 strain described here provides a useful tool to carry out such studies and highlights the potential of microbe-based PGP technology to benefit agriculture in an environmentally friendly and sustainable fashion.

AUTHOR CONTRIBUTIONS

ZTL, WJJ, and CD conceived the concept and designed the experiments. ZTL performed *in vitro* experiments, analyzed the

data, and drafted the manuscript. CD carried out transcriptome assembly and analyses. WJJ, WMJ, and BE conducted fungal identification experiments. ZL and AC provided scientific advice and discussed results. ZTL, CD, WJJ, WMJ, ZL, and AC reviewed the final version of the paper.

FUNDING

This research was supported in part by grants from AFRS, ARS, USDA and the Oak Ridge Institute for Science and Education (ORISE).

ACKNOWLEDGMENTS

We thank Dr. Frank M. Dugan for kindly providing *Cladosporium* fungi and also gratefully acknowledged Dr. Margaret Pooler, Research Leader, FNPR, ARS-USDA, for providing financial support. We also thank Dr. Tracy C. Leskey, Director, AFRS, ARS-USDA, for excellent scientific advice and support for this research and Mark A. Demuth, Elizabeth Lutton, and Dennis R. Bennett for their excellent technical assistance. The handling and movement of all fungi described in the current study were carried using Standard Operation Procedures in accordance with the Animal and Plant Health Inspection Service of the United States Department of Agriculture. Acquisition and movement of mentioned fungal materials were authorized under a permit (P526P-17-02230) issued by the agency.

SUPPLEMENTARY MATERIAL

The Supplementary Material for this article can be found online at: https://www.frontiersin.org/articles/10.3389/fpls.2018.01959/full#supplementary-material

FIGURE S1 | Neighbor-joining dendrogram of ITS sequences associated with TC09. The 249 bp ITS3/4 amplicon consensus sequence derived from PCR amplification using DNA samples of the fungal isolate was used to search GenBank database for homologous sequences. More than 148 sequence entries were retrieved and subject to phylogenetic analysis. Neighbor-joining method was used to reconstruct the phylogenetic tree with bootstrap values indicated for each branch. Sequence entries belonging to same species were labeled using identical colors. Asterisk indicates the caudal position of the fungal isolate within the monophyletic group *Cladosporium sphaerospermum*.

FIGURE S2 | Heat map depicting high (red) and low (blue) relative levels of differentially expressed genes (DEGs) in individual plants with and without exposure to TC09. Six-day-old tobacco seedlings were maintained under *in vitro* conditions with or without a 10-day exposure to TC09 and then subject to total RNA extraction using shoot tip tissues that include apical meristem and three to four young leaves. Heat map was generated by using CLC Genomics Workbench software program following transcriptome assembly using tomato transcriptome as a reference. Plant treatment type is marked by red (control) and blue (TC09-treated) line on top. There were four replicated individual plants for each treatment. Color-rendered scale of expression changes is marked on bottom.

FIGURE S3 | Colony of *Cladosporium sphaerospermum* TC09 on PDA medium **(A)**; and confocal microscopy micrographs of branching chains of conidia **(B)**; intercalary and terminal conidia **(C)**; and mycelium **(D)**.

TABLE S1 | List of all tobacco DEGs after exposure *in vitro* to TC09 for 10 days. Fold change in expression levels was calculated based on means of four biological replicates for both control and MVOC-treated plants. Genes were annotated using GO terms and tomato (*Solanum lycopersicum*) reference genes available on https://solgenomics.net.

REFERENCES

Ababutain, I. M. (2013). Effect of some ecological factors on the growth of *Aspergillus niger* and *Cladosprium sphaerospermum*. *Am. J. Appl. Sci.* 10, 159–163. doi: 10.3844/ajassp.2013.159.163

Ahemad, M., and Kibret, M. (2014). Mechanisms and applications of plant growth promoting rhizobacteria, current perspective. *J. King Saud. Univ. Sci.* 26, 1–20. doi: 10.1016/j.jksus.2013.05.001

Ahmad, F., Ahmad, I., and Khan, M. S. (2008). Screening of free-living rhizospheric bacteria for their multiple plant growth promoting activities. *Microb. Res.* 163, 173–181. doi: 10.1016/j.micres.2006.04.001

Audrain, B., Létoffé, S., and Ghigo, J. M. (2015). Airborne bacterial interactions: functions out of thin air? *Front. Microbiol.* 6:1476. doi: 10.3389/fmicb.2015.01476

Baez-Rogelio, A., Morales-Garcıa, Y. E., Quintero-Hernandez, V., and Munoz-Rojas, J. (2016). Next generation of microbial inoculants for agriculture and bioremediation. *Microb. Biotechnol.* 10, 19–21. doi: 10.1111/1751-7915.12448

Barea, J. M., Pozo, M. J., Azcon, R., and Azcon-Aguilar, C. (2005). Microbial co-operation in the rhizosphere. *J. Exp. Bot.* 56, 1761–1778. doi: 10.1093/jxb/eri197

Berg, G. (2009). Plant–microbe interactions promoting plant growth and health: perspectives for controlled use of microorganisms in agriculture. *Appl. Microbiol. Biotechnol.* 84, 11–18. doi: 10.1007/s00253-009-2092-7

Berg, G., and Smalla, K. (2009). Plant species and soil type cooperatively shape the structure and function of microbial communities in the rhizosphere. *FEMS Microbiol. Ecol.* 68, 1–13. doi: 10.1111/j.1574-6941.2009.00654.x

Bitas, V., McCartney, N., Li, N., Demers, J., Kim, J. E., Kim, H. S., et al. (2015). *Fusarium Oxysporum* volatiles enhance plant growth via affecting auxin transport and signaling. *Front. Microb.* 6:1248. doi: 10.3389/fmicb.2015.01248

Bourgaud, F., Hehn, A., Larbat, R., Doerper, S., Gontier, E., Kellner, S., et al. (2006). Biosynthesis of coumarins in plants: a major pathway still to be unravelled for cytochrome P450 enzyme. *Phtochem* 5, 293–308. doi: 10.1007/s11101-006-9040-2

Bourgaud, F., Olry, A., and Hehn, A. (2014). "Recent advances in molecular genetics of furanocoumarin synthesis in higher plants," in *Recent Advances in Redox Active Plant and Microbial Products*, eds C. Jacob, G. Kirsch, A. Slusarenko, P. Winyard, and T. Burkholz (Dordrecht: Spinger).

Breyne, P., Dreesen, R., Vandepoele, K., De Veylder, L., Van Breusegem, F., Callewaert, L., et al. (2002). Transcriptome analysis during cell division in plants. *PNAS* 99, 14825–14830. doi: 10.1073/pnas.222561199

Choi, D., Kim, J. H., and Lee, Y. (2008). Expansins in plant development. *Adv. Bot. Res.* 47, 47–97. doi: 10.1016/S0065-2296(08)00002-5

Chung, J. H., Song, G. C., and Ryu, C. M. (2016). Sweet scents from good bacteria: case studies on bacterial volatile compunds for plant growth and immunity. *Plant Mol. Biol.* 90, 677–687. doi: 10.1007/s11103-015-0344-8

Cordovez, V., Mommer, L., Moisan, K., Lucas-Barbosa, D., Pierik, R., Mumm, R., et al. (2017). Plant phenotypic and transcriptional changes induced by volatiles from the fungal root pathogen *Rhizoctonia solani*. *Front. Plant Sci.* 8:1262. doi: 10.3389/fpls.2017.01262

De Souza, R., Ambrosini, A., and Passaglia, L. M. P. (2015). Plant growth-promoting bacteria as inoculants in agricultural soils. *Genet. Mol. Biol.* 38, 401–419. doi: 10.1590/S1415-475738420150053

De-la-Peña, C., and Loyola-Vargas, V. M. (2014). Biotic interactions in the rhizosphere: a diverse cooperative enterprise for plant productivity. *Plant Physiol.* 166, 701–719. doi: 10.1104/pp.114.241810

Doerner, P., Jørgensen, J. E., You, R., Steppuhn, J., and Lamb, C. (1996). Control of root growth and development by cyclin expression. *Nature* 380, 520–523. doi: 10.1038/380520a0

Dugan, F. M., Braun, U., Groenwald, J. Z., and Crous, P. W. (2008). Morphological plasticity in *Cladosporium sphaerospermum*. *Persoonia* 21, 9–16. doi: 10.3767/003158508X334389

Dunaevsky, Y. E., Elpidina, E. N., Vinokurov, K. S., and Belozersky, M. A. (2005). Protease inhibitors in improvement of plant resistance to pathogens and insects. *Plant Mol.* 39, 702–708. doi: 10.1007/s11008-005-0076-y

Farag, M. A., Song, G. C., Park, Y. S., Audrain, B., Lee, S., Ghigo, J. M., et al. (2017). Biological and chemical strategies for exploring inter- and intra-kingdom communication mediated via bacterial volatile signals. *Nat. Protoc.* 12:1359. doi: 10.1038/nprot.2017.023

Fincheira, P., and Quiroz, A. (2018). Microbial volatiles as plant growth inducers. *Microbiol. Res.* 208, 63–75. doi: 10.1016/j.micres.2018.01.002

Jones, P., and Vogt, T. (2001). Glycosyltransferases in secondary plant metabolism: tranquilizers and stimulant controllers. *Planta* 213, 164–174. doi: 10.1007/s004250000492

Kai, M., and Piechulla, B. (2014). Impact of volatiles of the rhizobacteria *Serratia odorifera* on the moss *Physcomitrella patens*. *Plant Signal. Behav.* 5, 444–446. doi: 10.4161/psb.5.4.11340

Kanchiswamy, C. N., Malnoy, M., and Maffei, M. E. (2015a). Bioprospecting bacterial and fungal volatiles for sustainable agriculture. *Trends Plant Sci.* 20, 206–211. doi: 10.1016/j.tplants.2015.01.004

Kanchiswamy, C. N., Malnoy, M., and Maffei, M. E. (2015b). Chemical diversity of microbial volatiles and their potential for plant growth and productivity. *Front. Plant Sci.* 6:151. doi: 10.3389/fpls.2015.00151

Kim, J. S., Lee, J., Seo, S. G., Lee, C., Woo, S. Y., and Kim, S. H. (2015). Gene expression profile affected by volatiles of new plant growth promoting rhizobacteria, *Bacillus subtilis* strain JS, in tobacco. *Genes Genom.* 37, 387–397. doi: 10.1007/s13258-015-0267-4

Komaki, S., and Sugimoto, K. (2012). Control of the plant cell cycle by developmental and environmental cues. *Plant Cell Physiol.* 53, 953–964. doi: 10.1093/pcp/pcs070

Konno, K. (2011). Plant latex and other exudates as plant defense systems: roles of various defense chemicals and proteins contained therein. *Phytochem* 72, 1510–1530. doi: 10.1016/j.phytochem.2011.02.016

Kremer, R. J., and Souissi, T. (2001). Cyanide production by rhizobacteria and potential for suppression of weed seedling growth. *Curr. Microb.* 43, 182–186. doi: 10.1007/s002840010284

Lambers, H., Mougel, C., Jaillard, B., and Hinsinger, P. (2009). Plant-microbe-soil interactions in the rhizosphere: an evolutionary perspective. *Plant Soil* 321, 83–115. doi: 10.1007/s11104-009-0042-x

Lee, S., Yap, M., Behringer, G., Hung, R., and Bennett, J. W. (2016). Volatile organic compounds emitted by *Trichoderma* species mediate plant growth. *Fungal Biol. Biotechnol.* 3:7. doi: 10.1186/s40694-016-0025-7

Lemfack, M. C., Nickel, J., Dunkel, M., Preissner, R., and Piechulla, B. (2014). mVOC, a database of microbial volatiles. *Nucleic Acids Res.* 42, D744–D748. doi: 10.1093/nar/gkt1250

Li, N., Alfiky, A., Vaughan, M. M., and Kang, S. (2016). Stop and smell the fungi: fungal volatile metabolites are overlooked signals involved in fungal interaction with plants. *Fungal Biol. Rev.* 30, 134–144. doi: 10.1016/j.fbr.2016.06.004

Liu, X. M., and Zhang, H. (2015). The effects of bacterial volatile emission on plant abiotic stress tolerance. *Front. Plant Sci.* 6:774. doi: 10.3389/fpls.2015.00774

Minerdi, D., Bossi, S., Maffei, M. E., Gullino, M. L., and Garibaldi, A. (2011). *Fusarium oxysporum* and its bacterial consortium promote lettuce growth and expansin A5 gene expression through microbial volatile organic compound (MVOC) emission. *FEMS Microbiol. Ecol.* 76, 342–351. doi: 10.1111/j.1574-6941.2011.01051.x

Mironov, V., De Veylder, L., Van Maontagu, M., and Inze, D. (1999). Cyclin-dependent kinases and cell division in plants-the nexus. *Plant Cell* 11, 509–521.

Murashige, T., and Skoog, F. (1962). A revised medium for rapid growth and bio assays with tobacco tissue cultures. *Physiol. Plant.* 15, 473–497. doi: 10.1111/j.1399-3054.1962.tb08052.x

Nadeem, S. M., Ahmad, M., Zahir, Z. A., Javaid, A., and Ashraf, M. (2014). The role of mycorrhizae and plant growth promoting rhizobacteria (PGPR) in improving crop productivity under stressful environments. *Biotech. Adv.* 32, 429–448. doi: 10.1016/j.biotechadv.2013.12.005

Naznin, H. A., Kiyohara, D., Kimura, M., Miyazawa, M., Shimizu, M., and Hyakumachi, M. (2014). Systemic resistance induced by volatile organic compounds emitted by plant growth-promoting fungi in *Arabidopsis thaliana*. *PLoS One* 9:e86882. doi: 10.1371/journal.pone.0086882

Nieto-Jacobo, M. F., Steyaert, J. M., Salazar-Badillo, F. B., Nguyen, D. V., Rostás, M., Braithwaite, M., et al. (2017). Environmental growth conditions of *Trichoderma* spp. Affects indole acetic acid derivatives, volatile organic compounds, and plant growth promotion. *Front. Plant Sci.* 8:102. doi: 10.3389/fpls.2017.00102

Nitao, J. K., Berhow, M., Duval, S. M., Weisleder, D., Vaughn, S. F., Zangerl, A., et al. (2003). Characterization of furanocoumarin metabolites in parsnip webworm, *Depressaria pastinacella*. *J. Chem. Ecol.* 29, 671–682. doi: 10.1023/A:1022872704016

Noordermeer, M. A., Veldink, G. A., and Vliegenthart, J. F. G. (2001). fatty acid hydroperoxide lyase: a plant cytochrome P450 enzyme involved in would healing and pest resistance. *Chembiochem* 2, 494–504. doi: 10.1002/1439-7633(20010803)2:7/8<494::AID-CBIC494>3.0.CO;2-1

Ostrowski, M., and Jakubowska, A. (2014). UDP-glycosyltransferases of plant hormones. *Adv. Cell Biol.* 4, 43–60. doi: 10.2478/acb-2014-0003

Park, H. G., Managbanag, J. R., Stamenova, E. K., and Jong, S. C. (2004). Comparative analysis of common indoor *Cladosporium* species based on molecular data and conidial characters. *Mycotaxon* 89, 441–451.

Paul, D., and Park, K. S. (2013). Identification of volatile produced by *Cladosporium cladosporioides* CL-1, a fungal biocontrol agent that promotes plant growth. *Sensors* 13, 13969–13977. doi: 10.3390/s131013969

Peñuelas, J., Asensio, D., Tholl, D., Wenke, K., Rosenkranz, M., Piechulla, B., et al. (2014). Biogenic volatile emissions from the soil. *Plant Cell Environ.* 37, 1866–1891. doi: 10.1111/pce.12340

Pieterse, C. M. J., Zamioudis, C., Berendsen, R. L., Weller, D. M., VanWees, S. C. M., and Bakker, P. A. H. M. (2014). Induced systemic resistance by beneficial microorganisms. *Annu. Rev. Phytopathol.* 52, 347–375. doi: 10.1146/annurev-phyto-082712-102340

Rath, M., Mitchell, T. R., and Gold, S. E. (2018). Volatiles produced by *Bacillus mojavensis* RRC101 act as plant growth modulators and are strongly culture-dependent. *Microbiol. Res.* 208, 76–84. doi: 10.1016/j.micres.2017.12.014

Ryu, C. M., Farag, M. A., Hu, C. H., Reddy, M. S., Kloepper, J. W., and Pare, P. W. (2004). Bacterial volatiles induce systemic resistance in Arabidopsis. *Plant Physiol.* 134, 1017–1026. doi: 10.1104/pp.103.026583

Ryu, C. M., Farag, M. A., Hu, C. H., Reddy, M. S., Wei, H. X., Pare, P. W., et al. (2003). Bacterial volatiles promote growth in Arabidopsis. *PNAS* 100, 4927–4932. doi: 10.1073/pnas.0730845100

Saharan, B. S., and Nehra, V. (2011). Plant growth promoting rhizobacteria: a critical review. *Life Sci. Med. Res.* 21, 1–30.

Sampedro, J., and Cosgrove, D. J. (2005). The expansin superfamily. *Genome Biol.* 6:242. doi: 10.1186/gb-2005-6-12-242

Sánchez-López, A. M., Bahaji, A., De Diego, N., Baslam, M., Li, J., Muñoz, F. J., et al. (2016a). Plant response to fungal volatiles is triggering by mechanisms independent of plastid phosphoglucose isomerase. *Plant Physiol.* 172, 1989–2001. doi: 10.1104/pp.16.00945

Sánchez-López, A. M., Baslam, M., De Diego, N., Muñoz, F. J., Bahaji, A., Almagro, G., et al. (2016b). Volatile compounds emitted by diverse phytopathogenic microorganisms promote plant growth and flowering through cytokinin action. *Plant Cell Environ.* 39, 2592–2608. doi: 10.1111/pce.12759

Schulz, S., and Dickschat, J. S. (2007). Bacterial volatiles: the smell of small organisms. *Nat. Prod. Rep.* 24, 814–842. doi: 10.1039/b507392h

Sinclair, J. B., and Dhingra, O. D. (1995). *Basic Plant Pathology Methods*. Boca Raton, FL: CRC Press Inc.

Song, G. C., and Ryu, C. M. (2013). Two volatile organic compounds trigger plant self-defense against a bacterial pathogen and a sucking insect in cucumber under open field conditions. *Int. J. Mol. Sci.* 14, 9803–9819. doi: 10.3390/ijms14059803

Song, G. C., Ryu, S. Y., Kim, Y. S., Lee, J. Y., Choi, J. S., and Ryu, C. M. (2013). Elicitation of induced resistance against *Pectobacterium carotovorum* and *Pseudomonas syringae* by specific individual compounds derived from native Korean plant species. *Molecules* 18, 12877–12895. doi: 10.3390/molecules181012877

Sunesson, A. L., Vaes, W. H. J., Nilsson, C. A., Blomquist, G., Andersson, B., and Carlson, R. (1995). Identification of volatile metabolites from five fungal species cultivated on two media. *Appl. Environ. Microb.* 61, 2911–2918.

Tahir, H. A. S., Gu, Q., Wu, H., Raza, W., Hanif, A., Wu, L., et al. (2017). Plant growth promotion by volatile organic compounds produced by *Bacillus subtilis* SYST2. *Front. Microbiol.* 8:171. doi: 10.3389/fmicb.2017.00171

Thakore, Y. (2006). The biopesticide market for global agricultural use. *Ind. Biotechnol.* 2, 194–208. doi: 10.1089/ind.2006.2.194

The Tomato Genome Consortium (2012). The tomato genome sequence provides insights into fleshy fruit evolution. *Nature* 485, 635–641. doi: 10.1038/nature11119

Turner, S., and Meadows-Smith, H. (2016). Selection for rare biostimulant microorganisms - finding the proverbial needles in the rhizosphere haystack. *Acta Hort.* 1148, 105–108. doi: 10.17660/ActaHortic.2016.1148.13

van Loon, L. C. (2007). Plant responses to plant growth-promoting rhizobacteria. *Eur. J. Plant Pathol.* 119, 243–254. doi: 10.1007/s10658-007-9165-1

Wang, R. L., Peng, S. L., Zeng, R. S., Ding, L. W., and Xu, Z. F. (2009). Cloning, expression and wounding induction of beta-caryophyllene synthase gene from *Mikania micrantha* HBK and allelopathic potential of beta-caryophyllene. *Allelopathy J.* 24, 35–44.

Wardle, D. A., Bardgett, R. D., Klironomos, J. N., Setala, H., Van der Putten, W. H., and Wall, D. H. (2004). Ecological linkages between aboveground and belowground biota. *Science* 304, 1629–1633. doi: 10.1126/science.1094875

Weikl, F., Ghirardo, A., Schnitzler, J. P., and Pritsch, K. (2015). Sesquiterpene emissions from *Alternaria alternate* and *Fusarium oxysporum*: effects of age, nutrient availability, and co-cultivation. *Nature* 6:22152.

Weise, T., Kai, M., and Piechulla, B. (2013). Bacterial ammonia causes significant plant growth inhibition. *PLoS One* 8:e63538. doi: 10.1371/journal.pone.0063538

Whipps, J. (2001). Microbial interactions and biocontrol in the rhizosphere. *J. Exp. Bot.* 52, 487–511. doi: 10.1093/jxb/52.suppl_1.487

White, T. J., Bruns, T., Lee, S., and Taylor, J. W. (1990). "Amplification and direct sequencing of fungal ribosomal RNA genes for phylogenetics," in *PCR Protocols: A Guide to Methods and Applications*, 1st Edn, eds M. A. Innis, D. H. Gelfandm, J. J. Sninsky, and T. J. White (New York, NY: Academic Press), 315–322.

Zalar, P., de, Hoog GS, Schroers, H. J., Crous, P. W., Groenewald, J. Z., and Gunde-Cimerman, N. (2007). Phylogeny and ecology of the ubiquitous saprobe *Cladosporium sphaerospermum*, with descriptions of seven new species from hypersaline environments. *Stud. Mycol.* 58, 157–183. doi: 10.3114/sim.2007.58.06

Zhang, H., Kim, M. S., Krishnamachari, V., Payton, P., Sun, Y., Grimson, M., et al. (2007). Rhizobacterial volatile emissions regulate auxin homeostasis and cell expansion in Arabidopsis. *Planta* 226, 839–851. doi: 10.1007/s00425-007-0530-2

Zou, C., Li, Z., and Yu, D. (2010). *Bacillus megaterium* strain XTBG34 promotes plant growth by producing 2-pentylfuran. *J. Microbiol.* 48, 460–466. doi: 10.1007/s12275-010-0068-z

Conflict of Interest Statement: The authors declare that the research was conducted in the absence of any commercial or financial relationships that could be construed as a potential conflict of interest.

frontiers in Plant Science

ORIGINAL RESEARCH
published: 05 February 2019
doi: 10.3389/fpls.2019.00060

Foliar Application of Vegetal-Derived Bioactive Compounds Stimulates the Growth of Beneficial Bacteria and Enhances Microbiome Biodiversity in Lettuce

*Francesca Luziatelli[1], Anna Grazia Ficca[1], Giuseppe Colla[2], Eva Baldassarre Švecová[2] and Maurizio Ruzzi[1]**

[1] Department for Innovation in Biological, Agrofood and Forest Systems, University of Tuscia, Viterbo, Italy, [2] Department of Agriculture and Forest Sciences, University of Tuscia, Viterbo, Italy

Many studies on plant biostimulants and organic fertilizers have been focused on the ability of these products to increase crop productivity and ameliorate crop tolerance to abiotic stresses. However, little information is available on their effect on plant microbiota, whereas it is well known that microorganisms associated with plant play crucial roles on the health and productivity of their host. The aim of this study was to evaluate the effect of a vegetal-derived protein hydrolysate (PH), a vegetal-derived PH enriched with copper (Cu-PH), and a tropical plant extract enriched with micronutrients (PE) on shoot growth and the epiphytic bacterial population of lettuce plants and the ability of these products to enhance the growth of beneficial or harmful bacteria. The three plant-derived products enhanced shoot biomass of lettuce plants indicating a biostimulant effect of the products. Data obtained using culture-independent (Terminal Restriction Fragment Length Polymorphism and Next Generation Sequencing) and culture-dependent approaches indicated that foliar application of commercial products altered the composition of the microbial population and stimulated the growth of specific bacteria belonging to *Pantoea*, *Pseudomonas*, *Acinetobacter*, and *Bacillus* genus. Data presented in this work demonstrated that some of these strains exhibited potential plant growth-promoting properties and/or biocontrol activity against fungi and bacteria phytopathogens including *Fusarium*, *Trichoderma*, and *Erwinia* species. No indication of potential health risks associated to the enrichment of human or plant bacterial pathogens emerged by the analysis of the microbiota of treated and no-treated plants. Overall, the findings presented in this study indicate that the commercial organic-based products can enhance the growth of beneficial bacteria occurring in the plant microbiota and signals produced by these bacteria can act synergistically with the organic compounds to enhance plant growth and productivity.

Keywords: vegetal protein hydrolysates, *Lactuca sativa* L., plant microbiota, terminal restriction fragment length polymorphism, next generation sequencing, plant growth-promoting bacteria, biocontrol activity

OPEN ACCESS

Edited by:
Martine Dorais, Laval University, Canada

Reviewed by:
Antonio Ferrante, University of Milan, Italy
Andrea Ertani, University of Padova, Italy

****Correspondence:***
Maurizio Ruzzi
ruzzi@unitus.it

Specialty section:
This article was submitted to Crop and Product Physiology, a section of the journal Frontiers in Plant Science

Received: *29 September 2018*
Accepted: *16 January 2019*
Published: *05 February 2019*

Citation:
Luziatelli F, Ficca AG, Colla G, Baldassarre Švecová E and Ruzzi M (2019) Foliar Application of Vegetal-Derived Bioactive Compounds Stimulates the Growth of Beneficial Bacteria and Enhances Microbiome Biodiversity in Lettuce. Front. Plant Sci. 10:60. doi: 10.3389/fpls.2019.00060

INTRODUCTION

Plants provide a rich and diverse habitat which harbors a wide array of bacteria most of which contribute to the growth, and health of their plant hosts (Berendsen et al., 2012; Hirsch and Mauchline, 2012; Jackson et al., 2013; Leff and Fierer, 2013). Most microorganisms in the phyllosphere have ability to promote plant growth through different mechanisms that vary from changes in hormonal content, production of volatile compounds, increasing nutrient availability, or enhancing abiotic stress tolerance (Ruzzi and Aroca, 2015). In addition, some native plant epiphytic microbiota can be used for bio-control of foodborne pathogens (Lopez-Velasco et al., 2012).

Plant growth-promoting (PGP) activity of epiphytic microbes can be affected by the environmental conditions, including the exposure to biostimulants/fertilizers or to their degradation products (Timmusk et al., 2017; Thapa et al., 2018). Protein hydrolysates (PHs) and other plant extracts are widely used as plant biostimulants for their positive effects on plant growth and nutrition (Colla et al., 2014). In the last years, the use of biostimulants to promote plant growth has been widely studied (Parrado et al., 2007; Kowalczyk et al., 2008; Ertani et al., 2009; Gurav and Jadhav, 2013; Colla et al., 2015). Foliar and root applications have been shown to enhance the uptake of both macro and micronutrients (Ertani et al., 2009, 2012; Colla et al., 2015; Halpern et al., 2015) and to increase plant tolerance to environmental stress conditions. Biostimulant action of vegetal-derived products has been ascribed to the supply of bioactive compounds like amino acids, peptides, carbohydrates, humic substances, lignosulphonates, and phytohormones (Du Jardin, 2015). These bioactive compounds can be available in the plant-derived biostimulant or can be generated after foliar spray or substrate drench through the microbial activity.

Despite the use of biostimulants has been increasing and has become a common practice in the sustainable agriculture, little information is available on the effect of these products on the epiphytic bacterial microbiota. PHs and vegetal extracts-based products contain a wide range of compounds, as simple or complex carbohydrates and soluble organic nitrogen (Trouvelot et al., 2014), that can be utilized by both plants and bacteria as a source of carbon, nitrogen, and energy (Farrell et al., 2013). Therefore, foliar application of biostimulants can affect the epiphytic microbiota enhancing the development of bacteria that can be beneficial, neutral, or detrimental to plants (Colla et al., 2017a). Structural and functional modifications in the plant-associated microbiota have a crucial impact on the ecosystem, altering antagonistic and synergistic interactions among microorganisms and improving the fitness of the host by enhancing plant metabolic capacity, uptake of nutrients, and response of the plant to abiotic and biotic stresses (Kumar and Verma, 2018; Valencia et al., 2018). The exposure of plants to harsh environmental conditions can strongly influence the structure and composition of its microbiota, but this effect can be counteracted by enhancing the growth and survival of stress-tolerant PGP bacteria (Gouda et al., 2018). Therefore, growth stimulation of beneficial epiphytic microbes might be one of the different modes of action of some plant biostimulants.

In the case of amino acids and small peptide-based products, several studies on plant growth have been reported (Mladenova et al., 1998; Qurartieri et al., 2002). Kauffman et al. (2005) showed a growth-regulator activity of Foliar, an array of free amino acids and other organic constituents, on turf. The assessment of effects of foliar-applied agricultural products on plant epiphytic microbes guarantees the consumers harmless final product, especially for leafy vegetables such as lettuce. In lettuce, the diversity and abundance of epiphytic microbial community depend on the cultivation environment, i.e., phyllosphere microbiota from laboratory-grown plants is distinct from that colonizing plants grown in the field (Williams and Marco, 2014), and can be affected by the climatic variations such as radiation, rainfall, wind, and temperature (Medina-Martínez et al., 2015). The alteration of the composition of epiphytic microbial community of lettuce may lead to the promotion of beneficial microbes which can compete for the nutrient sources with enteric pathogens (Cooley et al., 2006) and perhaps phytopathogens.

We hypothesized that foliar applications of commercial products containing vegetal-derived bioactive compounds can enhance crop growth not only directly through the activity of signaling molecule such as peptides (e.g., short peptides such as root-hair promoting peptides), amino acids (e.g., glutamate), and phytohormones (e.g., auxins, cytokinins) but also indirectly by changing the microbial community in the phyllosphere. Vegetal-derived organic compounds sprayed on the leaf surface may be used as carbon and energy sources by beneficial microorganisms to synthesize new organic compounds including plant growth stimulating molecules (e.g., hormones) and/or toxic substances for plant pathogens. Moreover, vegetal-derived biostimulants are often enriched with copper or other micronutrients to improve plant nutrition and crop protection against pathogens. Several authors demonstrated that copper could alter the structure and function of the soil microbial communities, resulting in the selection of copper tolerant and copper resistant strains (Berg et al., 2012; Griffiths and Philippot, 2013; Nunes et al., 2016). Similarly, the addition of mineral elements to vegetal-derived biostimulants may also affect the microbial community in the phyllosphere and then the biostimulant activity of the product. To our knowledge, no information is available in the scientific literature on the influence of foliar applications of vegetal-derived bioactive compounds alone or enriched with micronutrients on bacterial community in the phyllosphere. Starting from the above considerations, a lettuce trial was carried out under greenhouse conditions to evaluate the influence of foliar sprays with three different commercial products containing vegetal-derived biostimulant compounds [tropical plant extract enriched with micronutrients (PE) "Auxym"; vegetal-derived PH "Trainer"; vegetal derived PH enriched with copper "Scudo"] on bacterial community from lettuce leaves in order to determine whether the product-mediated growth stimulation of certain epiphytic microbes contributes to the plant growth promoting properties of these products.

MATERIALS AND METHODS

Growing Conditions and Treatments

The trial was conducted, 2012, in a 300-m^2 polyethylene greenhouse situated at the Experimental Farm of Tuscia University, central Italy (latitude 42° 25′ N, longitude 12° 08′, altitude 310 m). Plants were grown under natural light conditions. The mean values of day/night air temperature and relative humidity and their standard deviations were 15.6 ± 1.3/22.7 ± 4.5°C, and 55.1 ± 8.2/80.0 ± 8.9%, respectively. Lettuce seeds (*Lactuca sativa* L. cv "Green Salad Bowl," SAIS S.p.A., Cesena, Italy) were sown on April 9 in polystyrene plug trays filled with vermiculite at a plant density of 720 plants m^{-2}. The floating raft system consisted of the polystyrene plug trays floating in plastic tanks with a constant volume of 60 L of aerated nutrient solution. An air compressor maintained the dissolved oxygen content above 6 mg/L. The composition of the nutrient solution in all treatments was: 10 mM NO_3-N, 1.5 mM P, 4.5 mM K, 10 mM Ca, 5.0 mM S, 2 mM Mg, 20 μM Fe, 9 μM Mn, 0.3 μM Cu, 1.6 μM Zn, 20 μM B, and 0.3 μM Mo. The electrical conductivity and pH of the nutrient solutions in all treatments were 2.0 ± 0.2 dS m^{-1} and 6.0 ± 0.3, respectively. To prevent large fluctuation in the nutrient concentrations, electrical conductivity, and pH, the nutrient solutions in all treatments were renewed from all tanks weekly.

After 14 days from emergence, plants were sprayed with 2.5 ml L^{-1} of a vegetal-derived PH ("Trainer"), or 1.0 ml L^{-1} of a copper-based fertilizer ("Scudo"), or 1 ml L^{-1} of a plant extract ("Auxym").

The plant extract "Auxym" (PE) is a commercial vegetal-derived biostimulant produced through water extraction and fermentation of tropical plant biomass. It contains mainly phytohormones with an auxin:cytokinin ratio 6:1, amino acids, vitamins, and microelements (**Table 1**; Colla et al., 2017b).

The legume-derived PH "Trainer" is a commercial biostimulant obtained through enzymatic hydrolysis of proteins derived from legume seeds. It contains mainly free amino acids, and soluble peptides with the following aminogram (g kg^{-1}): Ala (12), Arg (18), Asp (34), Cys (3), Glu (54), Gly (12), His (8), Ile (13), Leu (22), Lys (18), Met (4), Phe (15), Pro (15), Thr (11), Trp (3), Tyr (11), Val (14). The total content of micronutrients being below 0.05 g kg^{-1} can be considered negligible (**Table 1**). It has been shown that small peptides and single amino acids present in this PH exhibit auxin-like and gibberellin-like activities (Colla et al., 2014).

"Scudo" is a copper-based fertilizer (Cu-PH) containing copper complexed with peptides, and amino acids (90 g kg^{-1} of copper). Scudo (Cu-PH) contains free amino acids, and soluble peptides with the same composition of Trainer (PH; **Table 1**). Trainer, Scudo, and Auxym were manufactured by Italpollina S.p.A., Rivoli Veronese, Italy.

Foliar applications were repeated three times, at weekly intervals and, in each application, control plants were sprayed with the same amount of water used for the three vegetal-derived products. The four treatments were arranged in a randomized block design with three replicates per treatment (total of 12 plots). The number of plants per experimental plot was 84. Each plot included a polystyrene plug trays floating in a plastic tank. One day and seven days after the last treatment, six leaves (two from three independent plants) were sampled in each plot providing a total of 18 leaves per treatment (=6 leaves per plot × 3 replicates) per time point. Before the end of the trial (May 14, 35 days after sowing), the Soil-Plant Analysis Development (SPAD) index was recorded on lettuce leaves. A portable chlorophyll meter (SPAD-502, Minolta corporation, Ltd., Osaka, Japan) was used to measure the relative leaf chlorophyll concentration as a rational unit. Measurements were made at the central point of the leaflet between the midrib and the leaf margin of the second leaf starting from the apical shoot. Twenty random readings per plot were taken for each replicate and averaged to a single SPAD value; therefore, there were a total of three averaged SPAD values for each treatment. At end of the trial (May 14, 35 days after sowing), 20 plants per plot (single replicate) were harvested and the mean fresh weight of shoot biomass was determined from the 60 plants harvested from each treatment.

Isolation of Culturable Bacteria From Lettuce Phyllosphere

Epiphytic bacterial populations from treated and no-treated plants were recovered incubating six leaves per replicate in 20 mL of saline solution (0.9% w/v NaCl) for 60 min, under shaking condition (180 r/min). There were three replicates for each treatment with a total of 18 leaves per treatment per time point. Cell suspensions were serially diluted onto LB agar plates (Sambrook and Russel, 2001) for counting of predominant culturable bacteria and individual colonies were then picked and streaked on fresh LB plates for further characterization.

DNA Extraction

At each time point, metagenomic DNA used for T-RFLP and NGS analysis was prepared from three biological replicates for each treatment and from each biological replicate, two replicate extractions were performed. DNA was extracted from cells collected by centrifugation (13,000 r/min for 10 min) from saline solution used for leaf washing (six leaves for each replicate, two leaves from three independent plants per plot). Total DNA of culturable isolates was obtained from cells grown overnight on LB medium. DNA was extracted using PureLink Genomic DNA Mini Kit (Thermo Fisher Scientific, Italy) following the manufacturer protocol for Gram-positive bacteria[1]. The quantity and quality of isolated DNA was measured using a Qubit dsDNA HS Assay kit (Thermo Fisher Scientific, Italy) and agarose gel electrophoresis, respectively.

Molecular Identification of Culturable Bacteria

Two to four isolates for each morphotype were selected for the molecular analysis. The culturable isolates were characterized

[1]https://assets.thermofisher.com/TFS-Assets/LSG/manuals/purelink_genomic_man.pdf

TABLE 1 | Main components of products tested in the lettuce trial.

Class of compound	Tropical plant extract enriched with micronutrients (PE)	Vegetal-derived protein hydrolysate (PH)	Vegetal-derived protein hydrolysate enriched with copper (Cu-PH)
Phytohormones (mg kg^{-1})	Auxins (1.81) Cytokinins (0.29)	ND	ND
Organic nitrogen compound (g kg^{-1})	Amino acids and peptides (51.9)	Free amino acids and peptides (310)	Free amino acids and peptides (150)
Vitamins (g kg^{-1})	Niacin (3.3) Vitamin C (1.0) Vitamin E (0.4) Thiamine (0.3) Pyridoxine (0.3) Riboflavin (0.2)	ND	ND
Micronutrients (g kg^{-1})	Fe-EDTA (6.0) Mn-EDTA (6.0) Zn-EDTA (4.0) Cu-EDTA (2.0) B-H_3BO_3 (4.0)	Traces	Cu-complexed with amino acids and peptides (90) and traces of other micronutrients

ND = not detected.

by amplifying, sequencing, and analyzing the 16S rRNA gene. Universal primer 63F (5′-CAGGCCTAACACATGCAAGTC-3′) and 1389R (5′-ACGGGCGGTGTGTACAAG-3′) were used to generate amplicons (about 1400 bp) that were cloned into the pGEM-Teasy vector (Promega, Madison, WI, United States) and sequenced using MACROGEN commercial service (Amsterdam, Holland).

All 16S rRNA sequences from isolates with the same morphotype were identical and only sequences that could be shown to be derived from independent templates were analyzed. The 16S rRNA sequences were compared with those of all known bacterial species available in the GeneBank database[2] to identify potential phylogenetic relationships. All sequences were aligned using Clustal Omega (Sievers et al., 2011) and the unrooted phylogenetic tree was constructed using the neighbor-joining program contained in the PHYLIP phylogeny inference package (ver 3.6). The confidence values of the branches were determined by performing a bootstrap analysis based on 1000 replicates and the phylogenetic tree was displayed using iTOL (Letunic and Bork, 2006).

T-RFLP Analysis

To ensure that the Terminal Restriction Fragment Length Polymorphism (T-RFLP) analysis was not biased by biological sample, DNA extraction, and amplicon library preparation, two DNA pools, each consisting of equimolar DNA samples from biological replicates of each treatment, were prepared. Thus, the profile of the epiphytic bacterial community was derived analyzing 18 leaves from nine plants for each treatment. PCR amplification targeting the 16S rRNA gene was carried out using primer 8F (5′-AGAGTTTGATCCTGGCTCAG-3′), fluorescently labeled at the 5′ end with 6-FAM (6-carboxyfluorescein), and 1387R (5′-GGGCGGWGTGTACAAGGC-3′), with three replicates per DNA pool. PCR replicates were purified, using the Promega Wizard (Promega, Madison, WI, United States), quantified, using Qubit dsDNA HS Assay kit (Thermo Fisher Scientific, Italy), and pooled into a single tube to represent each amplicon library in equimolar amounts. Fluorescently labeled products were digested with 10 U of restriction enzyme *Ssp*I or *Avr*II (Thermo Fisher Scientific, Italy) by following the manufacturer's instructions. Digested products were purified and analyzed on an ABI3730 capillary sequencer in genotyping mode with the size standard ROX-labeled GS500. Only peaks that achieved a prevalence of more than 1% have been considered. Replicate T-RF profiles of each DNA pool and T-RF profiles of distinct DNA pools from samples of each treatment gave reproducible fingerprints. Consensus profiles were created as suggested by Dunbar et al. (2001), using the average values for peak heights. Total richness (S), Shannon's diversity index (H), and Simpson's evenness index (E) were calculated using PRIMER (v7, PRIMER-E Ltd., Plymouth, United Kingdom).

NGS Analysis

The V4 hypervariable region of the 16S rRNA gene was amplified using modified universal bacterial primer pairs 515F/806R designed for use with the Illumina platform (forward primer: 5′-TCGTCGGCAGCGTCAGATGTGTATAAGAGACAGGTGCCAGCMGCCGCGGTAA-3′; reverse primer: 5′-GTCTCGTGGGCTCGGAGATGTGTATAAGAGACAGGGACTACHVGGGTWTCTAAT-3′), with three replicates per DNA pool. PCR replicates were purified, quantified, and pooled in equimolar amounts as described before. Subsequently, amplicons were indexed and sequenced according to the Illumina MiSeq 16S Metagenomic Sequencing Library Preparation protocol[3]. Sequencing was performed on the Illumina MiSeq platform (Illumina, San Diego, CA, United States) at Molecular Digital Diagnostics S.r.L. (Viterbo, Italy).

The Quantitative Insights Into Microbial Ecology (QIIME, v1.9.0; Caporaso et al., 2010) software was used to analyze the 16S rRNA sequence generated from paired-end amplicon sequencing. Paired-end reads were merged with PEAR (Zhang et al., 2014), setting a *p*-value cutoff of 0.05. Chimeras were detected and

[2]https://www.ncbi.nlm.nih.gov/genbank/

[3]https://support.illumina.com/content/dam/illumina-marketing/documents/products/other/16s-metagenomics-faq-1270-2014-003.pdf

filtered from the paired-end reads using USEARCH (v6.1; Edgar, 2010). Operational taxonomic units (OTUs) were assigned to the reads using an open reference approach with UCLUST algorithm (Edgar, 2010) against the SILVA database release 132 that was clustered at 97% identity.

Based on the genus-level classification, principal component analysis (PCA) was performed to evaluate the similarity among various metagenomic communities. The PCA plots were displayed using PAST 3 (Hammer et al., 2001). The relative microbial abundance of all samples was summarized in a taxa plot.

Indole Acetic Acid Production

Estimation of extracellular indole acetic acid (IAA) was determined using the colorimetric method described by Patten and Glick (2002). Bacteria were grown for 16 h at 30°C, in LB medium (50 mL) without or with L-tryptophan (0.1% wt/vol). After growth, spent medium was recovered by centrifugation at 8000 r/min for 10 min and directly used for IAA quantification using Salkowski's reagent (0.5 M $FeCl_3$ in 35% perchloric acid). The mixture was incubated at room temperature for 30 min and absorbance of the developed pink color was read at 530 nm. IAA concentration in the culture was determined by using a calibration curve of pure IAA (Sigma–Aldrich, Italy), as a standard. All these experiments were carried out in triplicate.

Phosphate Solubilization

Phosphate solubilization activity was determined on NBRIP medium agar plates containing insoluble Pi. NBRIP medium contained (per liter of distilled water): glucose, 10 g; $MgCl_2 \cdot 6H_2O$, 5 g $MgSO_4 \cdot 7H_2O$, 0.25 g; KCl, 0.2 g; $(NH_4)_2SO_4$, 0.1 g; $Ca_3(PO_4)_2$, 5 g (Nautiyal, 1999). Plates were incubated for 2–5 days at 30°C. Colonies with clear halos were considered as phosphate solubilizing colonies. Solubilization index (SI) was calculated as: SI = (colony diameter + halo zone diameter)/colony diameter (Premono et al., 1996). All these experiments were carried out in triplicate.

Antimicrobial Activity

Evaluation of antimicrobial activity was performed on the following strains: *Erwinia amylovora* Ea273 strain (ATCC 49946); *Trichoderma reseii* DIBAF-10 an environmental strain isolated from poplar chips, *Trichoderma viride* T-67 strain (ATCC 28020), *Phytophthora cinnamomi* isolate 1, *Fusarium culmorum* isolate 485, *F. culmorum* isolate J1, *Fusarium oxysporum* isolate 2, and *Fusarium graminearum* isolate 3, were kindly supplied by Prof. G. Chilosi (DIBAF, University of Tuscia).

Production of diffusible compounds with antifungal activity was tested on Potato Dextrose Agar (PDA) using a dual-culture *in vitro* assay. First, a 5-mm-diameter mycelium disk from a 5-day-old fungal culture was placed on the surface of the agar plate in the center of the petri dish. Then, a bacterial suspension from an overnight culture on LB medium was streaked, on three sides, at a distance of about 3 cm from the fungus plug. Zones of inhibition were measured after 5 days of incubation at 30°C according to the method of Geels and Schippers (1983). Bacterial strains that caused an inhibition zone of at least 2 mm were judged as positive. All these experiments were repeated independently at least twice.

Production of bioactive volatile organic compounds (VOCs) with antimicrobial activity was assessed by the double plate technique. This analysis was carried out on *Bacillus* strains F13 and F14 which exhibited the best inhibition against phytopathogenic *Fusarium* and *Phytophthora* in dual-culture assay. One hundred microliters of a bacterial suspension from an overnight culture on LB medium was spread on a LB-agar petri dish and a 5-mm disk of a 5-days-old pure culture of the fungus was placed at the center of another Petri dish containing PDA. Both half plates were placed face to face, preventing any physical contact between the fungal pathogen and the bacterium, and sealed to isolate the inside atmosphere and prevent loss of volatiles formed during the growth. Each pair of plates was incubated at 30°C for 48 h and the growth of the pathogen was measured and compared to a control prepared in the same manner but without bacteria.

For the determination of the inhibitory effect of the bacterial isolates on pathogenic fungi, the percentage of inhibition of radial growth (PIRG %) was calculated according to the following formula $100 \times (R1 - R2)/R1$, where R1 and R2 are the radial growth of the pathogen in the absence and in the presence of the antagonist, respectively.

Antibacterial activity was assessed on plates according to Homma et al. (1989). Bacterial isolates were grown as spots on LB agar plates for 18 h and exposed to chloroform vapors for 30 min. After aeration, plates were covered with a suspension of *E. amylovora* Ea273 strain, obtained by mixing 5 mL of diluted LB soft agar (0.6%) with 200 μL of a stationary phase culture. Plates were incubated at 30°C until inhibition halos were detected.

Fungal and bacterial inhibition assays were repeated independently at least twice.

Nucleotide Sequence Accession Numbers

Sequences from independent templates/clones and amplicon libraries were deposited in GenBank under accession number: MH329697; MH338201; MH341118; MH375452; MH375453; MH375462; MH375472- MH375478; MH3755548; MH375601; MH375635; MH375635; MH375636; MH375453; MH376404; MH376429; MH376688; MH3756691; MH376690; MH376691; MH379797.

Statistical Analysis

Analysis of variance of the data was calculated using the software package, SPSS 10 for Windows, 2001. Tukey HSD test was performed at $p = 0.05$ on each of the significant variables measured. PCA was carried out compare NGS data obtained with samples collected 1 day after the last treatment. The PCA outputs include variable loading to each selected component and treatment component scores.

RESULTS

Effects on Plant Growth and Epiphytic Bacteria

Effects of vegetal-derived products on plant growth and composition of bacterial communities associated with lettuce leaves were determined on 5-weeks-old plants treated for 21 days with commercial extracts or hydrolysates of vegetal proteins. Leaf chlorophyll content expressed by the SPAD index was significantly increased by PE and PH (avg. 24.9) in comparison with Cu-PH and untreated control (18.9; **Table 2**). Fresh shoot biomass was also significantly enhanced by foliar applications of plant extracts or hydrolysates of vegetal proteins (avg. 6.69 g/plant) in comparison with untreated control (6.05 g/plant). After 1 day from the foliar application, the highest culturable aerobic epiphytic bacteria were observed in PE treatment whereas Cu-PH gave the lowest value. However, after 7 days from the foliar application the culturable aerobic epiphytic bacteria showed a different trend with the highest value in Cu-PH treatment (**Table 2**). Similarly, to fresh shoot biomass, leaf fresh weight sampled for the determination of abundance of culturable aerobic epiphytic bacteria was highest in lettuce plants treated with commercial products (**Table 2**).

Differences in the culturable count at 1 day were less pronounced when no-treated samples were compared to those collected from leaves of PE- or PH-treated lettuce. As shown in **Table 2**, the number of culturable epiphytic bacteria increased twofold in PE-treated plants (from $2.48 \pm 0.03*10^3$ to $4.92 \pm 0.02*10^3$ CFU/g of biomass) and had a slight decrease (about 0.3-fold) in samples from PH-treated plants (from $2.48 \pm 0.03*10^3$ to $1.76 \pm 0.05*10^3$ CFU/g of biomass).

In no-treated and in PE- or PH-treated plants, the abundance of culturable bacteria significantly decreased over the time and, 7 days after the last treatment, reached the same final value (between $0.92 \pm 0.01*10^3$ and $1.11 \pm 0.01*10^3$ CFU/g of biomass; **Table 2**).

Molecular Characterization of Culturable Bacteria

Based on colony morphology, we identified 23 morphotypes which were differentially distributed in the epiphytic population collected from leaves of treated and no-treated lettuce (**Table 3**). Only seven morphotypes were present in the aerobic bacterial population from no-treated control plants, while the larger number of morphotypes occurred in PH-treated lettuce (15 out of 23; **Table 3**).

For each morphotype, at least two independent colonies were characterized to the genus/species level using 16S rRNA gene as DNA barcode (see section "Materials and Methods"). Sequence data of 16S fragments were used to generate a phylogenetic tree to evaluate the genetic relatedness among these bacteria and known species (**Figure 1**). All strains exhibiting the same morphotype had identical 16S rRNA gene sequences (not shown). The 23 morphotypes belonged to six family and eight different genera of which *Pseudomonas*, *Bacillus*, and *Exiguobacterium* were the most recurrent. As shown in **Figure 1**, all *Exiguibacterium* strains clustered with *Exiguibacterium indicum*; *Bacillus* strains clustered into different clades corresponding to *Bacillus cereus* group (strain 5a, 5b, and F14), *Bacillus pumilus* (strain F12), and *Bacillus mojavensis* (strain F13); *Pseudomonas* strains could be organized in four different groups clustering with *Pseudomonas putida* (strain C3, C7, and F9), *Pseudomonas psychrotolerans* (strain F1G and F16), *Pseudomonas rhizosphaerae* (strain F4), and *Pseudomonas moraviensis* (strain F5), respectively (**Figure 1**).

Molecular Characterization of Bacterial Epiphytic Community

The structure of the total epiphytic bacterial community was evaluated using a molecular approach based on the T-RFLP of 16S rRNA gene (Osborn et al., 2000). To identify T-RFs in the community profiles, *Avr*II and *Ssp*I fingerprints of target gene from culturable bacteria reported in **Table 3** were generated. Completed digestion of PCR products gave T-RF of: 107 (218) bp for *Avr*II and 351 (354) bp for *Ssp*I on *Pseudomonas* DNA; 351 bp (*Ssp*I) on *Acinetobacter* and *Micrococcus* DNA; 259 (*Avr*II) and 355 bp (*Ssp*I) on *Enterobacter*/*Pantoea* DNA; 361 bp (*Ssp*I) on *Sphingobacterium* 16S gene. PCR product from *Bacillus* C5a and C5b generated a single peak of 174 bp after *Ssp*I digestion. Both enzymes do not generate detectable T-RFs using, as a template, DNA from *Exiguobacterium* and other *Bacillus* isolates.

As shown in **Figure 2**, *Ssp*I and *Avr*II digestions of amplicons from epiphytic bacterial community gave a total of 10 and 17

TABLE 2 | Crop parameters and cultivable epiphytic bacteria from lettuce plants treated and no treated with the three commercial products.

Treatment	Leaf SPAD index	Shoot fresh weight (g/plant)	Leaf fresh weight (g/leaf)	Aerobic cultivable bacteria [CFU (*10^3)/g biomass]	
				day after treatment	7 days after treatment
No-treated	18.0 ± 2.4^b	6.05 ± 0.28^b	1.03 ± 0.01^d	2.48 ± 0.03^b	1.11 ± 0.01^b
PE	24.6 ± 0.6^a	6.62 ± 0.20^a	1.23 ± 0.06^b	4.92 ± 0.02^a	0.92 ± 0.01^b
Cu-PH	19.9 ± 2.9^b	6.78 ± 0.14^a	1.27 ± 0.04^a	0.10 ± 0.05^c	17.90 ± 0.80^a
PH	25.3 ± 1.6^a	6.68 ± 0.21^a	1.19 ± 0.04^c	1.76 ± 0.05^b	0.98 ± 0.01^b
Significance[a]	*	**	**	**	**

PE = tropical plant extract; PH = protein hydrolysate; Cu-PH = copper-based PH. Values (± SD) with no letter in common significantly differ at $p \leq 0.05$ (Tukey HSD test).[a]**,** Significant at $P \leq 0.05$ and 0.01, respectively.*

TABLE 3 | Epiphytic bacteria isolated from no-treated and treated lettuce leaves and identified by 16S rRNA sequencing.

Taxonomic affiliation		Morphotype	Biostimulant				T-RF (bp)	
Family	**Genus**		**No-treated**	**PE**	**PH**	**Cu-PH**	***Ssp*I**	***Avr*II**
Enterobacteriaceae	*Pantoea*	C1	–	+	+	–	355	259
	Enterobacter	C4	–	+	+	–	355	259
		C6	–	–	+	–	355	259
Bacillaceae	*Exiguobacterium*	F11	+	+	+	–	No cut	
		F15	–	–	+	–	No cut	
		C2	+	+	+	–	No cut	
	Bacillus	C5a	–	–	+	–	174	No cut
		C5b	–	+	–	–	174	No cut
		F12	–	–	–	+	No cut	
		F13	–	+	+	+	No cut	
		F14	+	–	–	–	No cut	
Pseudomonadaceae	*Pseudomonas*	C3	+	+	+	+	351	107
		C7	+	+	+	+	351	107
		F1G	–	+	+	–	354	107
		F4	–	+	+	–	354	107
		F5	+	–	+	+	351	107
		F9	–	–	+	–	354	218
		F16	+	+	–	–	351	218
Moraxellaceae	*Acinetobacter*	F2	–	–	+	+	351	No cut
		F7	–	+	–	–	351	No cut
Sphingobacteriaceae	*Sphingobacterium*	F8	–	–	–	+	361	No cut
Micrococcaceae	*Micrococcus*	F3	–	–	–	+	351	No cut
		F10	–	–	–	+	351	No cut

(+) = present; (–) = absent/no-detectable.

peaks, respectively. Differences in the absolute number and in the relative height of discernible peaks could be seen comparing no-treated and treated samples, as well as samples collected 1 or 7 days after the last treatment. Differences in T-RF profiles of samples collected at 1 and 7 days were observed with both treated and no-treated plants. Three *Ssp*I T-RFs (351, 361, 364 bp) and one *Avr*II T-RF (107 bp) occurred in all samples, albeit the relative abundance (RA) of each of these phylotypes was significantly different in the single T-RFs pattern. In T-RF profiles of no-treated samples (1 and 7 days), a lower number of peaks were observed and with both enzymes 80% of the total peak area was associated to a single peak (351 bp in *Ssp*I profiles, 107 bp in *Avr*II profiles). In T-RF profiles of treated samples (1 and 7 days), specific additional peaks were observed at: 106, 355, and 356 bp for *Ssp*I, 120, 255, 259, and 261 bp for *Avr*II.

To describe the changes in the dominance among the phylotypes, the ecological diversity indices were calculated combining the data of both enzymes (**Figure 3**). The maximal number of species determined by T-RFLP analysis (15) was found in 1-day PH-treated samples and 7 days after the last treatment in samples from PE-treated plants (**Figure 3**). As shown in **Figure 3**, for PE and Cu-PH we observed an increase in species richness from 1 to 7 days. In contrast, in no-treated and PH-treated samples, we observed an overall reduction in the species richness over the time. In samples collected 1 and 7 days after the last treatment from PE- and PH-treated plants, the diversity (H) and evenness (E) were much higher than in no-treated plants (**Figure 3**). Interestingly, in samples collected 1 day after the last treatment with Cu-PH, we observed a reduction in the species richness (S), as well as an increase in the evenness compared to no-treated plants (**Figure 3**). These observations indicated that, on leaves of Cu-PH-treated plants, a lower number of species were present but a more equitable distribution in species abundance occurred.

To gain more insights about the effect of vegetal PHs on the structure of bacterial community of lettuce phyllosphere, samples collected one day after the last treatment were also analyzed using a next generation sequencing (NGS) approach. This analysis was only carried out on PH-treated and no-treated plants for two major reasons: the treatment with PH gave the highest species diversity (**Figure 3**); PH does not contain inorganic compounds, such as copper salts (Cu-PH) and micronutrients (PE), whose combined effect with vegetal PHs could not be easily uncoupled. NGS analysis based on the sequencing of V3–V4 region of 16S rDNA gene allowed us to generate a number of reads per sample comprised between 120,000 and 150,000 (not shown). Approximatively 90% of raw reads per sample passed merging, trimming, and chimera filtering steps and were analyzed for OTU search. In both samples, two phyla accounted for 90% of total sequence reads with the majority belonging to *Firmicutes* (74.7% of total bacteria in no-treated samples) or *Proteobacteria* (72.2% in samples from PH-treated plants:

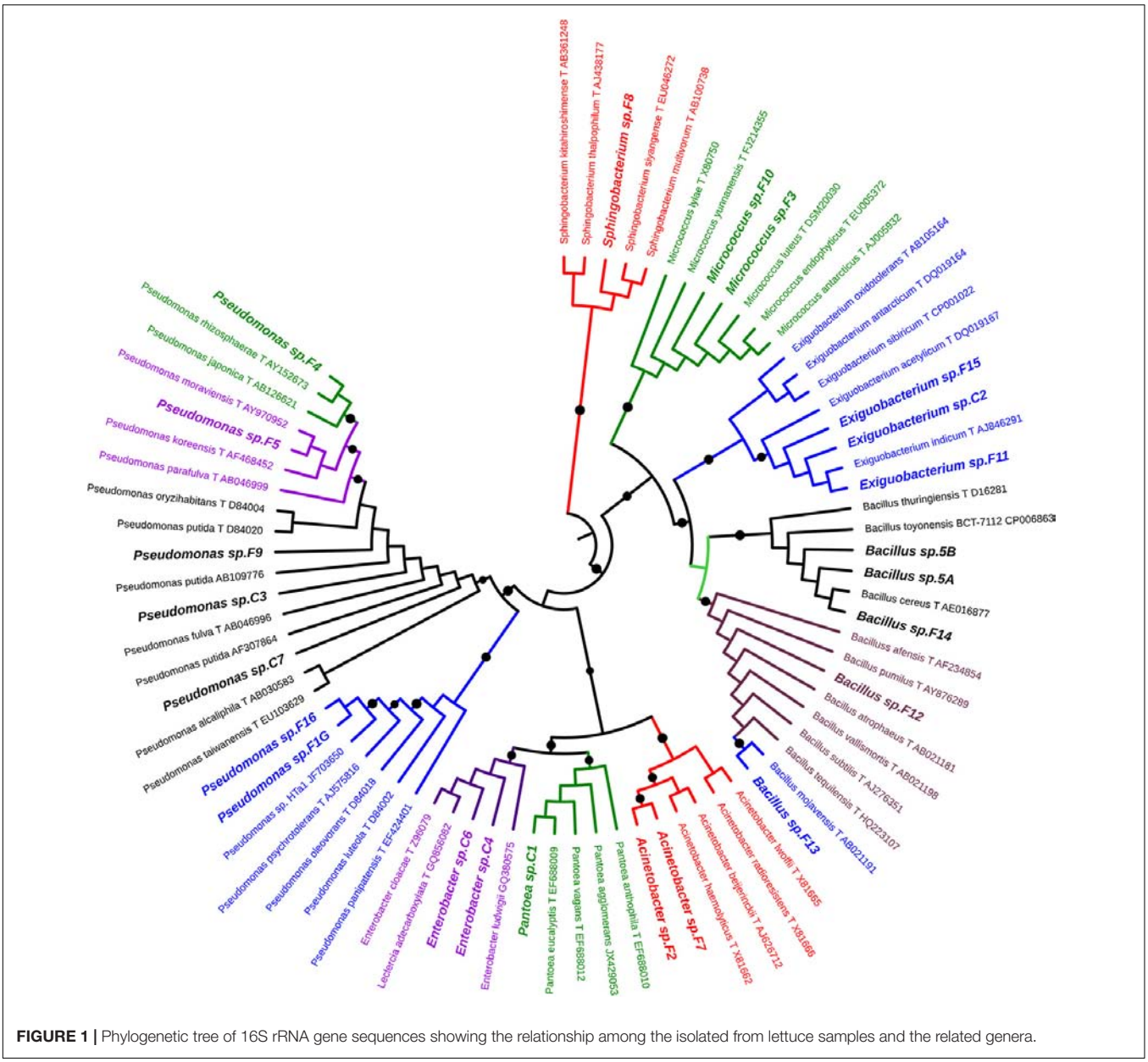


FIGURE 1 | Phylogenetic tree of 16S rRNA gene sequences showing the relationship among the isolated from lettuce samples and the related genera.

Figure 4). In PH-treated (compared to no-treated) samples, we observed a reduction of *Bacillales* (from 74.5 to 27.3% of total bacteria) and an increase in *Pseudomonadales* (from 21.5 to 44.4%) and *Enterobacteriales* (from 3.6 to 26.7%; **Figure 4**). Other orders that occurred as minor forms in PH-treated (and were not detectable in no-treated) samples comprised *Sphingomonadales*, *Flavobacteriales*, *Micrococcales*, and *Pasteurellales*.

A deeper phylogenetic classification of the reads at genus level (**Figure 4**) revealed that the most over-represented genera in PH-treated samples were *Bacillus* (25.5% of total bacteria), *Pantoea* (24.9%), *Pseudomonas* (24.6%), and *Acinetobacter* (19.3%). In no-treated samples, *Bacillus* and related genera represented 73.1% of total bacteria, major genera under the order of *Pseudomonadales* were *Acinetobacter* (15.8% of total bacteria) and *Pseudomonas* (5.4%), whereas *Enterobacteriales* included bacteria belonging to *Pantoea* genus (3.3%; **Figure 4**).

Principal component analysis analysis suggested substantial differences in the epiphytic microbial community between PH-treated and no-treated plants, as shown from the distribution in different zones of the PCA graph of data sets obtained from different samples (**Figure 5**). The two axes were involved in 99.9% of the total variance and could explain most variations in bacterial community structure. The first principal component (PC1) explained 78.4% and the second (PC2) 21.5% of the variance at genus level. Along the first axis, variability was mainly explained by an increase in the population of bacteria belonging

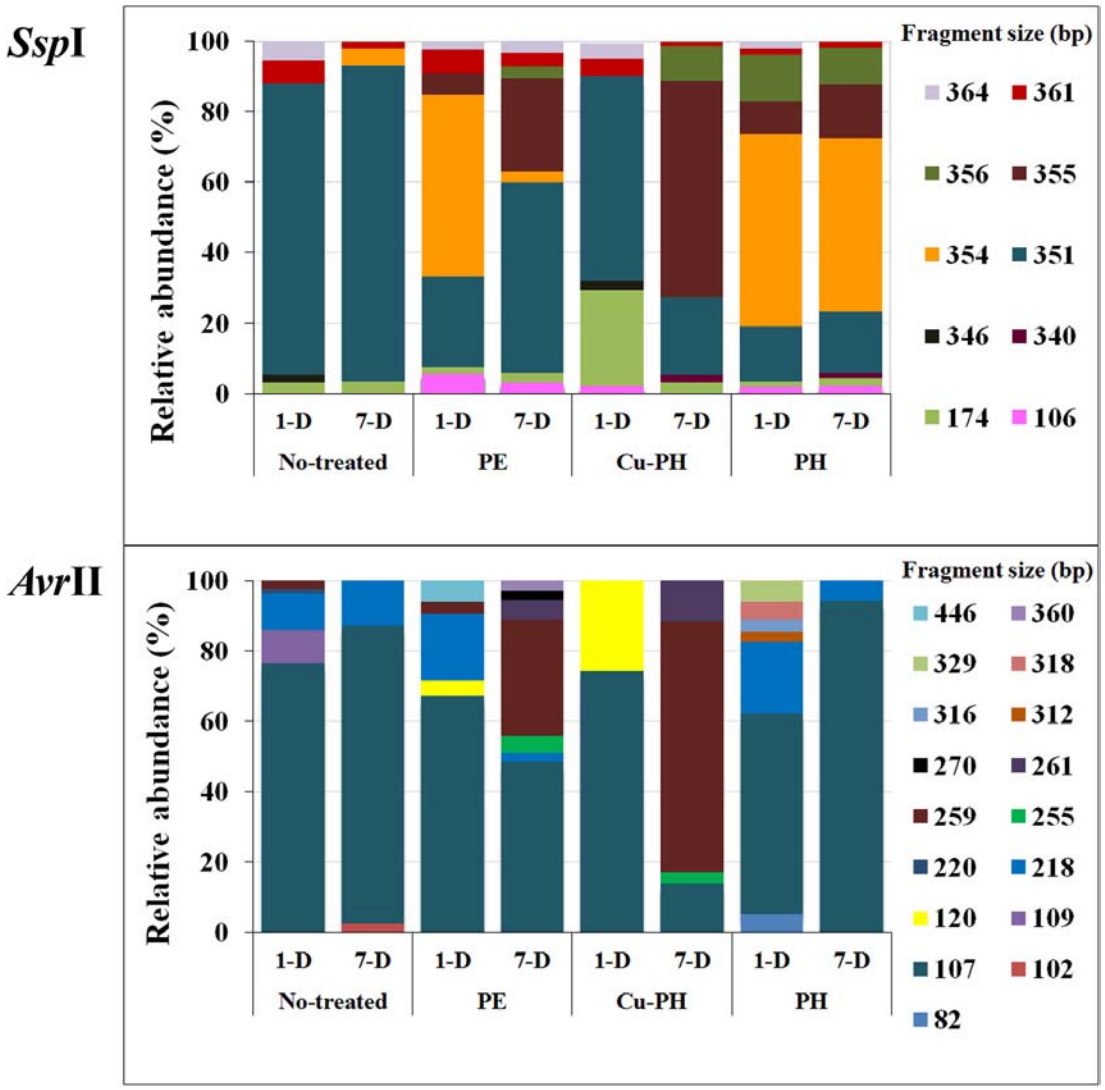


FIGURE 2 | Percentage of relative abundance of T-RFs after *Ssp*I and *Avr*II digestion among no-treated and PE-, Cu-PH-, and PH-treated samples at 1 and 7 days after the last treatment. The numbers indicate the size (in bp) of the T-RF fragments.

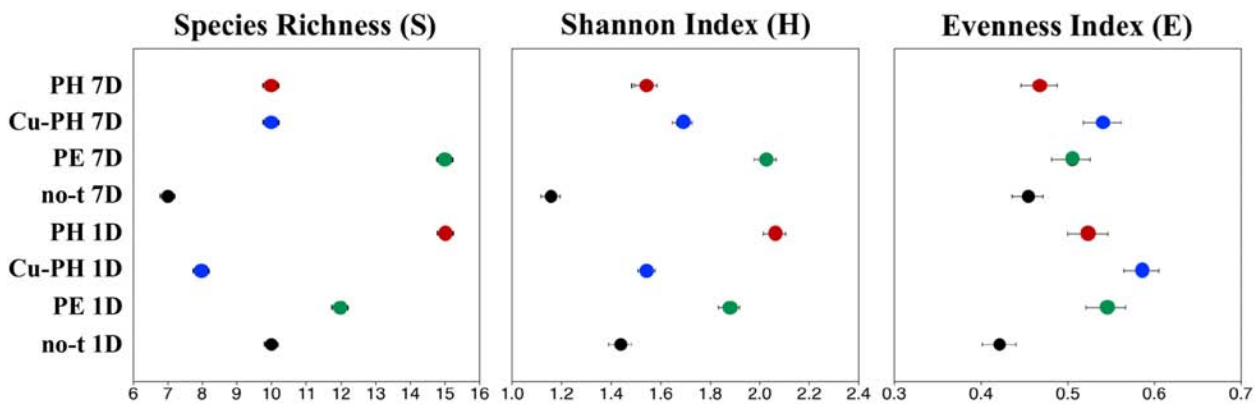


FIGURE 3 | Values for ecological diversity indices of total richness (S), Shannon–Weaver diversity (H), and evenness (E) obtained by using combined *Ssp*I and *Avr*II T-RFLP data of samples collected 1 or 7 days after the last treatment.

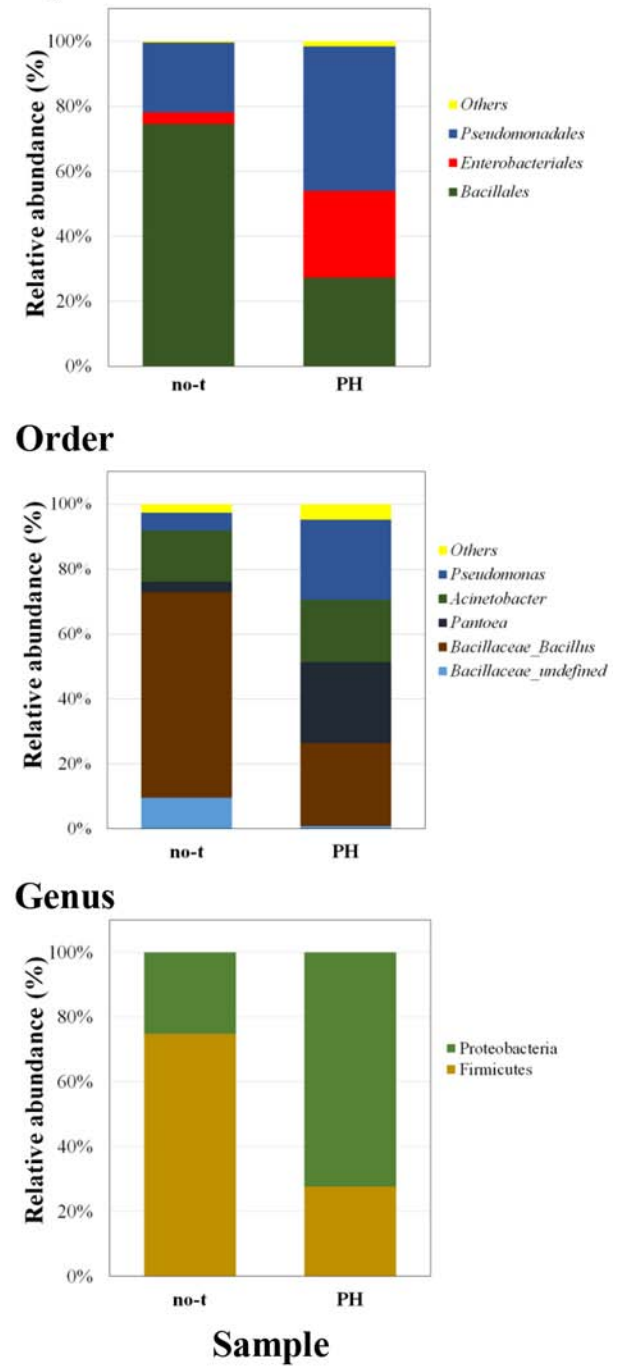


FIGURE 4 | Barplots illustrating the diversity and relative abundances at the phylum, order, and genus levels in PH-treated and no-treated samples collected 1 day after the last treatment. The taxonomy is constructed with the database Silva with a confidence threshold of 97%.

to *Pantoea* and *Pseudomonas* genus (**Figure 5**). Variability along the second PCA axis corresponded to a decrease in the RA of some bacteria belonging to *Bacillus* and *Acinetobacter* genus (**Figure 5**).

Phenotypic Characterization of Culturable Bacteria

Epiphytic isolates from lettuce plants were tested for features known to contribute to plant growth promotion, such IAA production and mineral phosphate solubilization, or plant growth protection against phytopathogens. Results reported in **Table 4** indicated that about 30% of all isolates (7 out of 23) were able to solubilize mineral phosphate, with a SI ranging between 2.5 and 4.8.

About 35% of isolates (8 out of 23) produced indoleacetic acid in tryptophan supplemented LB medium. The highest level of IAA was obtained with *Pantoea* strain C1 (>100 mg L^{-1}), and good levels of production were obtained with either *Pseudomonas* and *Micrococcus* (5–36 mg L^{-1}) or *Acinetobacter* strains (≤ 5 mg L^{-1}).

The antagonistic activity against phytopathogens was assayed using a dual culture technique (Oldenburg et al., 1996). Results reported in the **Table 4** indicated that most *Bacillus* strains had a high antagonist activity against *F. oxysporum* and *E. amylovora*. *Bacillus* strains F13 and F14 also exhibited strong inhibitory activity against *P. cinnamomi* (**Table 4**). *E. amylovora* was also strongly inhibited by *Pantoea* sp. C1 and *Micrococcus* sp. F3 strains. Biocontrol activity against fungi belonging to *Fusarium* and *Phytophthora* genus although at lower inhibitory levels were also observed with *Pseudomonas* strains F1G and F16. The latter one also exhibited antagonistic activity against *E. amylovora* (**Table 4**).

Antifungal Activity *in vitro*

The production of volatile metabolites active against fungi belonging to different *Fusarium* (*F. graminearum*, *F. culmorum*, and *F. oxysporum*) and *Trichoderma* (*T. viride* and *Trichoderma reesei*) species, was further investigated using *Bacillus* strains F13 and F14 which exhibited the best inhibition against phytopathogenic fungi in dual-culture assay.

Results from double plate assays indicated that both strains produce volatile compounds with strong inhibitory activity (higher than 65%) against these phytopathogens (**Figure 6**). With F13 strain, PIRG % against *Fusarium* strains varied between 72.2 ± 0.3 (vs *F. graminearum*) and 78.0 ± 0.9 (vs *F. culmorum* isolate J1), whereas no significant difference was observed between PIRG values against *T. viride* and *T. reesei* (about 75%; **Figure 6**).

Interestingly, the metabolites produced by *Bacillus* F13 strain were stable and remained biological active over a wide range of pH (between 2 and 10) and temperature (between 4 and 100°C), showing no loss of activity even after autoclaving (data not shown).

DISCUSSION

The primary object of this work was to evaluate the effect of foliar applications of commercial products containing vegetal-derived bioactive compounds on the structure of epiphytic bacterial community and unforeseen implications on useful/deleterious bacteria enrichment.

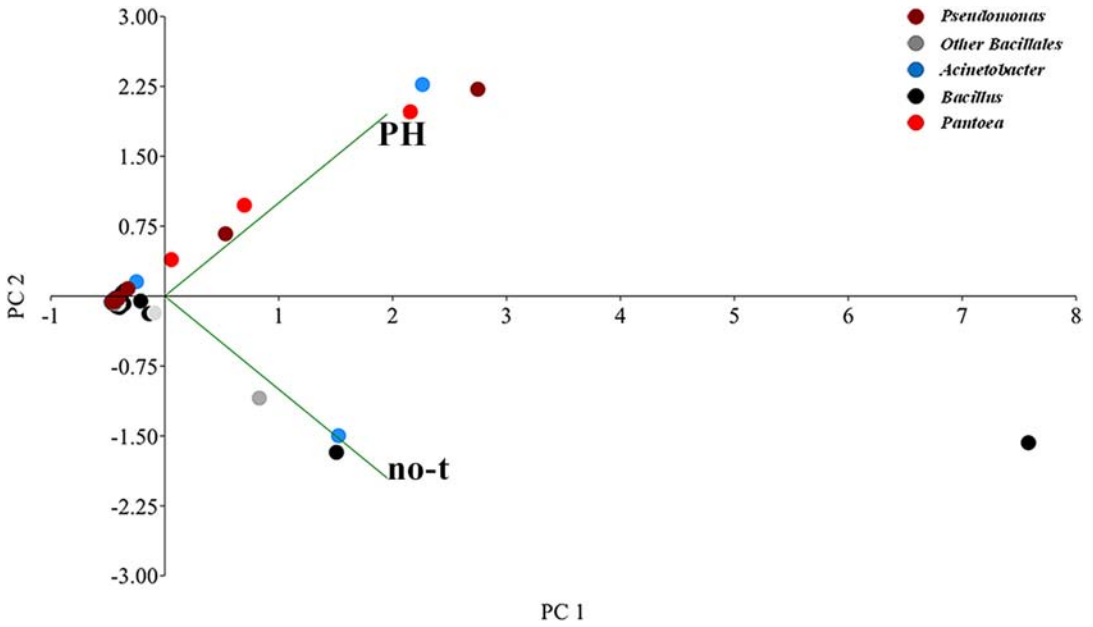


FIGURE 5 | Genus-level principal component analysis (PCA) comparison of the normalized bacterial OTU data. The PCA axes differentiate the PH-treated and no-treated samples according to their microbial composition. Different genera are denoted with different colors. Principal Component 1 and 2 explained 78.4 and 21.5% of the total variations, respectively.

TABLE 4 | Plant growth-promoting (PGP) activity and inhibitory effect on the growth of plant pathogens of culturable epiphytic bacteria isolated from treated and no-treated lettuce plants.

Taxonomic affiliation	Morphotype	PGP activity		Biocontrol activity (% of inhibition)		
		SI*	IAA§ (mg/L)	*F. oxysporum*	*P. cinnamomi*	*E. amylovora*
Pantoea	C1	0	106 ± 0.1	30–60%	<30%	>60%
Enterobacter	C4	0	0	0	0	0
	C6	0	0	0	0	0
Pseudomonas	F1G	4.3 ± 0.1	20 ± 0.1	30–60%	30–60%	0
	F4	0	0	0	0	0
	F5	4.8 ± 0.5	5.4 ± 0	<30%	<30%	0
	F9	0	0	<30%	<30%	0
	F16	3.0 ± 0.2	2.7 ± 0.1	30–60%	30–60%	30–60%
	C3	0	0	0	0	0
	C7	0	0	0	0	0
Acinetobacter	F2	2.5 ± 0.1	<5	0	0	0
	F7	0	<5	0	0	0
Sphingobacterium	F8	2.5 ± 0.1	0	0	0	0
Bacillus	C5a	0	0	>60%	<30%	>60%
	C5b	0	0	>60%	<30%	>60%
	F13	0	0	>60%	>60%	>60%
	F14	0	0	>60%	>60%	>60%
	F12	0	0	0	0	0
Exiguobacterium	F15	0	0	0	0	0
	F11	0	0	0	0	0
	C2	0	0	0	0	0
Micrococcus (2)	F3	2.7 ± 0.1	36 ± 0	<30%	<30%	>60%
	F10	4.7 ± 0	31 ± 0	0	0	0

**Phosphate solubilization index; § indole acetic acid production.*

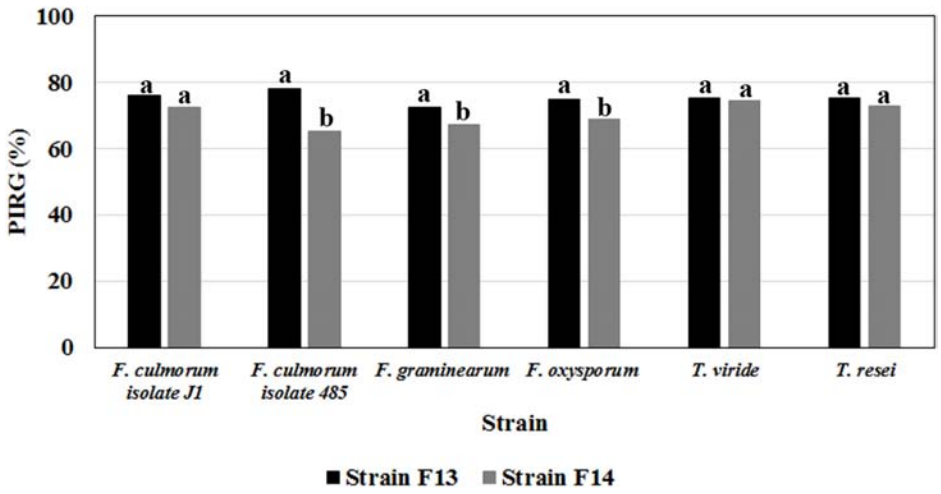


FIGURE 6 | Antifungal activities of volatile metabolites produced on LB medium by *Bacillus* strain F13 and F14 against mycelial cultures of different phytopathogenic *Fusarium* and *Trichoderma* species. Data are expressed in percentage of inhibition of radial growth (PIRG); values with no letter in common significantly differ at $p \leq 0.05$ (Tukey HSD test).

Foliar applications of the three commercial products increased fresh weights of shoots and leaves in lettuce plants compared to control treatment; moreover, leaf chlorophyll content expressed by the SPAD index was enhanced by foliar applications of PE and PH in comparison with Cu-PH and control treatments. These results agree with the previous findings on the effect of PH and PE on corn, tomato and spinach (Colla et al., 2014, 2017b; Rouphael et al., 2018). Bioactive compounds such as amino acids, peptides, and phytohormones supplied by the commercial products may be responsible for the increase of foliar biomass (**Table 2**). To better understand the effect of plant-derived bioactive products on leaf-associated bacteria, data obtained using different approaches were combined to have insight into the distribution of specific taxa and on the potential ability of strains belonging to these taxa to promote plant growth and/or suppress diseases. PE was the unique tested product that determined a significant twofold increase in the number of cultivable epiphytic bacteria compared to samples from no-treated plants (**Table 2**). This positive effect of PE on plant growth could be due to the stimulation of cell proliferation induced by signaling compounds (e.g., phytohormones, amino acids), the better protection of cells from oxidative damage resulting from the supply of antioxidant compounds (vitamins), the enhancement of plant metabolism arising from the supply of micronutrients (**Table 1**; Colla et al., 2017b) as well as of its microbiota. Similar evidences were obtained analyzing differences in the bacterial communities between treated and no-treated plants 1 day after the last treatment. Ecological diversity measures indicated a significant increase ($P < 0.05$) in species richness, evenness, and diversity indices in PE-treated (as well as in PH-treated) compared to no-treated plants (**Figure 3**). This microbial diversity persisted for at least a week (**Figure 3**), whereas no significant difference in the abundance of culturable bacteria was observed comparing treated (PE or PH) and no-treated plants 7 days after the last treatment (**Table 2**). In contrast, the use of copper-containing products, such as Cu-PH, had a transient negative effect on the epiphytic bacteria, as demonstrated by the reduction, at 1 day, of total aerobic count (25-fold compared to no-treated plants **Table 2**) and total species richness (1.25-fold vs no-treated control; **Figure 3**). A similar effect on the reduction of the bacterial biodiversity was also observed by Almeida et al. (2017) studying the dynamic of the bacterial community in sediments exposed to copper and the use of these bacterial communities resistant to copper to improve phytoremediation of copper-contaminated sediments. A comparison of T-RFLP profiles (**Figure 2**) and indices of diversity and evenness (**Figure 3**) of samples from no-treated and Cu-PH-treated lettuce also demonstrated that Cu-PH strongly affected the structure of the bacterial epiphytic community. In fact, in samples collected 1 day after the last treatment, we observed a significant increase ($P < 0.05$) in both Shannon–Weaver diversity and evenness when plants were treated with Cu-PH (**Figure 3**). In particular, the RA (% of total peak height) of the 351-bp peak in *Ssp*I T-RFLP profile decreased from 83 (no-treated) to 58% (Cu-PH), while RA of *Ssp*I peak sized 174 bp, the representative peak of *Bacillus* morphotypes C5a and C5b (**Table 3**), increased ninefold (from 3 to about 27%; **Figure 2**). A similar stimulatory effect was also observed with other minor forms that were undetectable in the *Ssp*I T-RFLP profile of no-treated samples, such as 106-, 355-, and 356-bp peak. The latter *Ssp*I peaks were also detected in T-RFLP profiles of samples collected from PE- and PH-treated plants, indicating that plant-derived bioactive compounds have the ability to stimulate the growth of specific groups of bacteria related to morphotypes C1, C4, and C6 (*Ssp*I peak of 355 bp) belonging to *Enterobacter*/*Pantoea* group (**Figure 1**) and to other not yet defined species (106- and 356-bp peaks). Interestingly, *Ssp*I peak sized 355 bp and the corresponding 259-bp *Avr*II peak became the most abundant ones (RA of 61 and 71%, respectively) in T-RFLP profiles obtained with samples collected from lettuce leaves 7 days after the last treatment with

Cu-PH (**Figure 2**). Therefore, we can conclude that the use of vegetal-PHs and plant extracts can determine the enrichment of epiphytic bacteria related to *Enterobacter/Pantoea* group (**Figure 1**), and that this effect can be enhanced combining these products with copper. Interestingly, *Enterobacter/Pantoea* group includes beneficial bacteria with PGP traits and biological control against phytopathogens (Taghavi et al., 2009; Madhaiyan et al., 2010; Dutkiewicz et al., 2016; Singh et al., 2018), as well as strains having the ability to interfere with the quorum-sensing which control biofilm and EPS formation in some food-borne pathogens, such as *Yersinia enterocolitica* (Gopu et al., 2016). The ability of copper-tolerant bacteria to promote plant growth was also observed by Liu et al. (2014) analyzing bacteria isolated from mine tailings. Our observation is also in agreement with current literature on phytoremediation indicating that bacteria with PGP traits can facilitate the removal of inorganic contaminants stimulating either plant growth or phytoremediation activity (Glick, 2010; Glick and Stearns, 2011; Ullah et al., 2015).

As shown in **Figure 3**, effects on the structure of the epiphytic bacterial community were also observed treating lettuce with PE and PH. These products selectively stimulated the growth of *Pseudomonas*-related bacteria that were not detected in no-treated samples at 1 day (*Ssp*I T-RF sized 354 bp; **Figure 2**). This conclusion was supported by the information that in T-RFLP profiles peaks corresponding to *Pseudomonas* morphotypes F1G and F4 (*Ssp*I T-RF of 354 bp and *Avr*II T-RF of 107 bp; **Table 2**) and morphotype F9 (*Ssp*I T-RF of 354 bp and *Avr*II T-RF of 218 bp; **Table 2**) were predominant (>50%) in PE- (1 day) and PH-treated (1 and 7 days) samples (**Figure 2**). Interestingly, strains F1G, F4, and F9 are related to members of different *P. putida* subclusters (**Figure 1**) that have been characterized for their ability to solubilize inorganic phosphate, such as *P. rhizosphaerae* (Kwak et al., 2015), enhance plant growth or antagonize fungal phytopathogens, such as *Pseudomonas fulva*/*Pseudomonas parafulva*/*P. putida* (Pena et al., 2016), fix nitrogen and enhance nutrient uptake, such as *P. psychrotolerans* (Liu et al., 2017).

Peaks corresponding to *Pseudomonas* morphotypes C3, C7, and F5 (*Ssp*I T-RF of 351 bp and *Avr*II T-RF of 107 bp) and morphotype F16 (*Ssp*I T-RF of 351 bp and *Avr*II T-RF of 218 bp; **Table 2**) were predominant in no-treated and Cu-PH-treated at 1 day (RA of 83 and 58%, respectively), remained the major peaks in no-treated samples at 7 days (RA of 90%) and became the most abundant ones in PE-treated samples at 7 days (RA of 54%; **Figure 2**). These peaks were also present in T-RFLP profiles of samples from PH-treated lettuce, whereas they were not the most predominant ones and their RA (16–18%) did not change over the time (**Figure 2**). In conclusion, treatments with hydrolysates of plant proteins lead to changing patterns of *Pseudomonas* populations which are quite complex to analyze and might lead to a specific enrichment of strains, such as morphotype F1G, showing plant promoting traits (**Table 4**).

As mentioned before, peaks corresponding to *Enterobacter*/*Pantoea* morphotypes (*Ssp*I T-RF of 355 bp and *Avr*II T-RF of 259 bp; **Table 2**) were also present in PE- and PH-treated samples and their RA increased, over the time (1→7 day after the last treatment), from 6 to 27% in samples from PE-treated plants and from 9 to 16% in those from PH-treated plants (**Figure 2**).

Comparing differences in the relative fluorescence of *Avr*II peaks specific for *Pseudomonas* (107 and 218 bp) and 351-bp *Ssp*I peak belonging to *Pseudomonas* and other species, and considering that vegetal-derived PHs and plant extracts analyzed in this work also stimulated the growth of cultivable *Acinetobacter* and *Micrococcus* strains with a *Ssp*I T-RF peak of 351 bp (**Table 3**), we can postulate that, in samples from treated plants, the relative high values of fluorescence associated with the 351-bp *Ssp*I peak (compared to the combined fluorescens of 107- and 218-bp peak in *Avr*II profile) reflects the presence in the epiphytic community, in addition to *Pseudomonas*, of a more abundant population of bacteria belonging to *Acinetobacter* and *Micrococcus* genus. It is worth pointing out that *Acinetobacter* and *Micrococcus* strains isolated from treated plants (**Table 3**) have PGP traits, such as ability to produce plant-related hormones (IAA) or solubilize inorganic phosphate (**Table 4**) and, therefore, their enrichment can be valuable for the plant.

Strains with PGP traits were enriched with all commercial products examined in this work, albeit each product stimulated the growth of a specific group of microorganisms. For example, *Pseudomonas* and *Pantoea* strains able to produce high levels of IAA were specifically stimulated by PH or PE (morphotypes C1, F1G, and F5), whereas *Micrococcus* sp. strains (F3 and F10) able to produce IAA and solubilize inorganic phosphate were specifically enriched on plants treated with Cu-PH (**Tables 3**, **4**). Other strains able to solubilize inorganic phosphate such as *Pseudomonas* sp. F1G or F5 were enriched by treatment with PE/PH or PH/Cu-PH, respectively.

The ability of vegetal-derived bioactive compounds to promote shifts in the composition of epiphytic bacterial communities, stimulating the growth of specific strains and increasing bacterial diversity, was confirmed by comparison of NGS data from no-treated and PH-treated plants (**Figure 4**). In fact, in samples collected 1 day after the last treatment, we observed that PH treatment altered the structure of the leaf-associated microbiome from phylum to genus level determining a reduction in the RA of bacteria belonging to *Bacillales* and, at the same time, an increase in the population of *Pseudomonas*, *Pantoea*, and other minor forms (**Figure 4**). Interestingly, the analysis at the level of individual OTUs demonstrated that PH specifically stimulates the growth of specific members of the epiphytic microbiota (**Figure 5**). This effect explains the increase in bacteria biodiversity, the alteration of the population of specific taxa, such as *Bacillus*, and the enrichment of rare species and specific strains which can play an important role in plant growth and protection (Shade et al., 2014; Jousset et al., 2017).

In agreement with data reviewed by Shafi et al. (2017), we demonstrated that microorganism belonging to *Bacillus* genus play an important role in lettuce microbiota as biocontrol agents (BCAs) against fungal and bacterial pathogens. Interestingly, the biocontrol activity is associated with strains related to *B. mojavensis* (F13) and *B. cereus* group (C5a, C5b, and F14; **Figure 1**), whose growth was stimulated by vegetal PHs and plant extracts (**Table 3**).

Among *Bacillus* strains isolated from treated plants, strain F13 exhibited the highest inhibitory activity (>71%) on the radial growth of all fungal pathogens tested in this work (**Figure 6**). The latter result is in agreement with data demonstrating that endophytic and epiphytic *B. mojavensis* strains have broad-spectrum antibacterial properties related to their ability to produce lipopeptides, surfactin and fengycin (Vágvölgyi et al., 2013; Kalai-Grami et al., 2014; Kim et al., 2015; Blacutt et al., 2016; Jasim et al., 2016). This wide range of metabolites can reduce pathogen attack by suppressing fungal growth or inducing the plant immune system (Khan et al., 2017). The ability of these bacteria to produce spore facilitates the use of *Bacillus* as bioinoculant.

CONCLUSION

The present study revealed that vegetal PHs and extracts containing vegetal-derived bioactive compounds can stimulate the growth of epiphytic bacteria with PGP and/or biological control activity against pathogens. Metagenomic analysis also demonstrated that these products can stimulate the growth of rare members of the microbial community that can promote the biostimulant effects of vegetal PHs and have direct and indirect effects on the ecosystem functioning and the plant health.

This is the first report indicating that the use of this class of biostimulants can enrich autochthonous bacterial strains able to enhance plant growth.

AUTHOR CONTRIBUTIONS

MR, GC, and FL conceived the study and wrote the manuscript. AF and EBŠ contributed to corrections and suggestions. GC and EBŠ performed experiments on plants. FL isolated the culturable bacteria and extracted the metagenomic DNA. AF, MR, and FL performed the analysis of T-RFLP and NGS data and performed the phenotypic and molecular characterization of culturable bacteria. All authors read and approved the final manuscript.

FUNDING

This research was partially supported by Italpollina S.p.A. (Rivoli Veronese, Italy).

REFERENCES

Almeida, C. M. R., Oliveira, T., Reis, I., Gomes, C. R., and Mucha, A. P. (2017). Bacterial community dynamic associated with autochthonous bioaugmentation for enhanced Cu phytoremediation of salt-marsh sediments. *Mar. Environ. Res.* 132, 68–78. doi: 10.1016/j.marenvres.2017.10.007

Berendsen, R. L., Pieterse, C. M., and Bakker, P. A. (2012). The rhizosphere microbiome and plant health. *Trends Plant Sci.* 17, 478–486. doi: 10.1016/j.tplants.2012.04.001

Berg, J., Brandt, K. K., Al-Soud, W. A., Holm, P. E., Hansen, L. H., Sørensen, S. J., et al. (2012). Selection for Cu-tolerant bacterial communities with altered composition, but unaltered richness, via long-term Cu exposure. *Appl. Environ. Microbiol.* 78, 7438–7446. doi: 10.1128/AEM.01071-12

Blacutt, A. A., Mitchell, T. R., Bacon, C. W., and Gold, S. E. (2016). *Bacillus mojavensis* RRC101 lipopeptides provoke physiological and metabolic changes during antagonism against *Fusarium verticillioides*. *Mol. Plant Microbe Interact.* 29, 713–723. doi: 10.1094/MPMI-05-16-0093-R

Caporaso, J. G., Kuczynski, J., Stombaugh, J., Bittinger, K., Bushman, F. D., Costello, E. K., et al. (2010). QIIME allows analysis of high-throughput community sequencing data. *Nat. Methods* 7, 335–336. doi: 10.1038/nmeth.f.303

Colla, G., Hoagland, L., Ruzzi, M., Cardarelli, M., Bonini, P., Canaguier, R., et al. (2017a). Biostimulant action of protein hydrolysates: unraveling their effects on plant physiology and microbiome. *Front. Plant Sci.* 8:2202. doi: 10.3389/fpls.2017.02202

Colla, G., Cardarelli, M., Bonini, P., and Rouphael, Y. (2017b). Foliar applications of protein hydrolysate, plant and seaweed extracts increase yield but differentially modulate fruit quality of greenhouse tomato. *Hortscience* 52, 1214–1220. doi: 10.21273/HORTSCI12200-17

Colla, G., Nardi, S., Cardarelli, M., Ertani, A., Lucini, L., Canaguier, R., et al. (2015). Protein hydrolysates as biostimulants in horticulture. *Sci. Hortic.* 196, 28–38. doi: 10.1016/j.scienta.2015.08.037

Colla, G., Rouphael, Y., Canaguier, R., Svecova, E., and Cardarelli, M. (2014). Biostimulant action of a plant-derived protein hydrolysate produced through enzymatic hydrolysis. *Front. Plant Sci.* 5:448. doi: 10.3389/fpls.2014.00448

Cooley, M. B., Chao, D., and Mandrell, R. E. (2006). *Escherichia coli* O157:H7 survival and growth on lettuce is altered by the presence of epiphytic bacteria. *J. Food Prot.* 69, 2329–2335. doi: 10.4315/0362-028X-69.10.2329

Du Jardin, P. (2015). Plant biostimulants: definition, concept, main categories and regulation. *Sci. Hortic.* 196, 3–14. doi: 10.1016/j.scienta.2015.09.021

Dunbar, J., Ticknor, L. O., and Kuske, C. R. (2001). Phylogenetic specificity and reproducibility and new method for analysis of terminal restriction fragment profiles of 16S rRNA genes from bacterial communities. *Appl. Environ. Microbiol.* 67, 190–197. doi: 10.1128/AEM.67.1.190-197.2001

Dutkiewicz, J., Mackiewicz, B., Lemieszek, M. K., Golec, M., and Milanowski, J. (2016). Pantoea agglomerans: a mysterious bacterium of evil and good. Part IV. Beneficial effects. *Ann. Agric. Environ. Med.* 23, 206–222. doi: 10.5604/12321966.1203879

Edgar, R. C. (2010). Search and clustering orders of magnitude faster than BLAST. *Bioinformatics* 26, 2460–2461. doi: 10.1093/bioinformatics/btq461

Ertani, A., Cavani, L., Pizzeghello, D., Brandellero, E., Altissimo, A., Ciavatta, C., et al. (2009). Biostimulant activity of two protein hydrolyzates in the growth and nitrogen metabolism of maize seedlings. *J. Plant Nutr. Soil Sci.* 172, 237–244. doi: 10.1002/jpln.200800174

Ertani, A., Pizzeghello, D., Baglieri, A., Cadili, V., Tambone, F., Gennari, M., et al. (2012). Agro-industrial residues and their biological activity on maize (*Zea mays* L.) metabolism. *J. Geochem. Explor.* 129, 103–111. doi: 10.1016/j.gexplo.2012.10.001

Farrell, M., Hill, P. W., Farrar, J., DeLuca, T. H., Roberts, P., Kielland, K., et al. (2013). Oligopeptides represent a preferred source of organic N uptake: a global phenomenon? *Ecosystems* 16, 133–145. doi: 10.1007/s10021-012-9601-8

Geels, F. P., and Schippers, B. (1983). Selection of antagonistic fluorescent *Pseudomonas* spp. and their root colonization and persistence following treatment of seed potatoes. *Phytopathol. Zeitschrift* 108, 193–206. doi: 10.1111/j.1439-0434.1983.tb00579.x

Glick, B. R. (2010). Using soil bacteria to facilitate phytoremediation. *Biotechnol. Adv.* 28, 367–374. doi: 10.1016/j.biotechadv.2010.02.001

Glick, B. R., and Stearns, J. C. (2011). Making phytoremediation work better: maximizing a plant's growth potential in the midst of adversity. *Int. J. Phytoremediation* 13, 4–16. doi: 10.1080/15226514.2011.568533

Gopu, V., Meena, C. K., Murali, A., and Shetty, P. H. (2016). Quorum quenching activity in the cell-free lysate of *Enterobacter ludwigii* isolated from beef and its effect on quorum sensing regulation in Yersinia enterocolitica. *RSC Adv.* 6, 21277–21284. doi: 10.1039/C5RA25440J

Gouda, S., Kerry, R. G., Das, G., Paramithiotis, S., Shin, H. S., and Patra, J. K. (2018). Revitalization of plant growth promoting rhizobacteria for sustainable development in agriculture. *Microbiol. Res.* 206, 131–140. doi: 10.1016/j.micres.2017.08.016

Griffiths, B. S., and Philippot, L. (2013). Insights into the resistance and resilience of the soil microbial community. *FEMS Microbiol. Rev.* 37, 112–129. doi: 10.1111/j.1574-6976.2012.00343.x

Gurav, R. G., and Jadhav, J. P. (2013). A novel source of biofertilizer from feather biomass for banana cultivation. *Environ. Sci. Pollut. Res. Int.* 20, 4532–4539. doi: 10.1007/s11356-012-1405-z

Halpern, M., Bar-Tal, A., Ofek, M., Minz, D., Muller, T., and Yermiyahu, U. (2015). The use of biostimulants for enhancing nutrient uptake. *Adv. Agron.* 130, 141–174. doi: 10.3389/fpls.2017.00597

Hammer, Ø, Harper, D. A. T., and Ryan, P. D. (2001). PAST: paleontological statistics software package for education and data analysis. *Palaeontol. Electron.* 4:9.

Hirsch, P. R., and Mauchline, T. H. (2012). Who's who in the plant root microbiome? *Nat. Biotechnol.* 30, 961–962. doi: 10.1038/nbt.2387

Homma, Y., Sato, Z., Hirayama, F., Konno, K., Shirahama, H., and Suzui, T. (1989). Production of antibiotics by *Pseudomonas cepacia* as an agent for biological control of soilborne plant pathogens. *Soil Biol. Biochem.* 21, 723–728. doi: 10.1016/0038-0717(89)90070-9

Jackson, C. R., Randolph, K. C., Osborn, S. L., and Tyler, H. L. (2013). Culture dependent and independent analysis of bacterial communities associated with commercial salad leaf vegetables. *BMC Microbiol.* 13:274. doi: 10.1186/1471-2180-13-274

Jasim, B., Mathew, J., and Radhakrishnan, E. K. (2016). Identification of a novel endophytic *Bacillus* sp. from *Capsicum annuum* with highly efficient and broad spectrum plant probiotic effect. *J. Appl. Microbiol.* 121, 1079–1094. doi: 10.1111/jam.13214

Jousset, A., Bienhold, C., Chatzinotas, A., Gallien, L., Gobet, A., Kurm, V., et al. (2017). Where less may be more: how the rare biosphere pulls ecosystems strings. *ISME J.* 11, 853–862. doi: 10.1038/ismej.2016.174

Kalai-Grami, L., Saidi, S., Bachkouel, S., Ben Slimene, I., Mnari-Hattab, M., Hajlaoui, M. R., et al. (2014). Isolation and characterization of putative endophytic bacteria antagonistic to *Phoma tracheiphila* and *Verticillium albo-atrum*. *Appl. Biochem. Biotechnol.* 174, 365–375. doi: 10.1007/s12010-014-1062-4

Kauffman, G. L. III., Kneivel, D. P., and Watschke, T. L. (2005). Growth regulator activity of Macro-Sorb foliar in vitro. *PGRSA Q.* 33, 134–141.

Khan, N., Maymon, M., and Hirsch, A. M. (2017). Combating *Fusarium* infection using *Bacillus*-based antimicrobials. *Microorganisms* 5:75. doi: 10.3390/microorganisms5040075

Kim, K. M., Jie, L., Youn, S. G., and Jae, S. K. (2015). Characterization of *Bacillus mojavensis* KJS-3 for the promotion of plant growth. *J. Life Sci.* 25, 910–916. doi: 10.5352/JLS.2015.25.8.910

Kowalczyk, K., Zielony, T., and Gajewski, M. (2008). "Effect of aminoplant and asahi on yield and quality of lettuce grown on rockwool," in *Biostimulators in Modern Agriculture. Vegetable Crops*, ed. Z. T. Dąbrowski Monographs (Warszawa: Wieś Jutra), 35–43.

Kumar, A., and Verma, J. (2018). Does plant-microbe interaction confer stress tolerance in plants: a review? *Microbiol. Res.* 207, 41–52. doi: 10.1016/j.micres.2017.11.004

Kwak, Y., Jung, B. K., and Shin, J. H. (2015). Complete genome sequence of *Pseudomonas rhizosphaerae* IH5T (= DSM 16299T), a phosphate-solubilizing rhizobacterium for bacterial biofertilizer. *J. Biotechnol.* 193, 137–138. doi: 10.1016/j.jbiotec.2014.11.031

Leff, J. W., and Fierer, N. (2013). Bacterial communities associated with the surfaces of fresh fruits and vegetables. *PLoS One* 8:e59310. doi: 10.1371/journal.pone.0059310

Letunic, I., and Bork, P. (2006). Interactive Tree Of Life (iTOL): an online tool for phylogenetic tree display and annotation. *Bioinformatics* 23, 127–128. doi: 10.1093/bioinformatics/btl529

Liu, R., Zhang, Y., Chen, P., Lin, H., Ye, G., Wang, Z., et al. (2017). Genomic and phenotypic analyses of *Pseudomonas psychrotolerans* PRS08-11306 reveal a turnerbactin biosynthesis gene cluster that contributes to nitrogen fixation. *J. Biotechnol.* 253, 10–13. doi: 10.1016/j.jbiotec.2017.05.012

Liu, W., Yang, C., Shi, S., and Shu, W. (2014). Effects of plant growth-promoting bacteria isolated from copper tailings on plants in sterilized and non-sterilized tailings. *Chemosphere* 97, 47–53. doi: 10.1016/j.chemosphere.2013.10.089

Lopez-Velasco, G., Tydings, H. A., Boyer, R. R., Falkinham, J. O., and Ponder, M. A. (2012). Characterization of interactions between *Escherichia coli* O157:H7 with epiphytic bacteria in vitro and on spinach leaf surfaces. *Int. J. Food Microbiol.* 153, 351–357. doi: 10.1016/j.ijfoodmicro.2011.11.026

Madhaiyan, M., Poonguzhali, S., Lee, J., Saravanan, V., Lee, K., and Santhanakrishnan, P. (2010). *Enterobacter arachidis* sp. nov., a plant growth-promoting diazotrophic bacterium isolated from rhizosphere soil of groundnut. *Int. J. Syst. Evol. Microbiol.* 60, 1559–1564. doi: 10.1099/ijs.0.013664-0

Medina-Martínez, M. S., Allende, A., Barberá, G. G., and Gil, M. I. (2015). Climatic variations influence the dynamic of epiphyte bacteria of baby lettuce. *Food Res. Int.* 68, 54–61. doi: 10.1016/j.foodres.2014.06.009

Mladenova, Y. I., Maini, P., Mallegni, C., Goltsev, V., Vladova, R., Vinarova, K., et al. (1998). Siapton – an amino-acid-based biostimulant reducing osmostress metabolic changes in maize. *Agro Food Ind. Hi Tech* 9, 18–22.

Nautiyal, C. S. (1999). An efficient microbiological growth medium for screening phosphate solubilizing microorganisms. *FEMS Microbiol. Lett.* 170, 265–270. doi: 10.1111/j.1574-6968.1999.tb13383.x

Nunes, I., Jacquiod, S., Brejnrod, A., Holm, P. E., Johansen, A., Brandt, K. K., et al. (2016). Coping with copper: legacy effect of copper on potential activity of soil bacteria following a century of exposure. *FEMS Microbiol. Ecol.* 92:fiw175. doi: 10.1093/femsec/fiw175

Oldenburg, K. R., Vo, K. T., Ruhland, B., Schatz, P. J., and Yuan, Z. (1996). A dual culture assay for detection of antimicrobial activity. *J. Biomol. Screen.* 1, 123–130. doi: 10.1177/108705719600100305

Osborn, A. M., Moore, E. R. B., and Timmis, K. N. (2000). An evaluation of terminal-restriction fragment length polymorphism (T-RFLP) analysis for the study of microbial community structure and dynamics. *Environ. Microbiol.* 2, 39–50. doi: 10.1046/j.1462-2920.2000.00081.x

Parrado, J., Escudero-Gilete, M. L., Friaza, V., Garcia-Martinez, A., Gonzales-Miret, M. L., Bautista, J. D., et al. (2007). Enzymatic vegetable extract with bioactive components: influence of fertiliser on the colour and anthocyanins of red grapes. *J. Sci. Food Agric.* 87, 2310–2318. doi: 10.1002/jsfa.2989

Patten, C. L., and Glick, B. R. (2002). Role of *Pseudomonas putida* indoleacetic acid in development of the host plant root system. *Appl. Environ. Microbiol.* 68, 3795–3801. doi: 10.1128/AEM.68.8.3795-3801.2002

Pena, A., Busquets, A., Gomila, M., Mulet, M., Gomila, R. M., Reddy, T. B. K., et al. (2016). High quality draft genome sequences of *Pseudomonas fulva* DSM 17717T, *Pseudomonas parafulva* DSM 17004T and *Pseudomonas cremoricolorata* DSM 17059T type strains. *Stand. Genomic Sci.* 11, 55. doi: 10.1186/s40793-016-0178-2

Premono, M. E., Moawad, A. M., and Vleck, P. L. G. (1996). Effect of phosphate solubilizing *Pseudomonas putida* on the growth of maize and its survival in the rhizosphere. Indones. *J. Crop Sci.* 11, 13–23.

Qurartieri, M., Lucchi, A., and Cavani, L. (2002). Effects of the rate of protein hydrolysis and spray concentration on growth of potted kiwifruit (*Actinidia deliciosa*) plants. *Acta Hort.* 594, 341–347. doi: 10.17660/ActaHortic.2002.594.42

Rouphael, Y., Giordano, M., Cardarelli, M., Cozzolino, E., Mori, M., Kyriacou, M. C., et al. (2018). Plant- and seaweed-based extracts increase yield but differentially modulate nutritional quality of greenhouse spinach through biostimulant action. *Agronomy* 8:126. doi: 10.3390/agronomy8070126

Ruzzi, M., and Aroca, R. (2015). Plant growth-promoting rhizobacteria act as biostimulants in horticulture. *Sci. Hortic.* 196, 124–134. doi: 10.1016/j.scienta.2015.08.042

Sambrook, J., and Russel, D. (eds) (2001). *Molecular Cloning: A Laboratory Manual*, 3rd Edn. New York, NY: Cold Spring Harbor Laboratory Press.

Shade, A., Jones, S. E., Caporaso, J. G., Handelsman, J., Knight, R., Fierer, N., et al. (2014). Conditionally rare taxa disproportionately contribute to temporal changes in microbial diversity. *mBio* 5:e1371-14. doi: 10.1128/mBio.01371-14

Shafi, J., Tian, H., and Ji, M. (2017). *Bacillus* species as versatile weapons for plant pathogens: a review. *Biotech. Biotechnol. Equip.* 31, 1–14. doi: 10.1080/13102818.2017.1286950

Sievers, F., Wilm, A., Dineen, D., Gibson, T. J., Karplus, K., and Li, W. (2011). Fast, scalable generation of high-quality protein multiple sequence alignments using Clustal Omega. *Mol. Syst. Biol.* 7:539. doi: 10.1038/msb.2011.75

Singh, R. P., Mishra, S., Jha, P., Raghuvanshi, S., and Jha, P. N. (2018). Effect of inoculation of zinc-resistant bacterium *Enterobacter ludwigii* CDP-14 on growth, biochemical parameters and zinc uptake in wheat (*Triticum aestivum* L.) plant. *Ecol. Eng.* 116, 163–173. doi: 10.1016/j.ecoleng.2017.12.033

Taghavi, S., Garafola, C., Monchy, S., Newman, L., Hoffman, A., Weyens, N., et al. (2009). Genome survey and characterization of endophytic bacteria exhibiting a beneficial effect on growth and development of poplar trees. *Appl. Environ. Microbiol.* 75, 748–757. doi: 10.1128/AEM.02239-08

Thapa, S., Ranjan, K., Ramakrishnan, B., Velmourougane, K., and Prasanna, R. (2018). Influence of fertilizers and rice cultivation methods on the abundance and diversity of phyllosphere microbiome. *J. Basic Microbiol.* 58, 172–186. doi: 10.1002/jobm.201700402

Timmusk, S., Behers, L., Muthoni, J., Muraya, A., and Aronsson, A.-C. (2017). Perspectives and challenges of microbial application for crop improvement. *Front. Plant Sci.* 8:49. doi: 10.3389/fpls.2017.00049

Trouvelot, S., Héloir, M., Poinssot, B., Gauthier, A., Paris, F., and Guillier, C. (2014). Carbohydrates in plant immunity and plant protection: roles and potential application as foliar sprays. *Front. Plant Sci.* 5:592. doi: 10.3389/fpls.2014.00592

Ullah, A., Heng, S., Munis, M. F. H., Fahad, S., and Yang, X. (2015). Phytoremediation of heavy metals assisted by plant growth promoting (PGP) bacteria: a review. *Environ. Exp. Bot.* 117, 28–40. doi: 10.1016/j.envexpbot.2015.05.001

Vágvölgyi, C., Sajben-Nagy, E., Bóka, B., Vörös, M., Berki, A., Palágyi, A., et al. (2013). Isolation and characterization of antagonistic *Bacillus* strains capable to degrade ethylenethiourea. *Curr. Microbiol.* 66, 243–250. doi: 10.1007/s00284-012-0263-8

Valencia, E., Gross, N., Quero, J. L., Carmona, C. P., Ochoa, V., Gozalo, B., et al. (2018). Cascading effects from plants to soil microorganisms explain how plant species richness and simulated climate change affect soil multifunctionality. *Glob. Change Biol.* 24, 5642–5654. doi: 10.1111/gcb.14440

Williams, T. R., and Marco, M. L. (2014). Phyllosphere microbiota composition and microbial community transplantation on lettuce plants grown indoors. *mBio* 5:e1564-14. doi: 10.1128/mBio.01564-14

Zhang, J., Kobert, K., Flouri, T., and Stamatakis, A. (2014). PEAR: a fast and accurate Illumina paired-end reAd mergeR. *Bioinformatics* 30, 614–620. doi: 10.1093/bioinformatics/btt593

Conflict of Interest Statement: The authors declare that the research was conducted in the absence of any commercial or financial relationships that could be construed as a potential conflict of interest.

frontiers in Plant Science

ORIGINAL RESEARCH
published: 08 February 2019
doi: 10.3389/fpls.2019.00047

Understanding the Biostimulant Action of Vegetal-Derived Protein Hydrolysates by High-Throughput Plant Phenotyping and Metabolomics: A Case Study on Tomato

Kenny Paul[1†], Mirella Sorrentino[1†], Luigi Lucini[2], Youssef Rouphael[3], Mariateresa Cardarelli[4], Paolo Bonini[5], Hélène Reynaud[6], Renaud Canaguier[7], Martin Trtílek[1], Klára Panzarová[1] and Giuseppe Colla[8,9]**

[1] Photon Systems Instruments (PSI, spol.sr.o.), Drásov, Czechia, [2] Department for Sustainable Food Process, Research Centre for Nutrigenomics and Proteomics, Università Cattolica del Sacro Cuore, Piacenza, Italy, [3] Department of Agricultural Sciences, University of Naples Federico II, Naples, Italy, [4] Centro di Ricerca Orticoltura e Florovivaismo, Consiglio per la Ricerca in Agricoltura e l'Analisi dell'Economia Agraria, Pontecagnano Faiano, Italy, [5] NGAlab, Tarragona, Spain, [6] Italpollina USA, Inc., Anderson, IN, United States, [7] Nixe, Valbonne, France, [8] Department of Agriculture and Forest Sciences, University of Tuscia, Viterbo, Italy, [9] Arcadia Srl, Rivoli Veronese, Italy

OPEN ACCESS

Edited by:
Jorge Almiro P. Paiva, Institute of Plant Genetics (PAN), Poland

Reviewed by:
Andrea Ertani, University of Padova, Italy
Giacomo Cocetta, University of Milan, Italy

***Correspondence:**
Klára Panzarová
panzarova@psi.cz
Giuseppe Colla
giucolla@unitus.it

[†] *These authors have contributed equally to this work*

Specialty section:
This article was submitted to Plant Breeding, a section of the journal Frontiers in Plant Science

Received: *02 November 2018*
Accepted: *14 January 2019*
Published: *08 February 2019*

Citation:
Paul K, Sorrentino M, Lucini L, Rouphael Y, Cardarelli M, Bonini P, Reynaud H, Canaguier R, Trtílek M, Panzarová K and Colla G (2019) Understanding the Biostimulant Action of Vegetal-Derived Protein Hydrolysates by High-Throughput Plant Phenotyping and Metabolomics: A Case Study on Tomato. Front. Plant Sci. 10:47. doi: 10.3389/fpls.2019.00047

Designing and developing new biostimulants is a crucial process which requires an accurate testing of the product effects on the morpho-physiological traits of plants and a deep understanding of the mechanism of action of selected products. Product screening approaches using omics technologies have been found to be more efficient and cost effective in finding new biostimulant substances. A screening protocol based on the use of high-throughput phenotyping platform for screening new vegetal-derived protein hydrolysates (PHs) for biostimulant activity followed by a metabolomic analysis to elucidate the mechanism of the most active PHs has been applied on tomato crop. Eight PHs (A–G, I) derived from enzymatic hydrolysis of seed proteins of *Leguminosae* and *Brassicaceae* species were foliarly sprayed twice during the trial. A non-ionic surfactant Triton X-100 at 0.1% was also added to the solutions before spraying. A control treatment foliarly sprayed with distilled water containing 0.1% Triton X-100 was also included. Untreated and PH-treated tomato plants were monitored regularly using high-throughput non-invasive imaging technologies. The phenotyping approach we used is based on automated integrative analysis of photosynthetic performance, growth analysis, and color index analysis. The digital biomass of the plants sprayed with PH was generally increased. In particular, the relative growth rate and the growth performance were significantly improved by PHs A and I, respectively, compared to the untreated control plants. Kinetic chlorophyll fluorescence imaging did not allow to differentiate the photosynthetic performance of treated and untreated plants. Finally, MS-based untargeted metabolomics analysis was performed in order to characterize the functional mechanisms of selected PHs. The treatment modulated the multi-layer

regulation process that involved the ethylene precursor and polyamines and affected the ROS-mediated signaling pathways. Although further investigation is needed to strengthen our findings, metabolomic data suggest that treated plants experienced a metabolic reprogramming following the application of the tested biostimulants. Nonetheless, our experimental data highlight the potential for combined use of high-throughput phenotyping and metabolomics to facilitate the screening of new substances with biostimulant properties and to provide a morpho-physiological and metabolomic gateway to the mechanisms underlying PHs action on plants.

Keywords: protein hydrolysates, integrative image-based high-throughput phenotyping, metabolomics, morpho-physiological traits, functional biostimulant characterization, ROS signaling

INTRODUCTION

Over the past decade, interest in plant biostimulants (PBs) has been on the rise, compelled by the growing interest of researchers, private industry and farmers in integrating these products in the array of environmentally friendly tools that secure improved crop productivity and yield stability under environmental stressors (Ertani et al., 2012, 2013; Haplern et al., 2015; Colla et al., 2017a; Yakhin et al., 2017; Rouphael et al., 2017a,c, 2018). Based on the new EU regulation, PBs are defined as '*CE marked products which stimulate plant physiological processes independently of the their nutrient content by improving one or more of the following characteristics of the plant rhizosphere or phyllosphere: nutrient use efficiency, tolerance to abiotic stress, crop quality, availability of confined nutrients in the soil and rhizosphere, humification and degradation of organic compounds in the soil*' (European Commission, 2016). Protein hydrolysates (PHs) are an important category of PBs which are produced by chemical, enzymatic or by combining chemical and enzymatic hydrolysis of proteins from animal or plant source (Ertani et al., 2009, 2017; Niculescu et al., 2009; Calvo et al., 2014; Colla et al. 2015, 2016, 2017a,b). Over the past 10 years, plant-derived PHs produced through enzymatic hydrolysis have received huge interest from farmers due to their high agronomic value and the lack of limitation in their application on organically produced crops (Colla et al., 2014; Nardi et al., 2016). PH-based biostimulants can be applied to plants through foliar application or soil/substrate drenching. PHs sprayed in foliar way reach mesophyll cells by absorption through cuticle, epidermal cells and stomata (Fernández and Eichert, 2009) while in drench application, the absorption occurs through root epidermal cells and gets redistributed through xylem (Subbarao et al., 2015). PHs can also be applied as seed treatments especially for field crops such as wheat, corn, and soybean (Rouphael et al., 2018). PH application stimulates plant uptake of macro and micronutrients and helps in rapid plant growth and biomass accumulation, interfering with the carbon and nitrogen metabolic activities (Ertani et al., 2009, 2016; Colla et al., 2017a). PHs can also improve crop tolerance to abiotic stresses such as drought, salinity, and thermal stress (Ertani et al., 2013; Lucini et al., 2015; Colla et al., 2017a). Therefore, improving metabolic and physiological traits by PH-based biostimulant treatments provides novel strategies for maximizing biomass yield (Dudits et al., 2016). Development of highly effective PH-based biostimulants requires an accurate evaluation of the effects of candidate products on morpho-physiological traits of selected crops during different developmental stages and environmental conditions. As conventional screening methods are time consuming, destructive (e.g., fresh and dry weight estimation), labor intensive and expensive, high-throughput plant phenotyping procedures were recently proposed as effective and high-precision tools for product screening in order to increase the probability of finding new bioactive substances in a more cost- and time-effective manner (Povero et al., 2016; Rouphael et al., 2018; Ugena et al., 2018). 'Phenomics' as a technological tool considers systematic management of complex traits in genome (G) × environment (E) interactions (Houle et al., 2010). Plant phenotyping systems are fully automated robotic systems usually installed in a controlled environment or in semi-controlled greenhouse conditions. The phenotyping platforms are designed to ensure not only non-invasive monitoring of plants in throughput of few up to several hundreds of plants, but also provide means for automated cultivation and handling of the plants such as automated watering/weighing or nutrient delivery units (Fahlgren et al., 2015; Großkinsky et al., 2015). High-throughput phenotyping systems, which can capture plant growth, morphology, color and photosynthetic performance using RGB and chlorophyll fluorescence (ChlF) imaging tools, are highly promising and essential tools for dissecting physiological components in product screening and for dynamic quantitative analysis of plant growth and physiological performance (Rahaman et al., 2015; Awlia et al., 2016; Rouphael et al., 2018). RGB imaging is used to estimate the true color of each pixel and by using image processing algorithms for identification of plant-derived pixels. For identified plant objects, morphological and geometrical features are quantified including color properties (Rahaman et al., 2015). The pixel number-based assessment of plant volume or total leaf area correlates with fresh and dry weight of above ground plant biomass and can be thus used to evaluate green/fresh weight of the plants without cutting and measuring them (Fehér-Juhász et al., 2014; Fahlgren et al., 2015). Further image-based automated phenotyping permits time-series measurements that are necessary to follow the progression of growth performance and stress responses on individual plants.

Chlorophyll fluorescence is a popular technique in plant physiology used for rapid non-invasive measurement of

photosystem II (PSII) activity. PSII activity is very sensitive to a range of biotic and abiotic factors and therefore the chlorophyll fluorescence technique is used as a rapid indicator of photosynthetic performance of plants in different developmental stages and/or in response to changing environment (Baker, 2008). The advantage of chlorophyll fluorescence measurements over other methods for monitoring stresses is that changes in chlorophyll fluorescence kinetic parameters often occur before other effects of stress are apparent (Murchie and Lawson, 2013). Chlorophyll fluorescence imagers integrated in high-throughput phenotyping platforms are becoming important tools for rapid screening for better photosynthetic performance and characterization of a plant's ability to harvest light energy, which is directly related to plant biomass formation and plant architecture (Tschiersch et al., 2017).

Nonetheless, the comprehension of biochemical processes and physiological functions underlying the changes observed at phenotype level is of primary relevance to scientifically demonstrate and support the use of plant biostimulants, likely providing some clues on the best scenarios where these products can be used. It is expected that in the near future, provided that a regulatory framework will be implemented at least in the EU and United States, the information on mechanism/mode(s) of action will support biostimulants authorization and implementation. In this regard, metabolomics is being proposed as a close link between an organism's genotype and phenotype (Lamichhane et al., 2018), including plant-environment interactions (Feussner and Polle, 2015). In fact, recent advances in metabolomics, data treatment and multi-variate statistics offer the possibility to achieve a rather inclusive phytochemical profile in biological systems, including plants, thus opening new opportunities (Meier et al., 2017; Tsugawa, 2018). This makes metabolomics a promising tool to elucidate, among others, the mode of action rather than the physiological processes involved in plant response to biostimulants.

Taking this background into consideration, the aim of this study was to unravel the morphological, physiological and biochemical mechanisms of action for protein hydrolysate-based biostimulants on tomato plants at early stage of growth (i.e., vegetative growth) by combining novel high-throughput plant phenotyping approach and metabolomics. Untreated and treated tomato plants were compared in terms of photosynthetic performance through kinetic chlorophyll fluorescence, and plant growth dynamics via RGB imaging by using high-throughput and non-invasive imaging technologies developed at Photon Systems Instruments (PSI, Czechia). Metabolomics analysis was performed to understand the mode of action of the best performing substances in improving plant growth. Evaluation of biostimulant activity at early growth stages of fruiting crops such as tomato can provide useful information for improving crop yield under field conditions. Crop traits like early vigor are associated with earliness of fruit maturity and high shoot biomass accumulation which have been often positively linked to increased yield of tomato crop (Kumar et al., 2015; Rouphael et al., 2017b). Finally, this study was also aimed to set up a methodology for screening plant biostimulants by combining an advanced phenotyping platform and metabolomic analysis.

MATERIALS AND METHODS

Plant Material and Growing Conditions

Seeds of tomato (*Solanum lycopersicum* L. - Hybrid F1 Chicco Rosso) were sown in trays with 100 ml size of pots containing freshly sieved soil (Substrate 2, Klasmann-Deilmann GmbH, Germany) watered to full soil-water holding capacity. Trays with seeds were stratified for 2 days at 4°C in the dark. Trays were then transferred to a climate-controlled chamber (FytoScope FS_WI, PSI, Drásov, Czechia) with cultivation conditions set at 16 h day/8 h night regime, temperature set at 22°C day/20°C night, relative humidity set at 60% and light intensity set at 250 μmol photons m^{-2} s^{-1} for cool-white LED and 5.5 μmol photons m^{-2} s^{-1} for far-red LED lighting (**Figure 1A**).

Plant Handling and Biostimulant Treatment

Prior initiation of automated phenotyping protocol, tomato plants were manually watered. Seven- and 14-day-old plants were watered to full saturation with fertilizers: 1.04 g L^{-1} calcium nitrate (15.5% N; 28% CaO), 0.04 g L^{-1} ammonium nitrate (34% N), 0.14 g L^{-1} monopotassium phosphate (52% P_2O_5, 34% K_2O), 0.18 g L^{-1} potassium sulfate (50% K_2O, 45%SO_3), 0.5 g L^{-1} magnesium sulfate (10%N, 16% MgO), and 0.5 ml L^{-1} FloraMicro (5% N, 1% K_2O, 5% Ca, 0.01% B, 0.001% Cu, 0.1% Fe, 0.05% Mn, 0.0008% Mo, 0.015% Zn).

Twenty-one-day-old plants reaching third true leaf stage were transplanted into 3 L pots filled with a mixture of Substrate 2 Klasmann soil and river sand in 3:1 ratio. Pots with soil mixture were prepared 1 day in advance of transplantation and were automatically watered in PlantScreen™ Modular System to ensure soil moisture reaching 60% container capacity. For optimizing container capacity, one set of soil pots was dried for 3 days at 80°C and another set was saturated with water and left to drain for 1 day before weighing 100% water holding capacity (Awlia et al., 2016). Following transplantation, plants were regularly watered to defined reference weight (60% container capacity) automatically in PlantScreen™ Modular System.

Plants were randomly distributed into nine groups with six biological replicates per group. Eight types of plant-derived protein hydrolysates (A–G, I) were provided by Italpollina Company (Rivoli Veronese, Italy). PHs were obtained by the advanced technology LISIVEG which is based on enzymatic hydrolysis of vegetal-derived proteins from different plant sources belonging to families of *Leguminosae* and *Brassicaceae*. Total nitrogen of each PH was as follow: 5.2% (A), 4.6% (B), 3.7% (C), 4.2% (D), 4.3% (E), 4.2% (F), 4.0% (G), 5% (I). PHs (A–G) were non-commercial products whereas I was a commercial biostimulant named 'Trainer®' derived from legume seeds. All PHs were foliarly sprayed in a water solution containing a non-ionic surfactant Triton X-100 at 0.1%. A control group sprayed with distilled water containing 0.1% Triton X-100 was also included. Foliage sprays were performed twice: 5 DAT (days after transplantation) referred to as Treatment 1 (T1) and 12 DAT

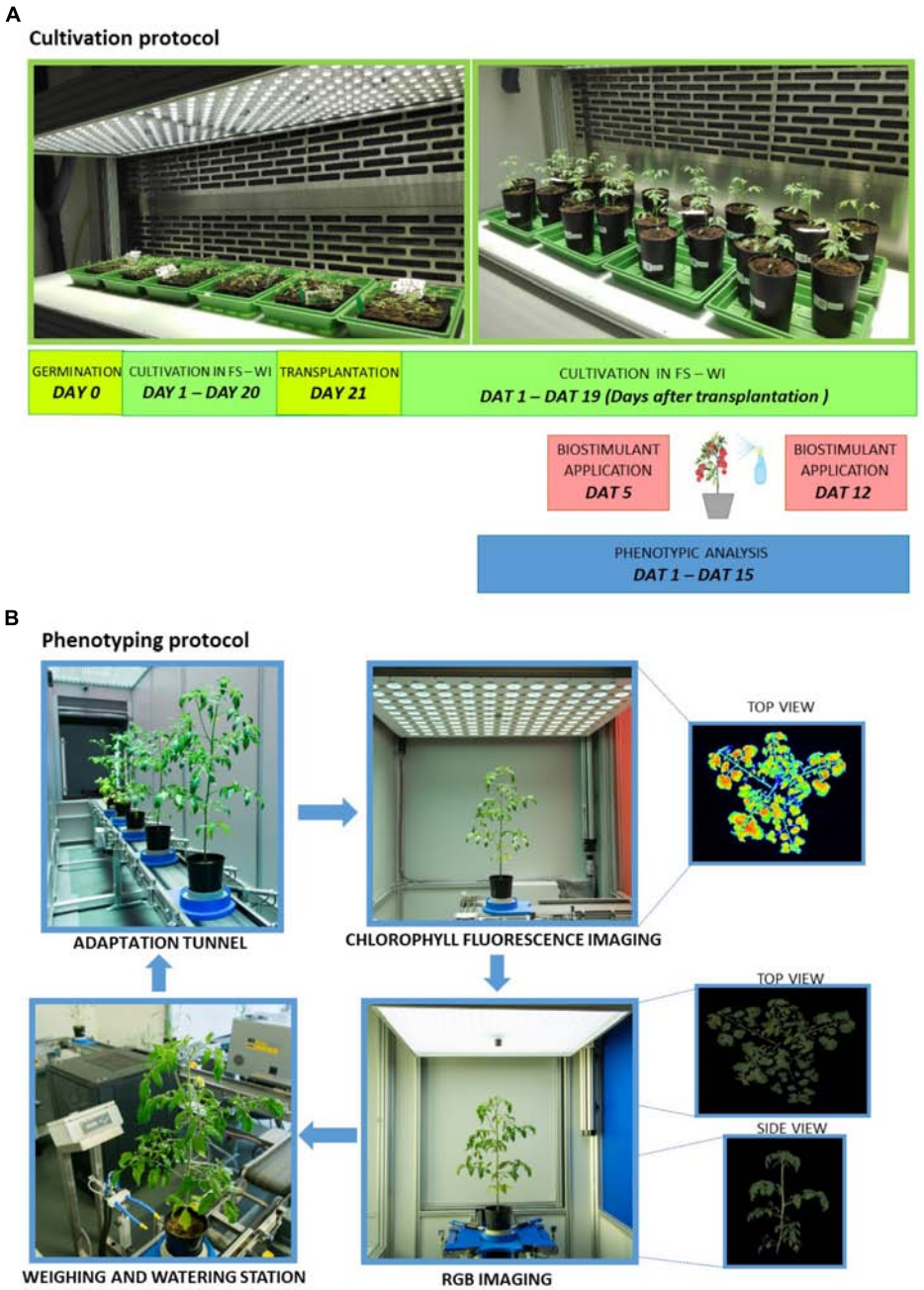


FIGURE 1 | Schematic overview of cultivation protocol and automated phenotyping protocol. **(A)** Plants were cultivated for 20 days prior to transplantation in control conditions (FS-WI, PSI, Czechia) and were further kept in the same conditions for the next 19 days (DAT, days after transplantation). Eight types of protein hydrolysates (A–G, I) plus control treatment were applied twice to tomato plants by spraying 5 and 12 days after transplantation. Plant phenotypic measurements were performed during the experiment using PlantScreen^{TM} Modular System installed in semi-controlled greenhouse environment conditions in PSI Research Center (PSI, Drásov, Czechia). **(B)** Plant handling and automated phenotyping protocol. Tomato plants were transferred to PlantScreen^{TM} Modular System and automated phenotyping protocol was initiated. Prior to and following the protein hydrolysates application, plants were regularly screened using the calibrated top and side view RGB camera and kinetic chlorophyll fluorescence camera for photosynthetic performance quantification. Plants were regularly watered and weighed using the automated watering and weighing station.

referred to as Treatment 2 (T2). For 24 h prior to and post spraying, humidity in the cultivation chamber was kept at 85% relative humidity. For foliar spray treatments, 2 ml of given PH was diluted in 500 ml distilled water with 0.1% Triton X-100 and 60 ml of solution was applied by homogenous foliar spray over the entire plant surface per plant replica. Soil of each pot was covered with aluminum foil during and upon spraying and was removed prior to the next phenotypical analysis in PlantScreen^{TM}

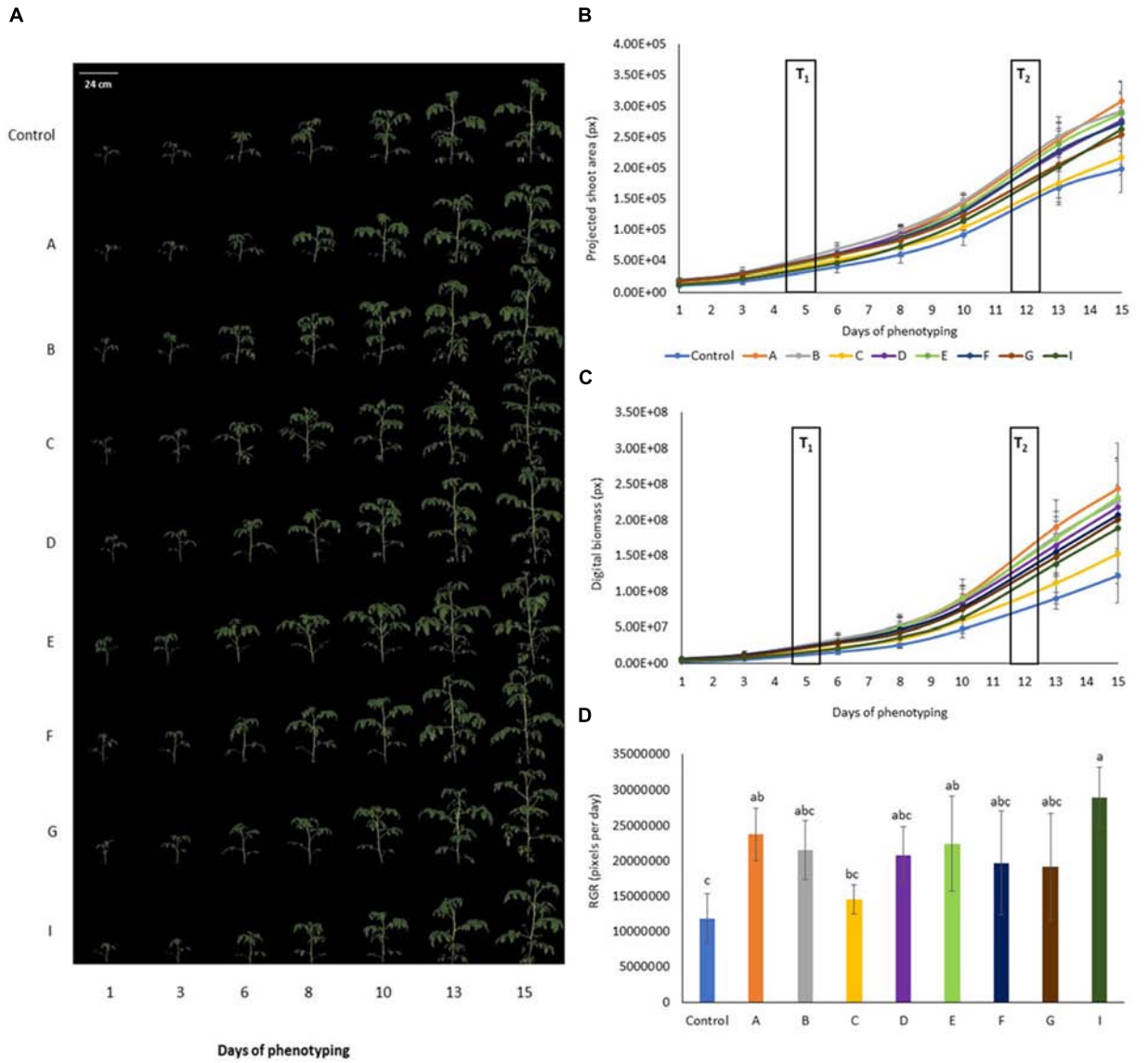


FIGURE 2 | Color segmented side view Red Green Blue (RGB) images of tomato plants prior to and upon PHs application. **(A)** Side view (120°) RGB image of the tomato plants over the time of phenotyping period (D1–D15). **(B)** Projected shoot area over time of phenotyping period. Values represent the average of six biological replicates per treatment. Error bars represent standard deviation. T1 and T2 correspond to days of protein hydrolysates application by foliar spraying. **(C)** Digital biomass quantified over time of phenotyping period. Values represent the average of six biological replicates per treatment. Error bars represent standard deviation. T1 and T2 correspond to days of protein hydrolysate application by foliar spraying. **(D)** Comparison of relative growth rate for the different treatments quantified over phenotyping period following the protein hydrolysate treatments (DAT 6–DAT 15). Values represent the average of six biological replicates per treatment. Error bars represent standard deviation.

Modular System (**Figure 1B**). Right after foliar spray treatment, plants were taken back to fytoscope FS-WI.

Phenotyping Protocol and Imaging Sensors

Plant phenotypic measurements were performed using PlantScreen^{TM} Modular System installed in semi-controlled greenhouse environment conditions in PSI Research Center (PSI, Drásov, Czechia). The platform was operated in a closed imaging loop that is within climatized environment with temperature ranging between 21 and 24°C. The platform is equipped with four robotic-assisted imaging units, automatic height measuring light curtain unit, an acclimation tunnel, and a weighing and watering unit. Plants set in individual transportation disks were transported to the individual units by a moving belt toward individual imaging and handling units. Twenty-two-day-old plants were randomly distributed into six batches, each batch containing 11 plants. Plant imaging started 1 DAT (day 1 of phenotyping) and continued until 15 DAT (day

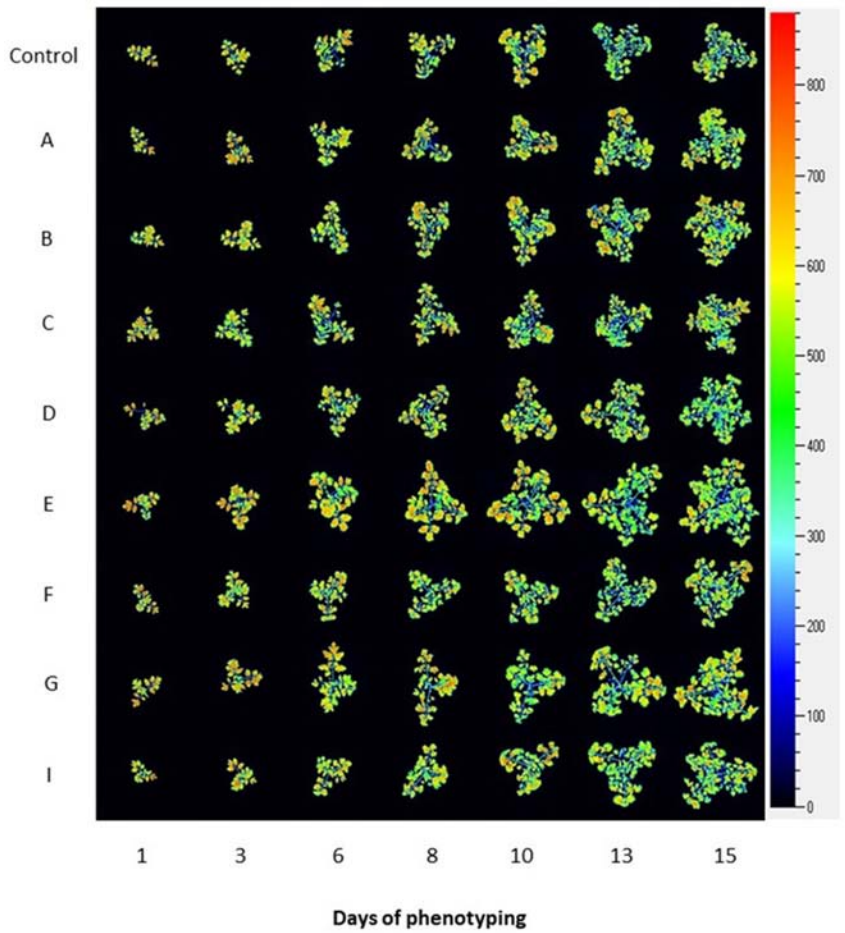


FIGURE 3 | Photosynthetic performance of tomato plants visualized by kinetic chlorophyll fluorescence imaging in all protein hydrolysate treatments. Representative images of chlorophyll fluorescence for tomato plants prior to and upon PHs treatment. False-color images of maximum fluorescence value (FM) for tomato plants over phenotyping period (days 1–15) are shown.

15 of phenotyping). Plants were imaged using the following protocol. Briefly, plants were manually transferred from the climate-controlled growth chamber to the manual loading station of the PlantScreen^{TM} Modular System, transported to the acclimation tunnel through an automatic height measuring unit and dark adapted in an acclimation tunnel for 15 min prior to imaging. Successively, plants were automatically phenotyped for around 30 min per batch using kinetic chlorophyll fluorescence imaging measurement for photosynthetic performance analysis and top view and multiple angle side view Red Green Blue (RGB) imaging for morphological and growth analysis. Finally, plants were automatically transported to the watering and weighing unit for maintaining precise soil water holding capacity at 60%. After the end of the phenotyping protocol, plants were manually moved back to the climate-controlled growth chamber until the subsequent phenotyping day. Using the automatic timing function of PlantScreen^{TM} Scheduler (PSI, Drásov, Czechia), the phenotyping protocol was programmed to always start at the same time of the diurnal cycle (after 3 h of illumination in the climate-controlled growth chamber). Phenotyping protocol was recorded twice prior to biostimulant application in days 1 and 3 (pre-T measurements); three times post first biostimulant application in days 6, 8, and 10 (post T1 application) and twice post second biostimulant application in days 13 and 15 (post T2 application). The acquired images were automatically processed using Plant Data Analyzer (PSI, Drásov, Czechia) and the raw data exported into CSV files were provided as input for further analysis.

Kinetic Chlorophyll Fluorescence Imaging

Kinetic chlorophyll fluorescence (ChlF) measurements were acquired using an enhanced version of the FluorCam FC-800MF pulse amplitude modulated (PAM) chlorophyll fluorometer (PSI, Czechia) (Awlia et al., 2016) with an imaging area in top view position of 800 mm × 800 mm as described in Tschiersch et al. (2017). Photosynthetic performance in the plants was assessed by quantifying the rate of photosynthesis at different photon irradiances using the light curve protocol (Henley, 1993; Rascher

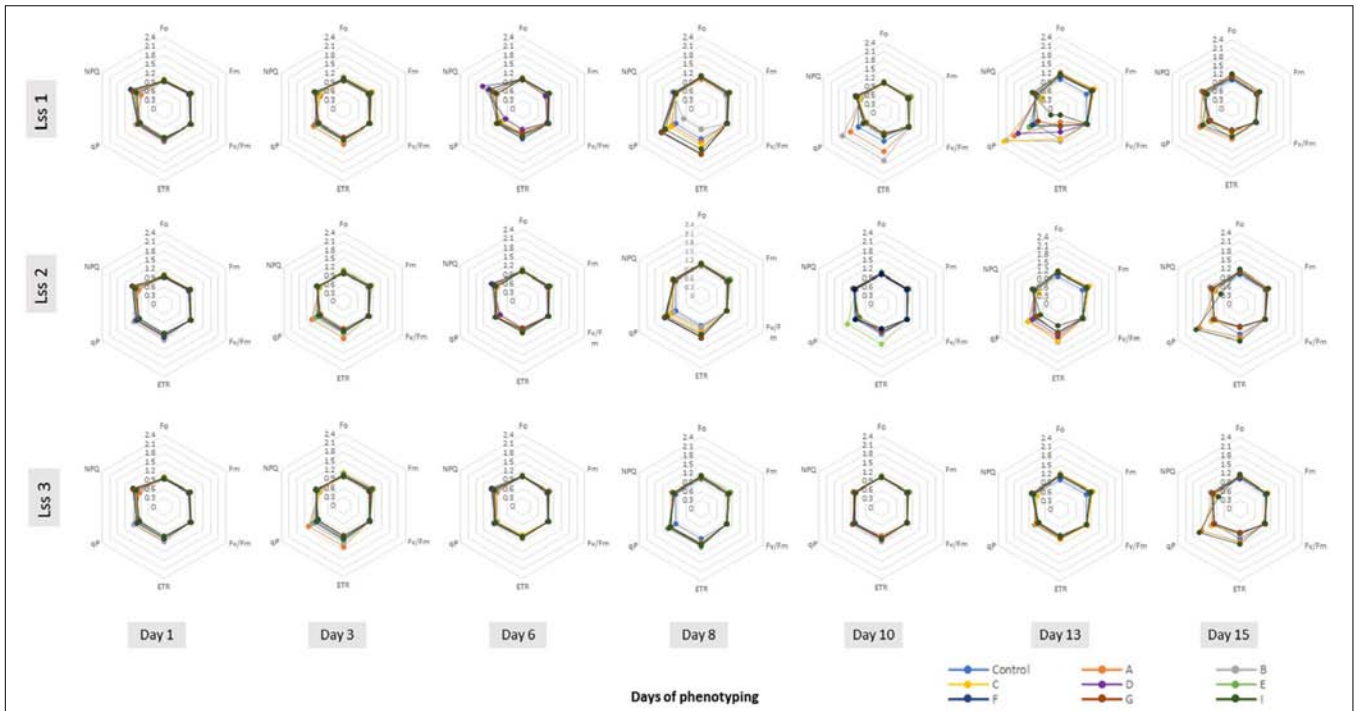


FIGURE 4 | Spider plots of photosynthetic parameters deduced from kinetic chlorophyll fluorescence imaging on whole plant level in all treatments. Minimal fluorescence in dark-adapted state (F_o), maximum fluorescence in dark-adapted state (F_m), maximum quantum yield of PSII photochemistry for the light-adapted state (F_v'/F_m'), the photochemical quenching coefficient that estimates the fraction of open PSII reaction centers (qP), steady-state non-photochemical quenching (NPQ) and electron transport rate (ETR) were measured using the light curve protocol for tomato plants prior to and upon PHs treatments. The data are shown for the protein hydrolysate treated plants after normalization to respective values obtained in the control treatment at various time points of phenotyping period. Data are mean of six independent plants per treatment. Lss1, Lss2 and Lss3 represent actinic photon irradiance measurements taken at 170, 620, and 1070 μmol photons m^{-2} s^{-1}, respectively.

et al., 2000) which was proven to provide detailed information on ChlF under stress, information on photosynthetic performance in many studies dealing with plants' stress and to quantify the rate of photosynthesis at different light irradiances (Digruber et al., 2018) (**Supplementary Figure S1**). Protocol described previously (Awlia et al., 2016) was optimized for the tomato plants from early to later developmental stage. Finally, three actinic light irradiances (Lss1- 170 μmol photons m^{-2} s^{-1}, Lss2 – 620 μmol photons m^{-2} s^{-1}, Lss3 - 1070 μmol photons m^{-2} s^{-1}) with a duration of 30 s in the light curve protocol were used to quantify the rate of photosynthesis.

Visible RGB Imaging

To assess digital biomass of the plants, RGB imaging was done from top view (RGB2) and side view from multiple angles (RGB1) (**Supplementary Figure S2**). The RGB imaging unit implemented in PlantScreen™ Modular System is a light isolated box equipped with a turning table with precise angle positioning, two RGB cameras (top and side) mounted on robotic arms and each supplemented with LED-based lighting source to ensure homogenous illumination of the imaged object. Imaged area in top view position is 800 mm × 800 mm, imaged area from side view is 1205 mm × 1005 mm (height × width). Here we acquired side view images from three different angles (0, 120, and 240°) for side view RGB analysis. RGB images (resolution 2560 pixels × 1920 pixels) of the plants were captured using the GigE uEye UI-5580SE-C - 5 Megapixels QSXGA Camera with 1/2″ CMOS Sensor (IDS, Germany) from top and side view. For side view projections, line scan mode was used with a resolution −2560 × 2956 px/scan, 200 lines per second. Lighting conditions, plant positioning and camera settings were fixed throughout the experiment. Raw RGB images were processed as described previously (Awlia et al., 2016) with some modifications for side view RGB image processing algorithms. Projected shoot area (PSA) for side view was calculated as average of plant specific pixels extracted from three side view images acquired from 0, 120, and 240° angles. PSA extracted from top and side view projections was used to estimate shoot biomass. Briefly side view and top view RGB images of the plants were used for calculation of plant volume, using the formula from Klukas et al. (2014):

$$V = \sqrt{A^2_{S\,(average)} \times A^2_t}$$

where A_s and A_t are the projected areas from side-view (at different angles) and top-view images, respectively. Volume was termed as "digital biomass," as reported in a work from Rahaman et al. (2017). Digital biomass was used to calculate relative growth

rate (RGR) between two timepoints T_1 and T_2 as follows:

$$RGR = (\ln W_2 - \ln W_1)/(T_2 - T_1)$$

In addition, height and width of the plants were calculated from the binary side view images. For shoot greenness evaluation, six hues of green were automatically generated using as input images all the original RGB images captured during the phenotyping period (DAT 1–DAT 15). These six most representative hues were selected and used to estimate the variations in shoot colors and are shown in RGB color scale as a percentage of the shoot area (pixel counts).

Sample Harvest

Ninteen DAT (19th day of phenotyping) plant material was harvested. For metabolomic analysis of tomato plants treated with biostimulants A, B, I, and control plants third and fourth fully expanded leaves from the top of each plant were harvested. The non-commercial biostimulants A and B were selected for the metabolomic analysis based on the higher morpho-physiological traits and were compared to the commercial biostimulant (I) as well as to the untreated control treatment. Final biomass of each plant was determined by measuring fresh weight and dry weight of remaining shoot in a ventilated oven at 65°C until constant weight.

Untargeted Metabolomics

Leaf samples (1.0 g) were extracted using an Ultra-Turrax (Ika T-25, Staufen, Germany), in 10 mL of 0.1% HCOOH in 80% aqueous methanol. The extracts were centrifuged (12,000 × *g*), then filtered through a 0.22 μm cellulose membrane directly into amber vials for analysis. Thereafter, untargeted metabolomics were carried out through an UHPLC chromatographic system coupled to a hybrid quadrupole-time-of-flight mass spectrometer (UHPLC/QTOF-MS). The metabolomic platform included a 1290 ultra-high-performance liquid chromatograph, a G6550 iFunnel Q-TOF mass spectrometer and a JetStream Dual Electrospray ionization source (all from Agilent technologies, Santa Clara, CA, United States). The analysis was carried out as previously described (Rouphael et al., 2016). Briefly, chromatographic separation was achieved in reverse phase mode, using an Agilent Zorbax Eclipse-plus C18 column (100 mm × 2.1 mm, 1.8 μm) and a linear gradient (5–95% methanol in water, 34 min run time) foe elution, with a flow of 220 μL min^{-1} at 35°C. The mass spectrometric acquisition was done in positive polarity and extended linear dynamic range SCAN (100–1000 *m/z*).

Features deconvolution and post-acquisition processing were done in Agilent Profinder B.06. After mass and retention time alignment, compounds annotation was achieved using the 'find-by-formula' algorithm based on monoisotopic accurate mass, isotopes spacing and isotopes ratio, with a mass accuracy tolerance of <5 ppm. The database PlantCyc 12.5 (Plant Metabolic Network[1]) was used for annotation purposes. Based on the strategy adopted, identification was carried out according to Level 2 (putatively annotated compounds) of COSMOS Metabolomics Standards Initiative[2].

A filter-by-frequency post-processing filter was applied to retain only those compounds that were present in 75% of replications within at least one treatment. The classification of differential compounds into biochemical classes was carried following PubChem (NCBI[3]) and PlantCyc information.

Data Management and Statistical Analysis

The data processing pipelines Plant Data Analyser (PSI, Drásov, Czechia) includes pre-processing, segmentation, feature extraction and post-processing. Values for projected shoot area were calculated from images taken in the visible light spectrum and correspond to volume estimation which were used as a proxy for the estimated biomass of the plants. Data were processed using MVApp application (mmjulkowska/MVApp: MVApp.pre-release_v2.0; Julkowska et al., unpublished). Using the MVApp, outliers were identified with the interquartile range rule as plants whose volume had a value 1.5 times away from the mean. Those plants were removed from the data set. Statistical differences between treatments and time points were determined by one-way analysis of variance (ANOVA) with *post hoc* Tukey's Honest Significant Difference (HSD) test (P-value < 0.05) performed using appropriate scripts in MVApp tool. Data are displayed as mean ± standard deviation of the six independent plants per treatment.

Interpretation of metabolomic data was formerly carried out using Mass Profiler Professional B.12.06 as previously described (Salehi et al., 2018). Briefly, compound abundance was Log2 transformed and normalized at the 75th percentile and baselined against the median. Unsupervised hierarchical cluster analysis was formerly carried out using the fold-change based heatmap, setting similarity measure as 'Euclidean' and 'Wards' linkage rule. Thereafter, the dataset was exported in SIMCA 13 (Umetrics, Malmo, Sweden), UV-scaled and elaborated for Orthogonal Projections to Latent Structures Discriminant Analysis (OPLS-DA) modeling. This latter multivariate supervised statistic allowed separating variance into predictive and orthogonal (i.e., ascribable to technical and biological variation) components. Outliers were excluded using Hotelling's T2 and adopting 95% and 99% confidence limits, for suspect and strong outliers, respectively. Model cross validation was done through CV-ANOVA (p < 0.01) and permutation testing (N = 300) was used to exclude overfitting. Model parameters (goodness-of-fit R^2Y and goodness-of-prediction Q^2Y) were also produced. Finally, Variable Importance in Projection (VIP) analysis was used to select the metabolites having the highest discrimination potential. A subsequent fold-change analysis was performed from VIPs to identify extent and direction of the changes in accumulation related to the biostimulants.

[1]http://www.plantcyc.org

[2]http://cosmos-fp7.eu/msi.html

[3]https://pubchem.ncbi.nlm.nih.gov/

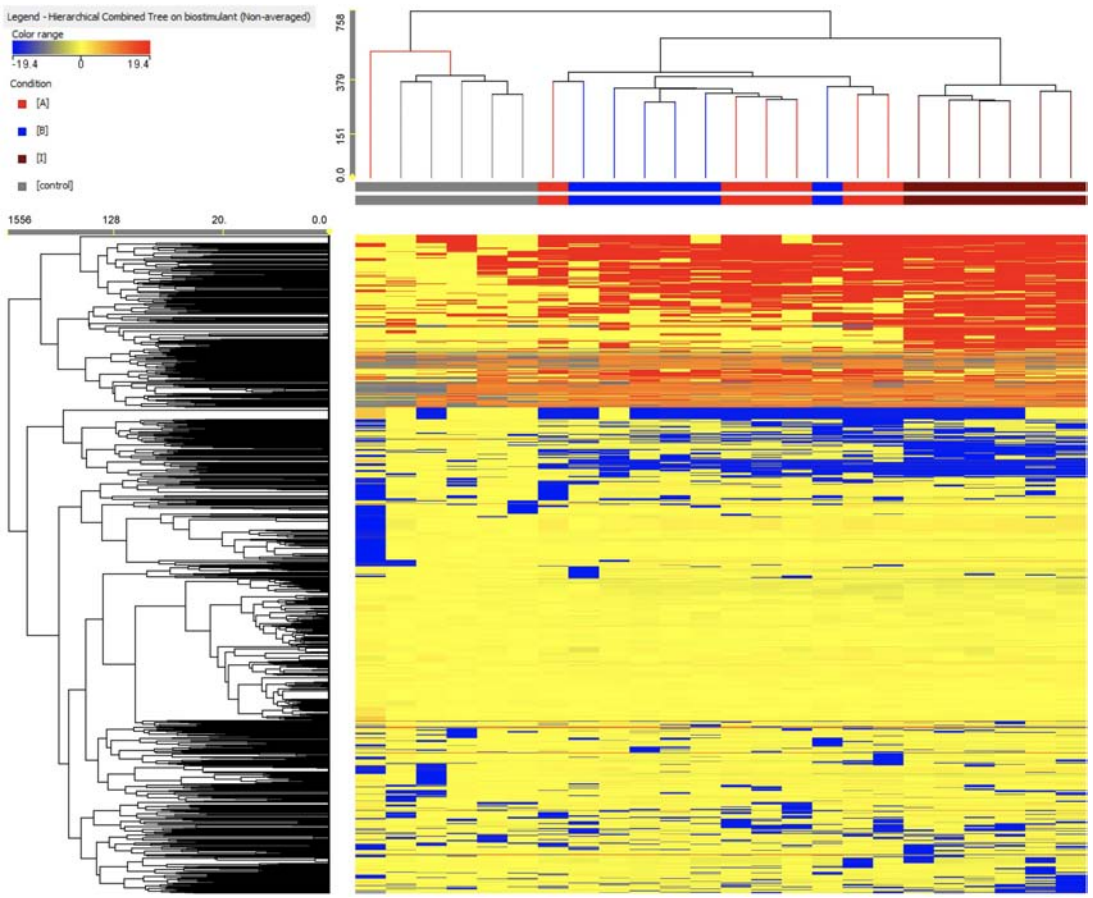


FIGURE 5 | Unsupervised hierarchical cluster analysis carried out from metabolomic profiles following application of selected protein hydrolysates. Dendrograms were produced on the basis of fold-change heat-maps using metabolites profile gained from UHPLC-ESI/QTOF untargeted metabolomic profiling. The Wards' linkage rule and Euclidean similarity were chosen to produce dendrograms.

RESULTS AND DISCUSSION

High-Throughput Phenotyping of Tomato Plant Growth

Visible light Red Green Blue (RGB) digital imaging based on using cameras sensitive in visible spectral range (400–750 nm) allows non-invasive dynamic quantification of shoot biomass, measurement of a wide range of plant morphological parameters and analysis of shoot color. Multiple angle side view images (**Figure 2** and **Supplementary Figure S2**) and simple image stacks acquired from top view were used to calculate plant volume that is an approximate of digital biomass of the plants throughout the cultivation period. Regularly acquired multiple time points measurements were used to asses dynamic changes in plant morphology, color and calculate growth rates.

In general, tomato plants treated with PHs showed better shoot biomass production in comparison with the untreated control plants (**Figure 2**). Top view projected shoot area was increased in tomato plants treated with PHs A and E post first foliar treatment (**Supplementary Table S1**). For A treatment this correlated with PSA extracted from multiple angle side view RGB images (**Supplementary Table S2**) with B treatment improving the PSA in period between the two foliar treatments. In terms of morphological features extracted from both top and side view images such as compactness, height and width of the plants, treatments A, B, D, E and F gave an increase of height and width of plants (**Supplementary Tables S3, S4**). The digital biomass of the plants sprayed with PHs increased (**Figure 2C**), especially in the case of A treatment where the improved growth performance was significantly compared to untreated control plants from the 8th day of phenotyping, 3 days post first foliar spraying, respectively (**Supplementary Table S5**). The same trend was recorded when the growth dynamics was considered by evaluating plant growth rates. We quantified relative growth rates from DAT 6–DAT 15 representing growth performance of the plants following the two PH treatments that were applied on DAT 5 and DAT 12 (**Figure 2D**). For A, E and I treatment, growth rates were improved when compared to control plants, however, the effect of A and E treatment could not be discriminated from the effects of the other PHs. Interestingly, the treatment I was identified as the one with highest growth rate among all PHs. Overall among all treatments, the best growth performance trend

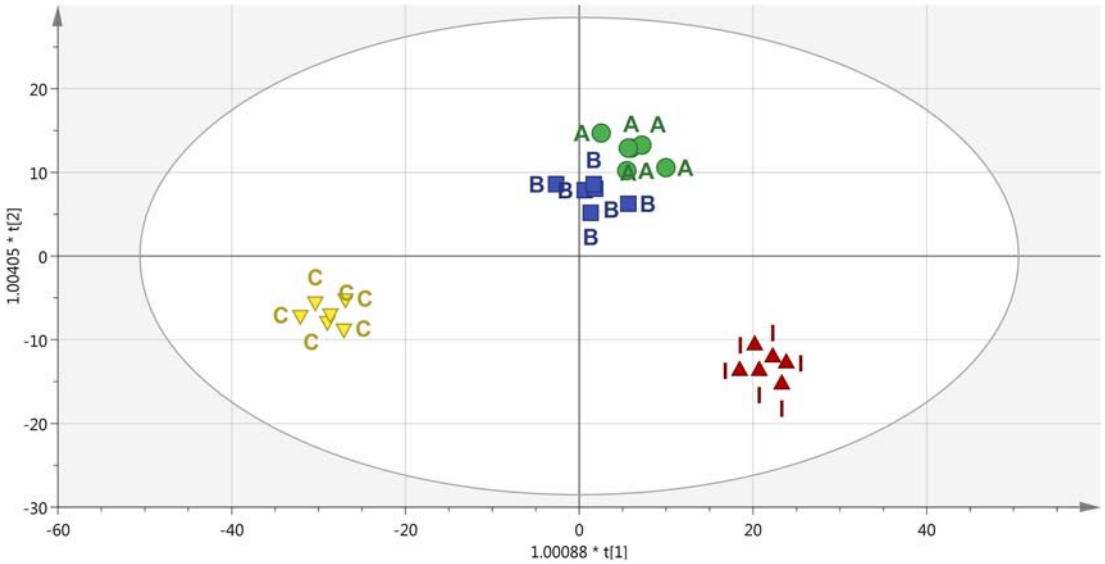


FIGURE 6 | Score plot of Orthogonal Projection to Latent Structures Discriminant Analysis (OPLS-DA) supervised modeling carried out on metabolomic profiles following application of selected protein hydrolysates. The variation between groups was separated into predictive and orthogonal components (i.e., that ascribable to technical and biological variation). The OPLS model was cross-validated using CV-ANOVA ($p < 0.01$) and permutation tested to exclude over fitting. Furthermore, the presence of outliers was investigated according to Hotelling's T2 method (i.e., the distance from the origin in the model) using 95 and 99% confidence limits for "suspect" and "strong" outliers, respectively. The pattern observed in the score plot was used to identify discriminant compounds based on Variable of Importance in projection (VIP) analysis.

in terms of biomass and growth rate was observed for tomato plants treated with treatment A, whereas tomato plants treated with PH named C were smaller with slower growth dynamics. This further correlated with analysis of dry and fresh weights of tomato shoots that were harvested following the end of the phenotyping period (r = 0.87* and 0.85* for fresh and dry weight, respectively).

We further evaluated the variation in shoot green colors over the phenotyping period by using greenness hue abundance automatically computed from color-segmented RGB images (**Supplementary Figure S3**). We calibrated the analysis algorithms by using RGB images from all treatments and all days of phenotyping as described previously in Awlia et al. (2016). Dynamic changes in green hues during the plant growth were observed, however, no significant differences in the green hues were detected between the treatments (**Supplementary Table S6**).

High-Throughput Phenotyping of Photosynthetic Performance in Tomato Plants

Chlorophyll fluorescence imaging has become one of the most powerful and popular tools in plant biology for rapid non-invasive measurement of Photosystem II (PSII) activity. Because PSII activity is very sensitive to a wide range of stimuli, chlorophyll fluorescence imaging can be used as rapid indicator of plant photosynthetic performance in different developmental stages, and in response to environmental changes (Murchie and Lawson, 2013).

To assess the physiological status of tomato plants treated with the biostimulants, we used the automated chlorophyll fluorescence imaging setup (**Figure 3** and **Supplementary Figure S1**) and quantified the rate of photosynthesis at different photon irradiances using the light curve protocol (Henley, 1993; Rascher et al., 2000). From the measured fluorescence transient states, the basic ChlF parameters were derived (i.e., F_o, F_m, F_t, and F_v), which were used to calculate range of parameters characterizing plant photosynthetic performance (i.e., F_v'/F_m', NPQ, qP, and ΦPSII) (for overview refer to Paul et al., 2011; Awlia et al., 2016; Tschiersch et al., 2017). In addition, ETR parameter was calculated which refers to photosynthetic electron transport rate of photosystem II and indicates the efficiency of linear electron flow route in the photosynthetic machinery for producing energy rich molecules ATP and NADPH.

We selected six of the parameters to characterize dynamically photosynthetic function of PSII in the tomato plants prior to and post biostimulant treatment: the minimal level of fluorescence measured in dark-adapted state (F_o), the maximum level of fluorescence measured in dark-adapted state (F_m), the maximum quantum yield of PSII photochemistry in the light-adapted state (F_v'/F_m'), the photochemical quenching coefficient that estimates the fraction of open PSII reaction centers (qP), steady-state non-photochemical quenching (NPQ) and PS II operating efficiency (ΦPSII) used for calculation of electron transport rate (ETR). ETR is a process correlated to the quantum yield of the CO_2 assimilation mechanisms and to the overall photosynthetic capacity of the plants (Genty et al., 1989). As shown in **Figure 4**, the selected fluorescence parameters varied partially between the individual days following the PH treatment, however, we could

not observe any trend among the treatments. In addition, we were not able to detect any significant changes in the ChlF parameters assessed (**Supplementary Table S7**). This was the case for all treatments at any photon irradiances used.

Kinetic chlorophyll fluorescence imaging used for non-invasive quantitative analysis of PSII fluorescence emission is especially suited to monitor physiological traits via changes in photochemistry. In the field of automated high-throughput phenotyping, PAM Chl fluorescence imaging is still not widely used in the imaging sensor platforms, however, a range of studies already demonstrated the broad potential of the technique to measure quantitatively physiological state of the plants and to diagnose the reactions of the plants to stress even before visible symptoms become apparent (Paul et al., 2011; Awlia et al., 2016; Tschiersch et al., 2017). Biostimulants have shown to increase photosynthetic efficiency, improve the efficiency of light utilization and dissipation of excitation energy in PSII antennae as well as an increase in photosynthetic pigments (Yakhin et al., 2017). The fact that in our case the application of the PHs did not result in improved photochemistry parameters, although the biomass of the biostimulant treated plants increased, might be associated with the beneficial action of PHs on stomatal conductance rather than on the PSII directly. This might improve net CO_2 assimilation rate and consequently biomass production. Another putative mechanism involved in the stimulation of plant growth and productivity of PH-treated tomato plants could be the occurrence of smaller and more responsive stomata that are proposed to be able to sustain higher photosynthetic activities (Rouphael et al., 2017d).

Metabolomics Analysis of Tomato Leaves for Understanding the Mode of Action of Selected PHs

A metabolomic approach was used, following phenotyping analysis, aimed to strengthen at the molecular level the effects of the PHs on morpho-physiological traits and plant growth. Indeed, the understanding of the mechanisms through which PHs act on a plant can effectively support their actual implementation into agricultural practices and possibly suggest specific contexts for their optimal and profitable use. With this aim, an untargeted UHPLC/QTOF-MS analysis was performed and multi variate statistics used to point out similarities/dissimilarities among metabolomic profiles of the PH-treated plants. The combination of a high-performance untargeted profiling, together with a rather comprehensive database (PlantCyc), resulted in a large dataset (overall, almost 1600 compounds annotated). A large chemical diversity was represented within the dataset, including compounds from a wide variety of biochemical classes and metabolic processes. The whole dataset, together with individual compounds' abundance and composite mass spectra, is provided in **supplementary material** (**Supplementary Table S8**).

As a first step of interpretation, a fold-change based hierarchical clustering was carried out (**Figure 5**). This unsupervised approach allowed producing two main clusters, one comprising all replications from the control and another including all PH-treated samples. In this latter, two further sub-clusters were evident, with products A and B being mixed together and with treatment I representing a separate sub-cluster. This unsupervised (i.e., naïve) classification of metabolomic profiles, based on individual fold-change values for each compound annotated, suggested that the PH treatments imposed a change in the plant metabolomic profile, and that treatments A and B induced a more comparable effect whereas treatment I had a more distinctive effect.

To better identify the specific responses induced in plants following the PH treatments, a supervised OPLS-DA multivariate modeling was carried out. This discriminant analysis approach allows discriminating among groups into score plot hyperspace, by separating predictive and orthogonal components (i.e., those components ascribable to technical and biological variation) of variance. Looking at the OPLS-DA score plot (**Figure 6**), the outcome of this supervised approach was in agreement with hierarchical clusters. Indeed, the control clustered in a separate region of score plot hyperspace, treatment with products A and B were separated but still closer to each other, and treatment I was confirmed to have the most distinctive profile. The model parameters of the OPLS-DA regression were excellent, being R^2Y and Q^2Y 0.94 and 0.63, respectively. The model was validated (CV-ANOVA $P = 0.009$) and overfitting could be excluded through permutation testing ($N = 100$). Furthermore, the Hotelling's T2 showed that suspect and strong outliers could be excluded. Given the more than acceptable model parameters, the variable selection method called VIP (Variable Importance in Projection) was used to explain the differences observed. The most discriminating compounds (i.e., the markers possessing a VIP score > 1.4) were exported and subjected to fold-change analysis against the control, to identify the trends of regulation altered by the treatments. The discriminant compounds, together with their VIP score and fold-change values, were grouped into chemical classes and are provided in **Table 1**. Interestingly, few biochemical classes included the most of discriminant metabolites. In more detail, low molecular weight phenolic compounds, poly-hydroxy fatty acids, membrane lipids (glyco- and phospholipids), hydroxy-carotenoids and phytohormones (polyamines) were the most represented.

From an overall perspective, the metabolomic changes imposed by the PH treatment can be correlated to relatively few processes, all of them converging toward the ROS-related plant signaling network. Among plant growth regulators, 1-aminocyclopropane-1-carboxylate (ACC), i.e., the direct precursor of ethylene, was found up accumulated in treated plants. Considering that ethylene is not detectable by our metabolomic approach, the increase of ACC suggests and increase in ethylene itself. The effects of ethylene on growth and development have been found to vary, depending on other phytohormone profile, CO_2 and light (Small and Degenhardt, 2018). Although usually related to senescence and fruit ripening, ethylene has been reported to play many other regulations in plants, including flowering and overall plant growth, cell division and root initiation, as well as modulation of secondary metabolites light (Schaller, 2012; Small and Degenhardt, 2018). In fact, at relatively low concentration,

TABLE 1 | Discriminant metabolites as identified by variables of importance in projection (VIP) analysis following OPLS-DA modeling on metabolomic profile of treated plants.

Class	Metabolite	VIP score		[A] vs. [control]		[B] vs. [control]		[I] vs. [control]	
		Score	*SE*	Log FC	Regulation	Log FC	Regulation	Log FC	Regulation
Phenolics	3.5-dihydroxyanisole	1.409	0.769						
	1.3.5-trimethoxybenzene	1.405	0.286	2.8	Up	5.5	Up	1.7	Up
	4-hydroxybenzaldehyde	1.418	r0.820						
	3.6.7.4′-tetramethylquercetagetin 3′-*O*-beta-D-glucoside	1.540	r0.883						
	3-phenylpropanoate	1.457	0.308	0.3	Up	5.5	Up	1.7	Up
	3-hydroxybenzaldehyde	1.418	0.820						
	Gallocatechin	1.372	0.548	0.2	Up	3.6	Up	4.5	Up
	Leucocyanidin	1.372	0.548	0.2	Up	3.6	Up	4.5	Up
	Epigallocatechin	1.372	0.548	0.5	Up	3.6	Up	4.3	Up
Glucosinolates	3-(7′-methylthio) heptylmalate	1.308	0.304	3.1	Up	1.2	Up	1.8	Up
	2-(7'-methylthio) heptylmalate	1.308	0.304	3.7	Up	1.2	Up	1.8	Up
Lipids	Oleate	1.367	0.497	−29.4	Down	0.2	Up	3.9	Up
	Colneleate	1.515	0.219	−3.9	Down	−4.0	Down	2.0	Up
	4-coumaryl alcohol	1.456	0.313	0.3	Up	5.5	Up	1.7	Up
	Germacra-1(10).4.11(13)-trien-12-ol	1.315	0.777	−8.7	Down	−5.1	Down	0.4	Up
	Dammarenediol II	1.428	0.899	6.2	Up	6.2	Up	6.0	Up
	1-16:0-2-18:3-diacylglycerol-trimethylhomoserine	1.365	0.919	1.0	Up	1.1	Up	0.7	Up
	1-16:0-2-18:2-digalactosyldiacylglycerol	1.394	1.122						
	Sitosterol	1.317	1.095	−0.5	Down	−1.1	Down	−0.5	Down
	(12Z.15Z)-9.10-epoxyoctadeca-12.15-dienoate	1.515	0.219	−3.9	Down	−4.0	Down	2.0	Up
	An epoxy-octadeca-dienoate	1.515	0.219	−3.9	Down	−4.0	Down	2.0	Up
	A dihydroxyoctadeca-dienoate	1.371	0.724	0.6	Up	−0.4	Down	1.5	Up
	9.10-12.13-diepoxyoctadecanoate	1.316	0.617	11.6	Up	1.2	Up	6.9	Up
	16-alpha-hydroxygypsogenate-28-beta-D-glucoside	1.319	0.684	0.6	Up	8.8	Up	1.5	Up
	2-hydroxyhexadecanoate	1.413	0.883						
	2-*trans*-6-*trans*-farnesyl monophosphate	1.397	0.571	4.5	Up	4.6	Up	0.7	Up
	Geranyl monophosphate	1.376	0.378	3.2	Up	3.1	Up	1.6	Up
	(9S)-HPODE/(13S)-HPODE	1.371	0.724	0.6	Up	−0.4	Down	1.5	Up
	3–beta;-D-galactosyl-sn-glycerol	1.369	1.015						
	A 2-acyl-sn-glycero-3-phosphoethanolamine (n-C14:1)	1.357	0.447	3.1	Up	9.4	Up	1.9	Up
	A 1-acyl-sn-glycero-3-phosphoglycerol (n-C14:1)	1.346	0.288	−0.4	Down	−0.4	Down	−1.8	Down
	3.4-dihydroxy-5-iall-trans/i-hexaprenylbenzoate	1.323	0.679	−3.1	Down	9.3	Up	6.1	Up
	4.4-dimethyl-5-alpha-cholest-7-en-3-beta-ol/4.4-dimethyl-5-alpha-cholesta-8-en-3-beta-ol	1.317	1.095	−0.5	Down	−1.1	Down	−0.5	Down
	1.2-dipalmitoyl-phosphatidylglycerol-phosphate	1.317	0.506	−9.4	Down	−7.5	Down	1.4	Up
	(6E)-8-oxogeranial	1.315	0.666	−1.8	Down	−1.8	Down	−1.5	Down

(Continued)

TABLE 1 | Continued

Class	Metabolite	VIP score		[A] vs. [control]		[B] vs. [control]		[I] vs. [control]	
		Score	*SE*	Log FC	Regulation	Log FC	Regulation	Log FC	Regulation
	(2E.6E)-farnesal	1.315	0.777	−8.7	Down	−5.1	Down	0.4	Up
	4-alpha-carboxy-4-beta-methyl-5-alpha-cholesta-8-en-3-beta-ol	1.312	0.670						
Carotenoids	4-methylocta-2.4.6-trienedial	1.456	0.313	0.6	Up	5.5	Up	1.7	Up
	5.6-epoxy-3-hydroxy-5.6-dihydro-12′-apo-beta;-caroten-12′-al	1.500	0.534	−0.28646278	Down	−1.0	Down	−1.3	Down
	18′-hydroxy-chi; chi;-caroten-18-oate	1.304	0.646	−9.2	Down	−1.5	Down	−1.5	Down
Hormones	1-aminocyclopropane-1-carboxylate	1.419	0.241	2.9	Up	2.9	Up	1.8	Up
	Salicylaldehyde	1.418	0.820						
Others	Triferuloyl spermidine	1.503	0.350	0.6	Up	2.8	Up	1.7	Up
	Sinapoyltyramine	1.516	0.366	0.6	Up	−0.4	Down	1.8	Up
	Thiamin	1.450	0.539	4.6	Up	4.5	Up	0.4	Up
	S-adenosyl 3-(methylthio) propylamine	1.431	0.440	−6.1	Down	9.2	Up	6.1	Up
	Methyl-1.4-benzoquinone	1.418	0.820						
	N-acetylneuraminate	1.384	0.363	−1.4	Down	−1.5	Down	−1.5	Down
	Menaquinol-8	1.367	0.491						
	Pyropheophorbide *a*	1.361	0.302	−1.1	Down	−1.0	Down	−1.2	Down

Discriminant metabolites (VIP > 1.4) are provided together with individual scores, their standard error (SE) and metabolite fold-change (FC) Log values, as compared to control; missing values denote fold-change values < 1.5.

ethylene has been reported to stimulate leaf growth (Dubois et al., 2018) and to promote yields (Habben et al., 2014). Scientific evidence suggests that such ethylene-dependent regulation of plant growth is related redox signaling pathways (Caviglia et al., 2018).

Notably, polyamine conjugates (namely sinapoyltyramine and triferuloyl spermidine, both up accumulated in treated plants) were additional plant growth regulators being induced by the treatments. Polyamines are preferentially detected in actively growing tissues and have been implicated in the control of cell division, embryogenesis, root formation, fruit development and ripening, and responses to biotic and abiotic stresses (Kumar et al., 1997; Gill and Tuteja, 2010; Agudelo-Romero et al., 2013; Rouphael et al., 2016). However, these metabolites are also reported to affect H_2O_2 signature under salt stress (Gémes et al., 2017) in a coordinate manner with ethylene (Hou et al., 2013).

Even though a clear trend could not be observed, a wide alteration of the profile of membrane lipids was observed in our experiments. Such modulation might be the consequence of the altered signature in signaling compounds and antioxidants. Nevertheless, it is important to consider that membrane lipids play an important role in secondary signaling cascades which control plant adaptation processes (Hou et al., 2016). The concurrent changes in antioxidant compounds such as phenolics and carotenoids, suggests a fine tuning of the ROS-mediated signaling in tomato following application of the biostimulants. Indeed, such secondary metabolites are well known to play a pivotal role in plant defense against oxidative stress (Shalaby and Horwitz, 2015; Lucini et al., 2018; Rouphael et al., 2018). Such interplay between polyamines, ROS and ethylene was reported to alleviate the decrease of plant biomass under stress conditions (Gémes et al., 2017) and might have had a role also in our experiments. Consistently with our findings, it is interesting to note that such support to biomass accumulation was not related to photosynthetic efficiency (Gémes et al., 2017) and was linked to the accumulation of phenolic compounds (Gémes et al., 2016).

Unlike mammals, plants produce the most of ROS in chloroplast, under a controlled multi-level antioxidant-scavenging system that includes thiols, antioxidant enzymes and low molecular weight antioxidants to manage their accumulation and transmit oxidative signals. While the concept that deleterious and irreversible oxidation driven by ROS is embed in literature, the scientific consensus is now shifting toward the recognition of the positive roles of ROS as essential components of chloroplast-nucleus retrograde signaling pathways (Foyer et al., 2017; Foyer, 2018). Since H_2O_2 is relatively more stable than superoxide and singlet oxygen (both having short half-lives), this compound is believed the likely candidate to diffuse over any distances within the cell. Such redox signals interact with the phytohormone signaling network to regulate plant growth and defense processes (Foyer, 2018). This production of ROS is essential not only to convey communication regarding the redox pressure within the electron transport chain, but also to trigger short-term genetic responses (Foyer, 2018).

Within this redox-mediated multi-layer signaling process, carotenoids (together with glutathione and tocopherols) are among the most effective $^1O_2$ scavengers; in fact, alteration in carotenoid oxidized forms has been recorded in our experiments. Coherently, the down accumulation of pheophorbide *a*, i.e., a precursor of chlorophylls, is a known process plant uses to control ROS production in the photosynthetic organs, given the fact that the photoreduction of oxygen to the superoxide radical is related to a reduced electron transport in PSI (Ghandchi et al., 2016) Although the link between the application of our PHs and biostimulants activity tomato could not be fully elucidated, a general consensus toward ROS-phytohormone interplay can be postulated, based on the differential metabolites identified by metabolomics. Such multi-level signaling might have played a role in determining the differences in growth observed through phenotyping.

CONCLUSION

The use of PBs in particular vegetal-derived protein hydrolysates (PHs) in agriculture has greatly increased in the last decade mostly due to their *multifaceted properties*. Highly efficient and effective PH-based biostimulant products can be obtained using the 'omics' sciences. A novel approach based on the use of high-throughput phenotyping technologies and metabolomics was successfully tested on tomato crop for identifying new PHs with biostimulant activity and for studying the PH effects on plants at metabolic level. Dynamic monitoring of plant performance by high-throughput phenotyping system has proven to be a powerful tool for substance screening on the basis of morpho-physiological traits quantification. The effects of PHs on tomato phenotype were more evident on digital biomass. Metabolomics followed by multivariate analysis allowed elucidating the metabolic signatures imposed by the specific PH treatments. PH treatments affected the metabolic profile of tomato leaves via the modulation of a complex signaling process that involved the direct precursor of ethylene and polyamine conjugates. The coordinated action of plant growth regulators together with antioxidant compounds such as carotenoids and phenolics, might have affected the ROS-mediated signaling pathways. Although further assays under defined conditions would strengthen our findings, the discriminant compounds pointed out by this approach suggest that treated plants might experience a metabolic reprogramming following the application of the tested biostimulants.

AUTHOR CONTRIBUTIONS

KP wrote the first draft of the manuscript and followed the phenotyping measurements and data interpretation. MS performed the big data analysis. LL and PB performed the metabolomics analysis, data interpretation, and wrote the metabolomic part. YR, MC, HR, RC, and MT were involved in data analysis, data interpretation, and writing. GC and KLP coordinated the whole project, provided the intellectual input, set up the experiment, and corrected the manuscript.

FUNDING

This work was supported by European Union's Horizon 2020 research and innovation program under the Marie Skłodowska-Curie grant agreement no. 675006. This work was also supported by Italpollina Company (Rivoli Veronese, Italy).

ACKNOWLEDGMENTS

We thank Jaromír Pytela for helping with the preparation of plant material for the phenotyping experiments at Photon Systems Instruments (PSI) Research Center (Drásov, Czechia) and Zuzana Benedikty for useful discussions during optimization phase of the phenotyping experiments at PSI (Drásov, Czechia), Petr Polach for helping with raw images re-processing at PSI (Drásov, Czechia).

SUPPLEMENTARY MATERIAL

The Supplementary Material for this article can be found online at: https://www.frontiersin.org/articles/10.3389/fpls.2019.00047/full#supplementary-material

FIGURE S1 | Schematic of the kinetic ChlF protocol in the PlantScreen™ Modular System. ChlF kinetics were captured with a PAM-based chlorophyll fluorometer. Images of the individual transient states were recorded. Corresponding frames were averaged for the measured parameters (F_o, F_m, F_m', F_t, and F_p) or calculated from the captured frames to compute the relative parameters such as F_v/F_m, ΦPSII, F_v'/F_m', NPQ, and others. Automated ChlF image processing consisted of image segmentation by mask application, background subtraction and feature extraction. The signals from all pixels of each segment were averaged at each given time point. MF refers to the measuring flash, and yellow arrows indicate the saturation pulses that transiently saturated the electron transport chain. Lss1, Lss2, and Lss3 represent actinic photon irradiance measurements taken at 170, 620, and 1070 μmol photons m^{-2} s^{-1}, respectively.

FIGURE S2 | Schematic of top and side view RGB image processing. Original RGB images were automatically processed using the PlantScreen™ Analyzer software to correct for barrel distortion caused by the fisheye lens, subtract the background and crop to isolate the plants within the imaged area, producing a binary (black and white) image. The binary images represent the plant surface (white) and background (black). Non-plant pixels, such as pots, were automatically removed to extract only plant pixels. Morphological analysis was conducted after separating the background from the plant shoot tissue. To evaluate color of plant shoot, RGB images were color-segmented to extract the green hues.

FIGURE S3 | Variation in shoot colors of tomato plants. Dynamic relative changes in greenness hue abundance over the phenotyping period in control tomato plants and plants treated with protein hydrolysates (A–G, I). The six most representative color hues are shown in RGB color scale as percentage of the shoot area (pixel counts) of six biological replicates per treatment.

TABLE S1 | Projected shoot area (PSA) of the tomato plants extracted from top view RGB images starting 3 days after the first PH application (day after transplanting, DAT = 8). Values are expressed as number of green pixels and represent the average of six biological replicates per treatment ± standard deviation. Within the same row and for the specified day different letters indicate significant difference according to one-way ANOVA *post hoc* Tukey's test ($p < 0.05$).

TABLE S2 | Projected shoot area (PSA) of the tomato plants extracted from multiple side view RGB images starting 3 days after the first PH application (day after transplanting, DAT = 8). Values are expressed as number of green pixels and

represent the average of six biological replicates per treatment ± standard deviation. Within the same row and for the specified day different letters indicate significant difference according to one-way ANOVA *post hoc* Tukey's test ($p < 0.05$).

TABLE S3 | Width of the tomato plants extracted from multiple side view RGB images starting 3 days after the first PH application (day after transplanting, DAT = 8). Values are expressed as number of green pixels and represent the average of six biological replicates per treatment ± standard deviation. Within the same row and for the specified day different letters indicate significant difference according to one-way ANOVA *post hoc* Tukey's test ($p < 0.05$).

TABLE S4 | Height of the tomato plants extracted from multiple side view RGB images starting 3 days after the first PH application (day after transplanting, DAT = 8). Values are expressed as number of green pixels and represent the average of six biological replicates per treatment ± standard deviation. Within the same row and for the specified day different letters indicate significant difference according to one-way ANOVA *post hoc* Tukey's test ($p < 0.05$).

TABLE S5 | Digital biomass of tomato plants treated with different protein hydrolysates starting 3 days after the first PH application (day after transplanting, DAT = 8). Values are expressed as number of green pixels and represent the average of six biological replicates per treatment ± standard deviation. Within the same row and for the specified day different letters indicate significant difference in digital biomass, according to one-way ANOVA *post hoc* Tukey's test ($p < 0.05$).

TABLE S6 | Variation in shoot colours of tomato plants treated with different protein hydrolysates at 15 days after transplanting. The values for 6 most representative colour hues are shown as percentage of the shoot area (pixel counts). Values represent the average of six biological replicates per treatment ± standard deviation. Within the same row and for the specified day different letters indicate significant difference according to one-way ANOVA *post hoc* Tukey's test ($p < 0.05$).

TABLE S7 | Photosynthetic performance of tomato plants at 15 days after transplanting. Photosynthetic parameters deduced from kinetic chlorophyll fluorescence imaging on whole plant level in all protein hydrolysate treatments. Minimal fluorescence in dark-adapted state (F_o), maximum fluorescence in dark-adapted state (F_m), maximum quantum yield of PSII photochemistry for the dark-adapted (F_v/F_m), the photochemical quenching coefficient that estimates the fraction of open PSII reaction centers (qP), steady-state non-photochemical quenching (NPQ) and electron transport rate (ETR) were measured using the light curve protocol for tomato plants prior and upon PHs application. Values represent the average of six biological replicates per treatment ± standard deviation. Within the same row and for the specified day different letters indicate significant difference according to one-way ANOVA *post hoc* Tukey's test ($p < 0.05$). Lss1, Lss2, and Lss3 represent actinic photon irradiance measurements taken at 170, 620, and 1070 μmol photons $m^{-2}s^{-1}$ PAR values, respectively.

TABLE S8 | List of compounds identified by UHPLC-ESI QTOF-MS metabolomics in tomato plants (Level 2 of COSMOS standards - http://cosmos-fp7.eu/msi.html).

REFERENCES

Agudelo-Romero, P., Erban, A., Sousa, L., Pais, M. S., Kopka, J., and Fortes, A. M. (2013). Search for transcriptional and metabolic markers of grape pre-ripening and ripening and insights into specific aroma development in three Portuguese cultivars. *PLoS One* 8:e60422. doi: 10.1371/journal.pone.0060422

Awlia, M., Nigro, A., Fajkus, J., Schmoeckel, S. M., Negrão, S., Santelia, D., et al. (2016). High-throughput non-destructive phenotyping of traits that contribute to salinity tolerance in *Arabidopsis thaliana*. *Front. Plant Sci.* 7:1414. doi: 10.3389/fpls.2016.01414

Baker, N. R. (2008). Chlorophyll fluorescence: a probe of photosynthesis *in vivo*. *Annu. Rev. Plant Biol.* 59, 89–113. doi: 10.1146/annurev.arplant.59.032607.092759

Calvo, P., Nelson, L., and Kloepper, J. W. (2014). Agricultural uses of plant biostimulants. *Plant Soil* 383, 3–41. doi: 10.1007/s11104-014-2131-8

Caviglia, M., Mazorra Morales, L. M., Concellón, A., Gergoff Grozeff, G. E., Wilson, M., Foyer, C. H., et al. (2018). Ethylene signaling triggered by low concentrations of ascorbic acid regulates biomass accumulation in *Arabidopsis thaliana*. *Free Rad. Biol. Med.* 122, 130–136. doi: 10.1016/j.freeradbiomed.2018.01.032

Colla, G., Cardarelli, M., Bonini, P., and Rouphael, Y. (2017a). Foliar applications of protein hydrolysate, plant and seaweed extracts increase yield but differentially modulate fruit quality of greenhouse tomato. *HortScience* 52, 1214–1220. doi: 10.21273/hortsci12200-17

Colla, G., Hoagland, L., Ruzzi, M., Cardarelli, M., Bonini, P., Canaguier, R., et al. (2017b). Biostimulant action of protein hydrolysates: unravelling their effects on plant physiology and microbiome. *Front. Plant Sci.* 8:2202. doi: 10.3389/fpls.2017.02202

Colla, G., Nardi, S., Cardarelli, M., Ertani, A., Lucini, L., Canaguier, R., et al. (2015). Protein hydrolysates as biostimulants in horticulture. *Sci. Hortic.* 196, 28–38. doi: 10.1016/j.scienta.2015.08.037

Colla, G., Rouphael, Y., Canaguier, R., Svecova, E., and Cardarelli, M. (2014). Biostimulant action of a plant-derived protein hydrolysate produced through enzymatic hydrolysis. *Front. Plant Sci.* 5:448. doi: 10.3389/fpls.2014.00448

Colla, G., Rouphael, Y., Lucini, L., Canaguier, R., Stefanoni, W., Fiorillo, A., et al. (2016). Protein hydrolysate-based biostimulants: origin, biological activity and application methods. *Acta Hortic.* 1148, 27–34. doi: 10.17660/actahortic.2016.1148.3

Digruber, T., Sass, L., Cseri, A., Paul, K., Nagy, A. V., Remenyik, J., et al. (2018). Stimulation of energy willow biomass with triacontanol and seaweed extract. *Ind. Crops Products* 120, 104–112. doi: 10.1016/j.indcrop.2018.04.047

Dubois, M., Van den Broeck, L., and Inzé, D. (2018). The pivotal role of ethylene in plant Growth. *Trends Plant Sci.* 23, 311–323. doi: 10.1016/j.tplants.2018.01.003

Dudits, D., Török, K., Cseri, A., Paul, K., Nagy, A. V., Nagy, B., et al. (2016). Response of organ structure and physiology to autotetraploidization in early development of energy willow (*Salix viminalis*). *Plant Physiol.* 170, 1504–1523. doi: 10.1104/pp.15.01679

Ertani, A., Cavani, L., Pizzeghello, D., Brandellero, E., Altissimo, A., Ciavatta, C., et al. (2009). Biostimulant activities of two protein hydrolysates on the growth and nitrogen metabolism in maize seedlings. *J. Plant. Nutr. Soil Sci.* 172, 237–244. doi: 10.1002/jpln.200800174

Ertani, A., Nardi, S., and Altissimo, A. (2012). Review: long-term research activity on the biostimulant properties of natural origin compounds. *Acta Hortic.* 1009, 181–188. doi: 10.17660/actahortic.2013.1009.22

Ertani, A., Pizzeghello, D., Francioso, O., Tinti, A., and Nardi, S. (2016). Biological activity of vegetal extracts containing phenols on plant metabolism. *Molecules* 21:205. doi: 10.3390/molecules21020205

Ertani, A., Schiavon, M., Muscolo, A., and Nardi, S. (2013). Alfalfa plant-derived biostimulant stimulate short-term growth of salt stressed *Zea mays* L. plants. *Plant Soil* 364, 145–158. doi: 10.1007/s11104-012-1335-z

Ertani, A., Schiavon, M., and Nardi, S. (2017). Transcriptome-wide identification of differentially expressed genes in Solanum Lycopersicon L. in response to an alfalfa-protein hydrolysate using microarrays. *Front. Plant Sci.* 8:1159. doi: 10.3389/fpls.2017.01159

European Commission (2016). *Proposal for a Regulation Laying Down rules on the Making Available on the Market of CE Marked Fertilizing Products and Amending Regulations (EC)1069/2009 and (EC)1107/2009.COM (2016)*. Brussels: European Commission, 157.

Fahlgren, N., Gehan, M. A., and Baxter, I. (2015). Lights, camera, action: high-throughput plant phenotyping is ready for a close-up. *Curr. Opin. Plant Biol.* 24, 93–99. doi: 10.1016/j.pbi.2015.02.006

Fehér-Juhász, E., Majer, P., Sass, L., Lantos, C., Csiszár, J., Turóczy, Z., et al. (2014). Phenotyping shows improved physiological traits and seed yield of transgenic wheat plants expressing the alfalfa aldose reductase under permanent drought stress. *Acta Physiol. Plant.* 92, 663–673. doi: 10.1007/s11738-013-1445-0

Fernández, V., and Eichert, T. (2009). Uptake of hydrophilic solutes through plant leaves: current state of knowledge and perspectives of foliar

fertilization. *Crit. Rev. Plant Sci.* 28, 36–68. doi: 10.1080/07352680902743069

Feussner, I., and Polle, A. (2015). What the transcriptome does not tell – proteomics and metabolomics are closer to the plants' patho-phenotype. *Curr. Opin. Plant Biol.* 26, 26–31. doi: 10.1016/j.pbi.2015.05.023

Foyer, C. H. (2018). Reactive oxygen species, oxidative signaling and the regulation of photosynthesis. *Environ. Exp. Bot.* 154, 134–142. doi: 10.1016/j.envexpbot.2018.05.003

Foyer, C. H., Ruban, A. V., and Noctor, G. (2017). Viewing oxidative stress through the lens of oxidative signalling rather than damage. *Biochem. J.* 474, 877–883. doi: 10.1042/BCJ20160814

Gémes, K., Kim, Y. J., Park, K. Y., Moschou, P. N., Andronis, E., Valassaki, C., et al. (2016). An NADPH-oxidase/polyamine oxidase feedback loop controls oxidative burst under salinity. *Plant Physiol.* 172, 1418–1431. doi: 10.1104/pp.16.01118

Gémes, K., Mellidou, I, Karamanoli, K., Beris, D., Park, K. Y., Matsi, T., et al. (2017). Deregulation of apoplastic polyamine oxidase affects development and salt response of tobacco plants. *J. Plant Physiol.* 211, 1–12. doi: 10.1016/j.jplph.2016.12.012

Genty, B., Briantais, J. M., and Baker, N. R. (1989). The relationship between the quantum yield of photosynthetic electron transport and quenching of chlorophyll fluorescence. *Biochim. Biophys. Acta* 990, 87–92. doi: 10.1016/s0304-4165(89)80016-9

Ghandchi, F. P., Caetano-Anolles, G., Clough, S. J., and Ort, D. R. (2016). Investigating the control of chlorophyll degradation by genomic correlation mining. *PLoS One* 11:e0162327. doi: 10.1371/journal.pone.0162327

Gill, S. S., and Tuteja, N. (2010). Polyamines and abiotic stress tolerance in plants. *Plant Signal. Behav.* 7, 26–33. doi: 10.4161/psb.5.1.10291

Großkinsky, D. K., Svensgaard, J., Christensen, S., and Roitsch, T. (2015). Plant phenomics and the need for physiological phenotyping across scales to narrow the genotype-to-phenotype knowledge gap. *J. Exp. Bot.* 66, 5429–5440. doi: 10.1093/jxb/erv345

Habben, J. E., Bao, X., Bate, N. J., DeBruin, J. L., Dolan, D., Hasegawa, D., et al. (2014). Transgenic alteration of ethylene biosynthesis increases grain yield in maize under field drought-stress conditions. *Plant Biotechnol. J.* 12, 685–693. doi: 10.1111/pbi.12172

Haplern, M., Bar-Tal, A., Ofek, M., Minz, D., Muller, T., and Yermiyahu, U. (2015). The use of biostimulants for enhancing nutrient uptake. *Adv. Agron.* 130, 141–174. doi: 10.1016/bs.agron.2014.10.001

Henley, W. J. (1993). Measurement and interpretation of photosynthetic light-response curves in algae in the context of photoinhibition and diel changes. *J. Phycol.* 29, 729–739. doi: 10.1111/j.0022-3646.1993.00729.x

Hou, Q., Ufer, G., and Bartels, D. (2016). Lipid signalling in plant responses to abiotic stress. *Plant Cell Environ.* 39, 1029–1048. doi: 10.1111/pce.12666

Hou, Z.-H., Liu, G., Hua, Hou, L., Xia, Wang, L., et al. (2013). Regulatory Function of polyamine oxidase-generated hydrogen peroxide in ethylene-induced stomatal closure in Arabidopsis thaliana. *J. Integr. Agric.* 12, 251–262. doi: 10.1016/S2095-3119(13)60224-5

Houle, D., Govindaraju, D. R., and Omholt, S. (2010). Phenomics: the next challenge. *Nat. Rev. Genet.* 11, 855–866. doi: 10.1038/nrg2897

Klukas, C., Chen, D., and Pape, J. M. (2014). Integrated analysis platform: an open-source information system for high-throughput plant phenotyping. *Plant Physiol.* 165, 506–518. doi: 10.1104/pp.113.233932

Kumar, A., Altabella, T., Taylor, M., and Tiburcio, A. F. (1997). Recent advances in polyamine research. *Trends Plant Sci.* 2, 124–130. doi: 10.1016/S1360-1385(97)01013-3

Kumar, P., Lucini, L., Rouphael, Y., Cardarelli, M., Kalunke, R. M., and Colla, G. (2015). Insight into the role of grafting and arbuscular mycorrhiza on cadmium stress tolerance in tomato. *Front. Plant Sci.* 6:477. doi: 10.3389/fpls.2015.00477

Lamichhane, S., Sen, P., Dickens, A. M., Hyötyläinen, T., and Orešiè, M. (2018). An overview of metabolomics data analysis: current tools and future perspectives. *Comp. Anal. Chem.* 82, 387–413. doi: 10.1016/bs.coac.2018.07.001

Lucini, L., Rouphael, Y., Cardarelli, M., Bonini, P., Baffi, C., and Colla, G. (2018). A vegetal biopolymer-based biostimulant promoted root growth in melon while triggering brassinosteroids and stress-related compounds. *Front. Plant Sci.* 9:472. doi: 10.3389/fpls.2018.00472

Lucini, L., Rouphael, Y., Cardarelli, M., Canaguier, R., Kumar, P., and Colla, G. (2015). The effect of a plant-derived protein hydrolysate on metabolic profiling and crop performance of lettuce grown under saline conditions. *Sci. Hortic.* 182, 124–133. doi: 10.1016/j.scienta.2014.11.022

Meier, R., Ruttkies, C., Treutler, H., and Neumann, S. (2017). Bioinformatics can boost metabolomics research. *J. Biotechnol.* 261, 137–141. doi: 10.1016/j.jbiotec.2017.05.018

Murchie, E. H., and Lawson, T. (2013). Chlorophyll fluorescence analysis: a guide to good practice and understanding some new applications. *J. Exp. Bot.* 64, 3983–3998. doi: 10.1093/jxb/ert208

Nardi, S., Pizzeghello, D., Schiavon, M., and Ertani, A. (2016). Plant biostimulants: physiological responses induced by protein hydrolyzed-based products and humic substances in plant metabolism. *Sci. Agric.* 73, 18–23. doi: 10.1590/0103-9016-2015-0006

Niculescu, M., Bajenaru, S., Gaidau, C., Simion, D., and Felipescu, L. (2009). Extraction of the protein components as amino-acids hydrolysates from chrome leather wastes through hydrolytic processes. *Rev. Chim.* 60, 1070–1078.

Paul, K., Deaik, Z., Csôsz, M., Purnhauser, L., and Vass, I. (2011). Characterization and early detection of tan spot disease in wheat in vivo with chlorophyll fluorescence imaging. *Acta Biol. Szeged* 55, 87–90. doi: 10.13140/2.1.3021.6320

Povero, G., Mejia, J. F., Di Tommaso, D., Piaggesi, A., and Warrior, P. (2016). A systematic approach to discover and characterize natural plant biostimulants. *Front. Plant Sci.* 7:435. doi: 10.3389/fpls.2016.00435

Rahaman, M. M., Ahsan, M. A., Gillani, Z., and Chen, M. (2017). Digital biomass accumulation using high-throughput plant phenotype data analysis. *J. Integr. Bioinform.* 14:20170028. doi: 10.1515/jib-2017-0028

Rahaman, M. M., Chen, D., Gillani, Z., Klukas, C., and Chen, M. (2015). Advanced phenotyping and phenotype data analysis for the study of plant growth and development. *Front. Plant Sci.* 6:619. doi: 10.3389/fpls.2015.00619

Rascher, U., Liebig, M., and Luttge, U. (2000). Evaluation of instant light-response curves of chlorophyll fluorescence parameters obtained with a portable chlorophyll fluorometer on site in the field. *Plant Cell Environ.* 23, 1397–1405. doi: 10.1046/j.1365-3040.2000.00650.x

Rouphael, Y., Cardarelli, M., Bonini, P., and Colla, G. (2017a). Synergistic action of a microbial based biostimulant and a plant-derived protein hydrolysate enhances lettuce tolerance to alkalinity and salinity. *Front. Plant Sci.* 8:131. doi: 10.3389/fpls.2017.00131

Rouphael, Y., Colla, G., Giordano, M., El-Nakhel, C., Kyriacou, M. C., and De Pascale, S. (2017b). Foliar applications of a legume-derived protein hydrolysate elicit dose dependent increases of growth, leaf mineral composition, yield and fruit quality in two greenhouse tomato cultivars. *Sci. Hort.* 226, 353–360. doi: 10.1016/j.scienta.2017.09.007

Rouphael, Y., Colla, G., Graziani, G., Ritieni, A., Cardarelli, M., and De Pascale, S. (2017c). Phenolic composition, antioxidant activity and mineral profile in two seed-propagated artichoke cultivars as affected by microbial inoculants and planting time. *Food Chem.* 234, 10–19. doi: 10.1016/j.foodchem.2017.04.175

Rouphael, Y., Colla, G., Bernardo, L., Kane, D., Trevisan, M., and Lucini, L. (2016). Zinc excess triggered polyamines accumulation in lettuce root metabolome, as compared to osmotic stress under high salinity. *Front. Plant Sci.* 7:842. doi: 10.3389/fpls.2016.00842

Rouphael, Y., Kyriacou, M., Vitaglione, P., Giordano, M., Pannico, A., Colantuono, A., et al. (2017d). Genotypic variation in nutritional and antioxidant profile among iceberg lettuce cultivars. *Acta Sci. Pol. Hortorum Cultus* 16, 37–45. doi: 10.24326/asphc.2017.3.4

Rouphael, Y., Spìchal, L., Panzarova, K., Casa, R., and Colla, G. (2018). High-throughput plant phenotyping for developing novel biostimulants: from lab to field or from field to lab? *Front. Plant Sci.* 9:1197. doi: 10.3389/fpls.2018.01197

Salehi, H., Chehregani, A., Lucini, L., Majd, A., and Gholami, M. (2018). Morphological, proteomic and metabolomic insight into the effect of cerium dioxide nanoparticles to *Phaseolus vulgaris* L. under soil or foliar application. *Sci. Tot. Envion.* 616, 1540–1551. doi: 10.1016/j.scitotenv.2017.10.159

Schaller, G. E. (2012). Ethylene and the regulation of plant development. *BMC Biol.* 10:9. doi: 10.1186/1741-7007-10-9

Shalaby, S., and Horwitz, B. A. (2015). Plant phenolic compounds and oxidative stress: integrated signals in fungal–plant interactions. *Curr. Genet.* 61, 347–357. doi: 10.1007/s00294-014-0458-6

Small, C. C., and Degenhardt, D. (2018). Plant growth regulators for enhancing revegetation success in reclamation: a review. *Ecol. Engin.* 118, 43–51. doi: 10.1016/j.ecoleng.2018.04.010

Subbarao, S. B., Aftab Hussain, I. S., and Ganesh, P. T. (2015). Biostimulant activity of protein hydrolysate: influence on plant growth and yield. *J. Plant Sci. Res.* 2:125.

Tschiersch, H., Junker, A., Meyer, R. C., and Altmann, T. (2017). Establishment of integrated protocols for automated high throughput kinetic chlorophyll fluorescence analyses. *Plant Methods* 13:54. doi: 10.1186/s13007-017-0204-4

Tsugawa, H. (2018). Advances in computational metabolomics and databases deepen the understanding of metabolisms. *Curr. Opin. Biotechnol.* 54, 10–17. doi: 10.1016/j.copbio.2018.01.008

Ugena, L., Hýlová, A., Podlešáková, K., Humplík, J. F., Doležal, K., De Diego, N., et al. (2018). Characterization of biostimulant mode of action using novel multitrait high-throughput screening of *Arabidopsis* germination and rosette growth. *Front. Plant Sci.* 9:1327. doi: 10.3389/fpls.2018.01327

Yakhin, O. I., Lubyanov, A. A., Yakhin, I. A., and Brown, P. H. (2017). Biostimulants in plant science: a global perspective. *Front. Plant Sci.* 7:2049. doi: 10.3389/fpls.2016.02049

Conflict of Interest Statement: MT is the owner and CEO of PSI (Photon Systems Instruments), Drásov, Czechia, and KLP is an employee of his company. KP is an ex. employee of PSI and MS is a Ph.D. student, both conducted the experiments at PSI. RC is the Director of Nixe Company. HR is an employee of Italpollina Company (Anderson, United States) who provided the eight types of plant-derived protein hydrolysates (A–G, I). GC is a member of the spin-off Company Arcadia Srl approved by University of Tuscia, Italy.

The remaining authors declare that the research was conducted in the absence of any commercial or financial relationships that could be construed as a potential conflict of interest.

frontiers in Plant Science

ORIGINAL RESEARCH
published: 27 March 2019
doi: 10.3389/fpls.2019.00371

Flavonoid, Nitrate and Glucosinolate Concentrations in *Brassica* Species Are Differentially Affected by Photosynthetically Active Radiation, Phosphate and Phosphite

Libia Iris Trejo-Téllez[1†], Elías Estrada-Ortiz[1†], Fernando Carlos Gómez-Merino[1], Christine Becker[2], Angelika Krumbein[3] and Dietmar Schwarz[3]*

[1] Colegio de Postgraduados, Montecillo, Mexico, [2] Department of Crop Protection, Hochschule Geisenheim University, Geisenheim, Germany, [3] Leibniz Institute of Vegetable and Ornamental Crops, Großbeeren, Germany

We evaluated the effects of phosphate (Pi-deficiency: 0.1 mM; Pi-sufficiency: 0.5 mM), phosphite (low-Phi: 0.1 mM; medium-Phi: 0.5 mM; and high-Phi: 2.5 mM), and two mean daily photosynthetically active radiations (lower PAR: 22.2 mol · m^{-2} · d^{-1}; higher PAR: 29.7 mol · m^{-2} · d^{-1}), as well as their interactions, on flavonoid, nitrate and glucosinolate (GL) concentrations and growth characteristics in hydroponically grown *Brassica campestris* cv. Mibuna Early and *Brassica juncea* cv. Red Giant. As expected, higher PAR increased dry matter and contrariwise decreased number of leaves but only in *B. campestris*. Total flavonoid and individual flavonoid compounds increased with the higher PAR value in *B. campestris*. Pi-sufficiency resulted in a lower quercetin concentration in both species, the isorhamnetin and total flavonoid concentrations in *B. campestris*, and the cyanidin concentration in *B. juncea*, in comparison to Pi-deficiency. Similarly, Pi-sufficient plants exhibited lower GL concentration, especially alkyl-GLs in *B. campestris* and alkenyl-GLs and an aryl-GL in *B. juncea*. Pi did not affect the nitrate concentration in either species, and nor did Phi influence the flavonoid concentrations in either species. In *B. campestris*, medium Phi (0.5 mM) increased the 1-methoxyindol-3-ylmethyl GL concentration by 28.3%, as compared to that observed at low Phi. In *B. juncea,* high Phi level increased the but-3-enyl-GL concentration by 18.9%, in comparison to values recorded at medium Phi. *B. campestris* plants exposed to higher PAR increased total flavonoids concentration. In both *Brassica* species, higher PAR stimulated the alkyl-, alkenyl-, and indole-GLs. The interaction of lower PAR and increasing Phi significantly decreased flavonoid concentration in *B. juncea*, whereas increasing Phi at higher PAR increased such concentration in this species. The same combination reduced the concentration of 2-phenylethyl- and indol-3-ylmethyl-GL in *B. juncea*. The highest indol-3-ylmethyl-GL concentration was observed when Pi was deficient combined with medium Phi in *B. juncea*. Thus, PAR, Pi and Phi may modulate flavonoid, GL and nitrate concentrations in *Brassica* species, which may be a useful tool to improve the nutraceutical quality of these leafy vegetables if properly managed.

Keywords: Brassicaceae, biostimulation, secondary metabolites, nutraceutics, PAR, phosphorus (deficiency, uptake)

OPEN ACCESS

Edited by:
Youssef Rouphael, University of Naples Federico II, Italy

Reviewed by:
Jim Tokuhisa, Virginia Tech, United States
Luis Alonso Valdez-Aguilar, Universidad Autónoma Agraria Antonio Narro, Mexico

***Correspondence:**
Dietmar Schwarz
schwarz@igzev.de

† These authors have contributed equally to this work

Specialty section:
This article was submitted to Plant Nutrition, a section of the journal Frontiers in Plant Science

Received: *20 September 2018*
Accepted: *11 March 2019*
Published: *27 March 2019*

Citation:
Trejo-Téllez LI, Estrada-Ortiz E, Gómez-Merino FC, Becker C, Krumbein A and Schwarz D (2019) Flavonoid, Nitrate and Glucosinolate Concentrations in Brassica Species Are Differentially Affected by Photosynthetically Active Radiation, Phosphate and Phosphite. Front. Plant Sci. 10:371. doi: 10.3389/fpls.2019.00371

INTRODUCTION

Solar radiation is a key environmental signal that regulates most forms of life on Earth. Among the relevant bands along the solar radiation spectrum, the visible one (photosynthetically active radiation, PAR) makes up 43.9% of total solar radiation, energy, and its interaction with other environmental cues, including nutrient supply, may affect primary and secondary metabolism in plants (Fallovo et al., 2009, 2011). Particularly, it is well documented that sulfur and phosphorus (P) deficiencies stimulate the synthesis of secondary metabolites such as flavonoids and glucosinolates (GLs) (Lunde et al., 2008; Pant et al., 2015).

Phosphorus is one of the primary nutrients required by plants, making up about 0.2% of their dry biomass (Herrera-Estrella and López-Arredondo, 2016). This macronutrient is an essential component of biomolecules such as sugar phosphates, phospholipid, phosphoproteins, enzymes, and energy-rich compounds such as ATP and NADP, as well as the nucleic acids DNA and RNA, thus playing a pivotal role in genetic heredity, membrane structure, signal perception and transduction, and metabolism (López-Arredondo et al., 2014; Trejo-Téllez and Gómez-Merino, 2018). The demand for P is supplied by the phosphate form of P (Pi; $H_2PO_4^-$ or HPO_4^{2-}), which is the sole P-containing nutrient important for optimal plant performance (López-Arredondo et al., 2014). Alternatively, an analog of Pi, phosphite (Phi; $H_2PO_3^-$ or HPO_3^{2-}) is gaining momentum as a novel biostimulant in agriculture, improving yield and quality of crops, as well as inducing diverse mechanisms of tolerance against stress factors (Gómez-Merino and Trejo-Téllez, 2015, 2016). In the same way as Pi, the Phi molecule displays a tetrahedral structure with formal charge of −3. Nevertheless, instead of having four oxygen (O) atoms distributed evenly at the points of the structure bonded to the P atom located in the center found in Pi, the Phi ion has only three O atoms with a hydrogen (H) atom bonded strongly to the P atom. Hence, Phi is defined an isostere of the Pi anion, in which a H atom replaces one of the O atoms bound to the P atom (Varadarajan et al., 2002; Gómez-Merino and Trejo-Téllez, 2015). The lack of an O atom in Phi and the corresponding charge distribution of the structure significantly changes the nature and reactivity of the resultant molecule. For instance, such changes give Phi increased mobility in plant tissues through both the xylem and the phloem. Such high mobility allows Phi to be absorbed and translocated within the plant more readily than Pi (Ratjen and Gerendas, 2009; Jost et al., 2015). Moreover, Phi-containing salts exhibit a higher solubility than that of their analogous Pi-containing ones, which render Phi uptake by leaf and root a more efficient process (Trejo-Téllez and Gómez-Merino, 2018).

Phi may modify nitrogen (N) metabolism in plants and induce nitrate accumulation in edible tissues (Thao et al., 2009). Nitrate itself is relatively non-toxic but its metabolites may produce adverse physiological effects (Santamaria, 2005). Indeed, Phi is not a proper P-source for plant nutrition, and when applied at high concentrations, it may cause deleterious effects on different physiological processes including photosynthesis (Thao et al., 2008; Zambrosi et al., 2011). Another factor affecting photosynthesis is radiation. For instance, chlorophyll fluorescence decreases with increasing sunlight (Gómez et al., 1998). Furthermore, daily changes in nitrate reductase and carbonic anhydrase activities are antagonistic during the onset of natural radiation (Gómez et al., 1998). Importantly, if Phi is not properly used, it may negatively affect nutrient use efficiency and the whole metabolism (Zambrosi et al., 2011; Ramezani et al., 2017). Conversely, a proper application of Phi may induce positive responses, including an enhanced biosynthesis of secondary metabolites involved in antioxidant responses (Estrada-Ortiz et al., 2013, 2016).

Secondary metabolites produced by plants exhibit enormous structural variation, and consequently display a wide range of biological activities (O'Connor, 2015). Based on their biosynthetic origins, plant secondary metabolites encompass three major groups: (1) flavonoids and allied phenolic and polyphenolic compounds; (2) terpenoids; and (3) N-containing alkaloids and sulfur-containing compounds, including GLs (Crozier et al., 2006). In recent years, flavonoids and GLs have been the focus of much research due to their potential as health-promoting phytochemicals. Flavonoids display antioxidant ability and antimicrobial properties and may reduce the risk of cardiovascular diseases and various types of cancer and chronic diseases (Lee et al., 2007). Among them, the flavonoid quercetin has shown the greatest benefits to human health (Knekt et al., 2002; Williams et al., 2004). Furthermore, flavonoids have a great number of functions in plants. For example, the colorless flavonoids accumulate in the outermost layers of plants, absorbing UV radiation and thus preventing its harmful effects on the internal tissues (Gould and Lister, 2006). Some flavonoids have defense functions against herbivores (Roland et al., 2013), and may modulate the activity of auxin-transporting *P*-glycoproteins as well as that of regulatory proteins such as phosphatases and kinases (Peer and Murphy, 2007).

Flavonoid synthesis can be affected by climate conditions such as temperature and radiation. A reduction in PAR has been associated with low concentrations of flavonoids (Agati and Tattini, 2010; Fallovo et al., 2011). Another factor affecting flavonoids biosynthesis is Pi, since Pi deficiency increases their accumulation (Bariola et al., 1999; Stewart et al., 2001; Misson et al., 2005). Pi deficiency induces important changes in several primary and secondary metabolic pathways. Alteration of secondary metabolism in root tissues under Pi deficiency seems to enhance Pi uptake. Regardless of the physiological mechanism involved, flavonoids biosynthesis is enhanced in nutrient-poor conditions and can help plants to cope with unfavorable environments (Malusà et al., 2006).

Glucosinolates are mainly found in the family Brassicaceae. Some GLs and their breakdown products have attracted intense research because of their cancer-preventing attributes (Plate and Gallaher, 2006; Stoin et al., 2007). The effect of P on the production of GLs such as isothiocyanates is relatively insignificant in mustard (*Sinapis alba* cv. Ida Gold) and radish (*Raphanus sativa* cv. Colonel), and these crops differ significantly in their ability to uptake and accumulate P (Brown et al., 2008). However, P deficiency does increase the total GL concentration in *B. campestris* L. subsp. *chinensis* var. *communis* at normal

light intensity, though this effect is not significant with low light intensity (Yang et al., 2009).

Many previous studies have focused on the influence of N and sulfur on GLs biosynthesis (e.g., Schonhof et al., 2007; Fallovo et al., 2011), because these nutrients have a strong impact as amino acid precursors and intermediaries involved in this biosynthetic process. In addition, during the biosynthesis of GLs, there is a high P demand for the formation of phosphorylated cofactors such as uridine diphosphate glucose (UDPG) and co-substrates such as 3′-phosphoadenosine 5′-phosphosulfate (PAPS) (Wittstock and Halkier, 2002). While Pi is the sole source of P important for plant nutrition, Phi is emerging as a novel biostimulant, and may improve some quality attributes in horticultural crops. However, information is missing on the effect of PAR, Pi and Phi and their interactions on the biosynthesis of secondary metabolites such as flavonoids and GLs. Moreover, it is not well understood whether these factors affect the accumulation of nitrate in edible tissues. Hence, the primary aim of this research was to evaluate the main effect of PAR, Pi, Phi, and their interactions, on the concentrations of flavonoids, GLs, and nitrate in two *Brassica* species differing in their concentration and composition of these secondary metabolites (Fallovo et al., 2011). Due to the wide spectrum of health-promoting substances, the two *Brassica* species are important for human consumption.

MATERIALS AND METHODS

Plant Material, Experimental Conditions, and Treatments

Two experiments were conducted in a hydroponic system, under greenhouse conditions (covered with conventional glass), at the Leibniz Institute of Vegetable and Ornamental Crops located in Großbeeren, Germany (13° 20′ east longitude and 52° 22′ north latitude), considering two mean daily PAR levels: the first experiment was carried out from 29 August to 23 September 2013 resulting in 29.7 mol · m^{-2} · d^{-1} (higher level) and the second one from 30 September to 30 November 2013 resulting in 22.2 mol · m^{-2} · d^{-1} (lower level). All other climate conditions were the same in both experiments, conducted at average temperature of 17.4°C, average relative humidity of 77%, and average CO_2 concentration of 400 μmol · mol^{-1}. For each experiment, seeds of *B. juncea* cv. Red Giant and *B. campestris* cv. Mibuna Early were germinated in rockwool cubes. Twenty-two days after germination, plants with 2 and 3 true leaves respectively, were transplanted into a recirculating nutrient solution system supported by 7.5-m-long gullies, having 44 plants per gully, being half of each cultivar; spacing between plants was 0.15 m. Eighteen gullies were placed within the greenhouse in a completely randomized experimental design with 12 treatments, each replicated three times.

A factorial experiment, resulting from the treatment combinations of three study factors, namely PAR, Pi, and Phi, was conducted. Pi and Phi were applied in the nutrient solution. The Pi factor was tested at deficiency (0.1 mM) and sufficiency (0.5 mM) levels. Phi was tested at low (0.1 mM), medium (0.5 mM) and high (2.5 mM) levels. Phosphate was obtained from phosphoric acid and the Phi from phosphorous acid, both reagent grade (Carl Roth GmbH, Karlsruhe, Germany). The other nutrients were the same in both experiments and added to the nutrient solution at concentrations (mM) as follows: NO_3^- 7.82, NH_4^+ 0.33, K^+ 3.93, Ca^{2+} 1.95, Mg^{2+} 0.77, SO_4^{2-} 0.77; the nutrient solution was supplemented with micronutrients at concentrations (μM) as follows: Fe^{2+} 40.0, Mn^{3+} 5.0, Zn^{2+} 4.0, BO_3^{3-} 30.0, Cu^{2+} 0.5, MoO_4^{2-} 0.5. Electrical conductivity was kept at 2 dS · m^{-1} when preparing the nutrient solution with demineralized water in both experiments and changed once a week. The pH was controlled and kept between 5.5 and 6.0.

Preparation of Samples for Analyses

At commercial maturity (25 and 35 days after transplanting for experiments at 29.7 and 22.2 mol m^{-2} daily mean PAR, respectively), 10 plants were harvested from each treatment and its replications. Commercial maturity for *B. campestris* was defined by the presence of at least 49 leaves and for *B. juncea* of 7 to 8 leaves. For *B. juncea*, a leaf was randomly taken from each of the 10 harvested plants, and then the midrib was cut off and discarded. In *B. campestris*, the petiole was cut off from each of the 10 harvested plants, and then 100-150 g samples were taken in duplicate from each treatment and its replications.

Samples for analysis of flavonoids and GLs were frozen at −20°C in a Poron-brand freezer (Erfurt, Germany). Already-frozen samples were lyophilized in a Christ-brand freeze drier (Martin Christ, Osterode, Germany) for about 1 week. Following this, the samples were finely milled in a Retsch-brand grinder (F. Kurt Retsch GmbH & Co., Haan, Germany) and stored for subsequent analysis.

Samples used for nitrate analysis were dried in a Binder-brand drying oven for 72 h at 70°C, and then finely milled in the same Retsch grinder and stored for subsequent analysis.

Growth Characteristics and Nitrate Analysis

The number of plant leaves was determined at harvest. After harvest, leaves were dried in a Binder-brand drying oven for 72 h at 70°C to determine leaf dry matter.

Nitrate concentrations were measured potentiometrically in plant tissue extracts with a nitrate ion plus Sure-Flow1 electrode (Orion-Research, Beverly, MA, United States).

Analyses of Flavonoids

Flavonoids were determined as their aglycones after acid hydrolysis (Fallovo et al., 2011). To do this, 0.25 g of the lyophilized plant material were weighed, and 20 mL of aqueous methanol (62.5%) and 5 mL of HCl (8 M) added. Then it was held at reflux for 2 h in a hot water bath (100°C). After this time, the extract was cooled by immersing it in cold water, and it was adjusted to 50 mL with 50% methanol and sonicated (Bandelin Sonorex RK 100, Berlin, Germany) for 5 min. Subsequently, a sample of the previously homogenized extract was passed through a PTFE filter (0.45 μm, polytetrafluoroethylene; Roth, Karlsruhe, Germany) and placed in a vial for later analysis by HPLC-DAD-ESI-MS (Agilent, Waldbronn, Germany).

The composition and concentration of flavonoids were determined using an 1100 series HPLC (Agilent, Waldbronn, Germany) equipped with a diode array detection system. The extracts were separated on a Prodigy column (ODS 3, 150 × 3.0 mm, 5 μm, 100 Å) (Phenomenex, Aschaffenburg, Germany) with a C18 security guard (ODS 3, 4 × 3.0 mm, 5 μm, 100 Å) at a temperature of 25°C using a water/acetonitrile gradient (Th Geyer GmbH, Renningen, Germany). Solvent A consisted of 99.5% water and 0.5% acetic acid (VWR International, Dresden, Germany), while solvent B was 100% acetonitrile. In this analysis, the following gradient was used: 30–35% B (5 min), 35–39% B (12 min), 39–90% B (5 min), isocratic 90% B (5 min), 90–30% B (5 min), isocratic 30% B (5 min). The injection volume was 50 μL, using a flow rate of 0.3 mL min^{-1}, using a wavelength of 370 nm (for quercetin, kaempferol and isorhamnetin) and 520 nm (for cyanidin) for quantification. Dihydroquercetin, kaempferol, isorhamnetin and cyanidin (Carl Roth GmbH, Karlsruhe, Germany) were used as standards for external calibration curves and compound identification based on retention time and characteristic MS signals. The deprotonated molecular ions $[M\text{-}H]^-$ with m/z 315, 301 and 285 for isorhamnetin, quercetin and kaempferol respectively, and the molecular ion $[M]^+$ with m/z 287 for cyanidin were detected by HPLC-DAD-ESI-MS, using Agilent 1100 series MSD equipment, with ESI as a source of ions in negative mode and positive mode, respectively. Nitrogen was used as drying gas (12 L min^{-1}, −350°C) and nebulizing gas (40 psi).

Analyses of Glucosinolates

Desulfo-glucosinolate profiles and concentrations were derived using a modified HPLC protocol (Krumbein et al., 2005). Twenty mg of previously lyophilized plant material were weighed and finely milled, in duplicate, and placed in 2-mL plastic tubes, to which 750 μL of 70% methanol at 70°C were added, shaken for 10 min at 1400 rpm and at 70°C in a DITABIS unit (Model MHL 23, Pforzheim, Germany). Samples were subsequently centrifuged for 5 min at 4500 rpm and at 20°C (Centrifuge Heraeus, D-37520, Osterode, Germany). The resulting supernatants were placed in a plastic tube. The residues were extracted twice more with 500 μL of 70% methanol at 70°C, shaken for 5 min at 1400 rpm and at 70°C; subsequently they were centrifuged for 5 min at 4500 rpm at 20°C, and the supernatants were collected in the same plastic tube used in the previous step. The supernatants were added to a SPE column, which was pre-conditioned with 500 μL of DEAE Sephadex A-25 ion exchanger suspension (Sigma-Aldrich Chemie GmbH, Sweden). Prior to sample loading, the column was first equilibrated in 2 M acetic acid, then pre-treated by the addition of 1 mL aliquots of 6 M imidazole formate (Carl Roth GmbH, Germany) in 30% (v/v) formic acid, followed by two washes with 1 mL deionized water. The column was washed twice with 1 mL 20 mM sodium acetate buffer pH 4.0 (Sigma-Aldrich Chemie GmbH, Germany), and 75 μL purified *Helix pomatia* aryl sulfatase (Roche Diagnostics GmbH, Germany) was loaded and left to stand for 12 h. The desulfo-glucosinolates were eluted from the columns with two applications of 0.5 mL of ultrapure water, then placed in tube filters (Costar Spin-X 0.22 μm of cellulose acetate in 2-mL polypropylene tubes, Corning Incorporated, United States) and centrifuged for 5 min at 3000 rpm at 20 °C for reading. The analysis was performed using a 1290 Infinity HPLC (Agilent Technologies, Germany) with a Poroshell 120 EC-C18 column (2.1 × 100 mm 2.7 Micron, Agilent Technologies, United States) at a temperature of 30°C using a water/acetonitrile gradient (Th. Geyer GmbH, Renningen, Germany). Solvent A consisted of 100% water, and solvent B consisted of 40% acetonitrile in water. The following gradient was used: 99.5% A, 0.5% B (12 min); 50.5% A, 49.5% B (3 min); 0.5% A, 99.5% B (3 min); 99.5% A, 0.5% B (1 min). The injection volume was 5 μL, using a 0.4 mL min^{-1} flow and a 229 nm wavelength for quantification. The GL concentrations were calculated using prop-2-enyl-glucosinolate as standard for the external calibration curve and the response factor of each compound relative to prop-2-enyl-GL. Total GLs were determined from the sum of individual GLs. Each analysis was performed in duplicate. Desulfo-glucosinolates were identified by HPLC-ESI-MS2 using Agilent 1100 series (Agilent Technologies, Germany) operating in the positive ionization mode, based on the protonated molecular ions $[M + H]^+$ and the fragment ions corresponding to $[M + H\text{-glucose}]^+$, identified by Zimmermann et al. (2007).

The **Supplementary Material S1** summarizes the GLs assessed in this study, indicating the belonging group, trivial name, IUPAC nomenclature, semi systematic names used in texts, and abbreviations used in tables and figures.

Statistical Analysis

The Shapiro–Wilk and Kolmororov–Smirnov procedures were performed to verify that the data had a normal distribution, and the Levene, O'Brien and Bartlet tests were conducted to verify the homogeneity of variances. Then a multifactorial ANOVA of all data obtained was performed. Data were analyzed using PAR, Pi, and Phi as study factors. Means were compared using Tukey's test ($P \leq 0.05\%$). In addition, regression analysis was performed to determine the relationship between flavonoids and nitrate, for which SAS 9.3 statistical software was used (SAS, 2011).

RESULTS

Leaf Number and Biomass

The leaf number and dry biomass of leaves at harvest were affected by PAR only in *B. campestris* (**Supplementary Materials S2, S3**). Leaf number was higher in plants treated with lower PAR with an average value of approximately 79 leaves, exceeding in 18 leaves to the means observed in plants exposed to higher PAR (**Figure 1A**). Conversely, leaf dry matter was 10.6% higher in plants grown at higher PAR in comparison to those grown at lower PAR (**Figure 1B**).

Flavonoid Concentrations

PAR significantly affected concentrations of quercetin, kaempferol, isorhamnetin, and total flavonoids in both *Brassica* species evaluated. In addition, Pi significantly influenced the concentrations of quercetin, isorhamnetin, and total flavonoids in *B. campestris*. In *B. juncea*, Pi only affected quercetin and cyanidin

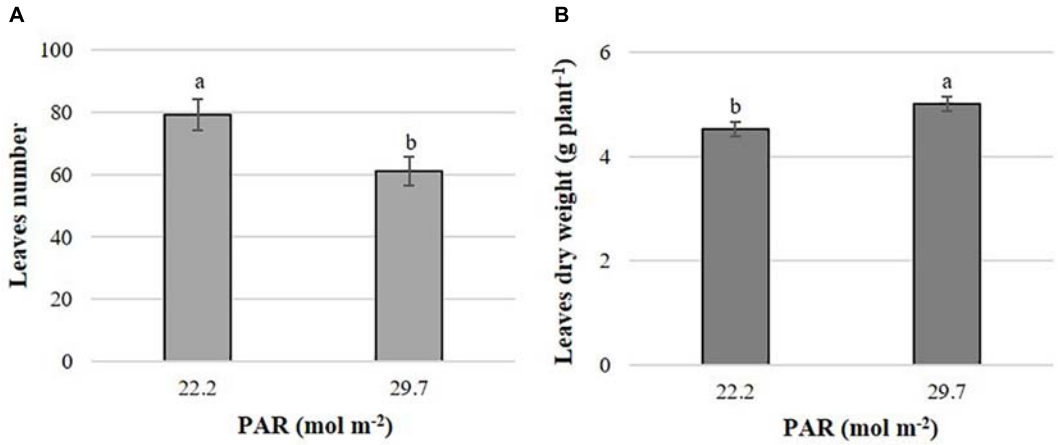


FIGURE 1 | Effects of mean daily photosynthetically active radiation (PAR) on leaf number **(A)** and leaf dry matter **(B)** in *B. campestris* cv. Mibuna Early. Means ± SD with different letters in each subfigure indicate significant differences at $P \leq 0.05$ (Tukey's test).

concentrations. Interestingly, PAR × Phi interaction modified all flavonoids quantified in *B. juncea* (**Supplementary Material S4**).

The effects of PAR on flavonoid concentrations were different between *Brassica* species evaluated. In *B. campestris*, increasing PAR elevated the concentrations of quercetin, kaempferol, isorhamnetin, and total flavonoids by 121.4, 24.4, 61.1, and 40.3%, respectively, in comparison to the lower PAR applied (**Figure 2**). At high Phi, *B. juncea* exposed to higher PAR displayed higher quercetin concentrations in comparison to plants exposed to lower PAR, while no other interactions occurred. Plants exposed to lower PAR exhibited higher concentrations of kaempferol than those exposed to higher PAR independent of the Phi concentration applied. Isorhamnetin and total flavonoid concentrations were higher in plants exposed to lower PAR at any level of Phi tested. Cyanidin concentrations were the highest in plants exposed to higher PAR at high Phi (**Table 1**).

Deficient Pi in the nutrient solution resulted in a significant increase of total flavonoids at 14.3%, especially in *B. campestris*, which included increases of quercetin and isorhamnetin at 25.2 and 25.6% in comparison to sufficient Pi tested, respectively. However, in *B. juncea* deficient Pi only increased the concentrations of quercetin and cyanidin by 15.6 and 20.1%, respectively, in comparison to the application of sufficient Pi (**Figure 3**).

The PAR × Phi interaction was significant for flavonoids concentrations in *B. juncea* (**Table 1**). Quercetin, kaempferol, isorhamnetin, cyanidin and total flavonoids had their highest concentrations at low Phi and higher PAR. Conversely, at lower PAR, all flavonoids but cyanidin reduced their concentrations when Phi levels increased in the nutrient solution.

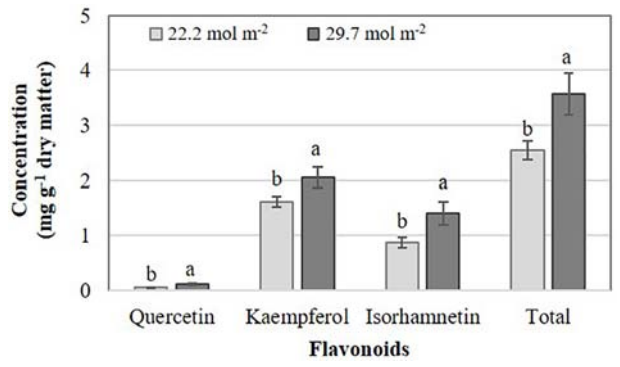


FIGURE 2 | Effects of mean daily photosynthetically active radiation (PAR) on flavonoid concentration in *B. campestris* cv. Mibuna Early. Means ± SD with different letters in each variable analyzed indicate significant differences at $P \leq 0.05$ (Tukey's test).

Nitrate Concentrations

The *Brassica* species evaluated displayed different responses to the factors studied regarding nitrate concentrations in edible tissues. Between the two species, *B. campestris* showed stronger effects. Phi as main factor did not affect nitrate concentration in either species (**Supplementary Material S5**).

Increasing the PAR value from 22.2 to 29.7 mol m^{-2} decreased nitrate concentrations in edible tissues by 35.5%, but only in *B. juncea* (**Figure 4A**). Phi only affected nitrate concentrations in *B. campestris*, decreasing it by 15.3% in plants exposed to medium Phi, with respect to those exposed to low Phi (**Figure 4B**).

In *B. campestris*, the PAR × Pi interaction significantly affected the nitrate concentration in leaves (**Figure 5**). Nitrate concentration was generally higher in plants at lower PAR. At lower PAR it increased with increasing Pi but decreased at higher PAR.

The rest of the interactions had no significant effects on nitrate concentrations in ether species evaluated (**Supplementary Material S5**).

Relationship Between Total Flavonoids and Nitrate Concentrations

A significant negative relationship between total flavonoids and nitrate concentrations was analyzed for *B. campestris* with

TABLE 1 | Mean daily photosynthetically active radiation (PAR) and phosphite (Phi) affecting flavonoid concentration (mg g^{-1} dry matter) in *B. juncea* cv. Red Giant.

PAR (mol m^{-2})	Phi (mM)	Quercetin	Kaempferol	Isorhamnetin	Cyanidin	Total flavonoids
22.2	0.1	0.232 ± 0.02ab	2.50 ± 0.10a	1.36 ± 0.103a	0.66 ± 0.054ab	4.75 ± 0.24a
	0.5	0.227 ± 0.01ab	2.35 ± 0.12ab	1.23 ± 0.094ab	0.67 ± 0.044ab	4.48 ± 0.23ab
	2.5	0.194 ± 0.02b	2.27 ± 0.07ab	1.17 ± 0.053ab	0.57 ± 0.089b	4.20 ± 0.15abc
29.7	0.1	0.219 ± 0.01b	1.73 ± 0.18c	1.02 ± 0.057b	0.56 ± 0.059b	3.53 ± 0.26c
	0.5	0.250 ± 0.04ab	1.80 ± 0.14c	1.10 ± 0.092b	0.71 ± 0.137ab	3.87 ± 0.33bc
	2.5	0.287 ± 0.01a	2.04 ± 0.14bc	1.15 ± 0.069ab	0.85 ± 0.062a	4.33 ± 0.21ab

Means ± SD with different letters in each column indicate statistically significant differences at $P \leq 0.05$ (Tukey's test).

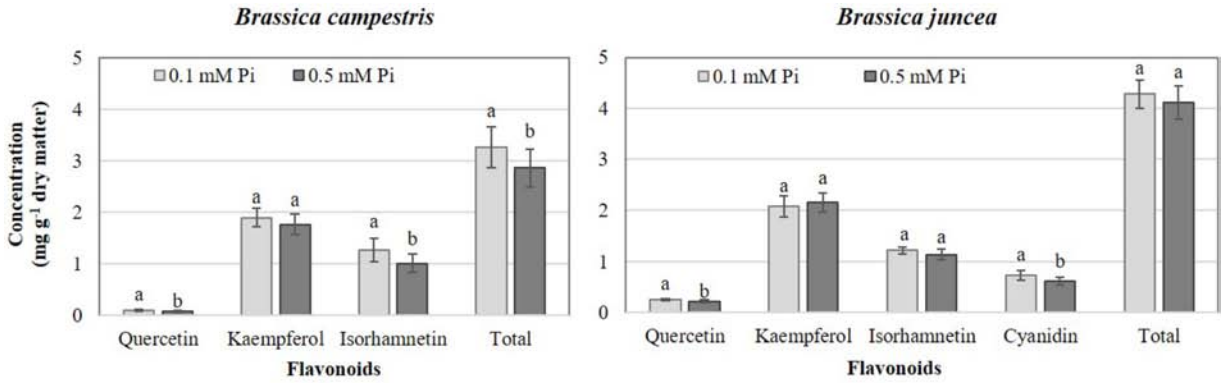


FIGURE 3 | Effects of phosphate (Pi) on flavonoid concentration in two *Brassica* species. Means ± SD with different letters in each subfigure and different letters indicate significant differences at $P \leq 0.05$ (Tukey's test).

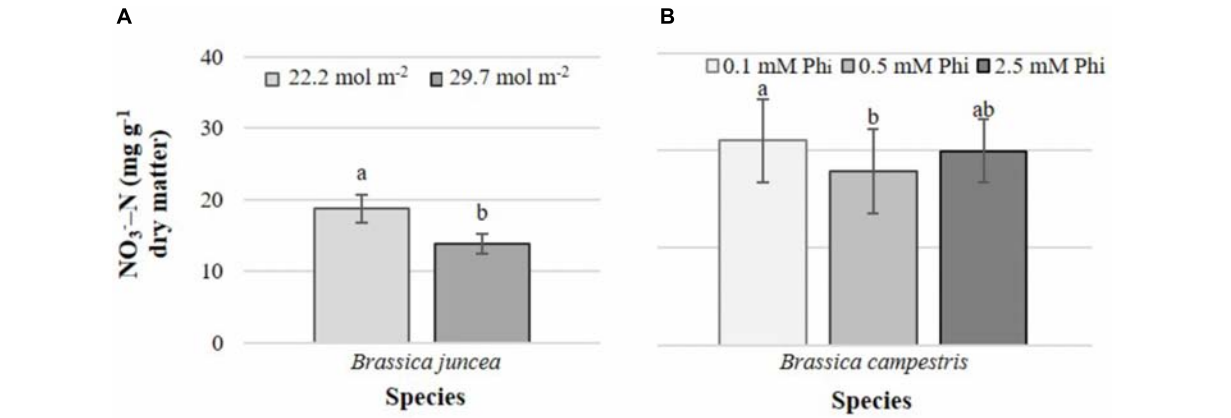


FIGURE 4 | Effects of mean daily photosynthetically active radiation (PAR) and phosphite (Phi) on nitrate concentration in *B. juncea* cv. Red Giant **(A)** and *B. campestris* cv. Mibuna Early **(B)**, respectively. Means ± SD with different letters in each subfigure indicate significant differences at $P \leq 0.05$ (Tukey's test).

$R^2 = 0.576$ (**Figure 6**). The same relationship was non-significant in *B. juncea* ($R^2 = 0.279$).

Study Factors and Their Interactions Influencing Glucosinolate Concentrations

Effects of PAR were significant in *B. campestris* for total alkyl-GL (only detected in this species), total alkenyl-GL, and the aryl-GL (2-phenylethyl) (**Supplementary Material S6a**). Among alkyl-GLs, plants exposed to higher PAR displayed concentrations of 5-methylsulfinylpentyl-GL 87.7% higher than plants exposed to lower PAR. Interestingly, plants exposed to higher compared with lower PAR exhibited 89.4% higher concentrations of the three alkenyl-GLs identified (2-hydroxybut-3-enyl-, but-3-enyl-, and pent-4-enyl-GL), and 96.1 and 101.1% higher concentrations of the indole-GLs, 1-methoxyindol-3-ylmethyl- and indol-3-ylmethyl-GL. Conversely, the higher PAR level significantly

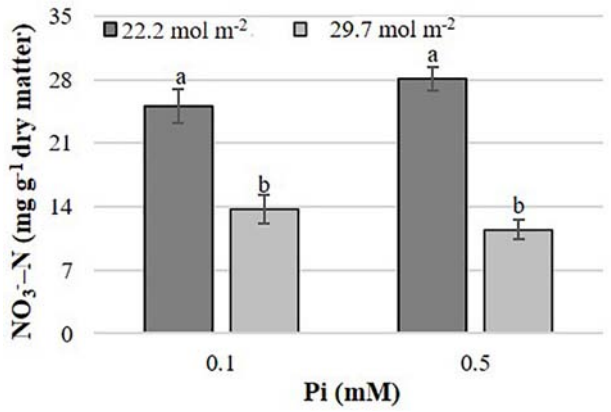


FIGURE 5 | Effects of mean daily photosynthetically active radiation (PAR) and phosphate (Pi) on nitrate concentration in *B. campestris* cv. Mibuna Early. Means ± SD with different letters indicate significant differences at $P \leq 0.05$ (Tukey's test).

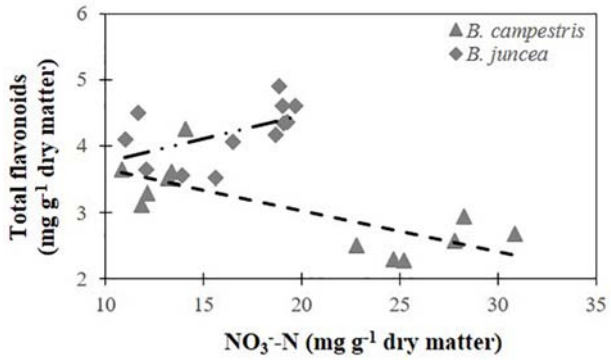


FIGURE 6 | Relationship between total flavonoid concentration and nitrate concentration in two *Brassica* species. Total flavonoids in *B. juncea* cv. Red Giant = 3.040+ 0.071 · (NO_3^--N) (R^2 = 0.279). Total flavonoids in *B. campestris* cv. Mibuna Early = 4.269 – 0.062 · (NO_3^--N) (R^2 = 0.576*). *Significant at $P \leq 0.05$.

reduced the concentration of 2-phenylethyl-GL, which was 23.4% lower than that observed in plants grown under lower PAR level (**Figure 7A**).

Increasing the PAR level from 22.2 to 29.7 mol m^{-2} raised the concentration of indol-3-ylmethyl-GL and 4-hydroxyindol-3-ylmethyl-GL in *B. juncea*. However, at higher PAR, the concentration of 1-methoxyindol-3-ylmethyl-GL fell by 51.5% in comparison to lower PAR. Within the group of alkenyl-GLs, prop-2-enyl-GL (propenyl) and but-3-enyl-GL (butenyl) were increased by 52 and 30.4%, respectively (**Supplementary Material S6b** and **Figure 7B**).

In *B. campestris,* Pi-deficient plants increased the concentrations of two alkyl-GLs, namely 4-methylsulfinylbutyl-GL and 5-methylsulfinylpentyl-GL, by 144.3 and 64.3%, respectively, in comparison to Pi-sufficient plants. Among the indole-GLs, Pi differentially affected the concentrations of 1-methoxyindol-3-ylmethyl-GL, being reduced by 17.3% in Pi-deficient plants, in comparison to Pi-sufficient plants (**Supplementary Material S6a** and **Figure 7A**).

In *B. juncea*, Pi significantly increased the concentrations of prop-2-enyl-GL (9.2%), total alkenyl-GLs and the aryl-GL (2-phenylethyl-GL) comparing Pi-deficient with Pi-sufficient plants (**Supplementary Material S6b**). All other GLs evaluated in this species were also increased under Pi-deficiency (**Figure 7B**).

The concentrations of 1-methoxyindol-3-ylmethyl-GL, and 4-methoxyindol-3-ylmethyl-GL were significant influenced by Phi in *B. campestris* (**Supplementary Material S6a**). Regarding indole-GLs, plants exposed to medium Phi significantly increased by 28.3% the concentration of 1-methoxyindol-3-ylmethyl-GL, in comparison to plants treated with low Phi (**Figure 7A**).

In *B. juncea*, Phi only had effect on but-3-enyl-GL concentration (**Supplementary Material S6b**). Indeed, the highest concentrations were recorded in plants treated with high Phi. The concentrations were 18.9% higher in comparison to those in plants grown at medium Phi (0.5 mM) (**Figure 7B**).

The PAR × Pi interaction affected the concentrations of 2-hydroxybut-3-enyl-, 4-methoxyindol-3-ylmethyl-GL, and total indole-GLs in *B. campestris*. In this species, the PAR × Phi interaction predominantly affected the concentrations of 5-methylsulfinylpentyl-, 2-hydroxybut-3-enyl-, and but-3-enyl-GL. The Pi × Phi interaction only influenced the concentration of total alkyl-GLs, and 4-methylsulfinylbutyl-GL belonging to this group. The PAR × Pi × Phi interaction did not influence the concentrations of GLs in *B. campestris* (**Supplementary Material S6a**).

In *B. juncea*, the PAR × Pi interaction influenced only the concentration of 4-hydroxyindol-3-ylmethyl-GL. The concentrations of prop-2-enyl-, total alkenyl-, aryl-(2-phenylethyl-GL), indol-3-ylmethyl-, 4-hydroxyindol-3-ylmethyl-, and total-GLs, were affected by PAR × Phi. The Pi × Phi interaction significantly affected the indol-3-ylmethy-GL concentration. In the same way, the interaction PAR × Pi × Phi influenced indol-3-ylmethyl-GL, total indole-GLs and total-GLs (**Supplementary Material S6b**).

At higher compared with lower PAR, we observed a concomitant increase in the concentrations of the aryl-GL, alkyl-, alkenyl-, indole-, and total GLs in *B. campestris* (**Figure 8A**). In *B. juncea*, Pi-deficient plants had increased concentrations of alkenyl-GLs and the aryl-GL, by at least 8.8% compared to the values observed in Pi-sufficient plants (**Figure 8B**).

In *B. campestris* the concentrations of some GLs were significantly affected by the interactions PAR × Pi, PAR × Phi and Pi × Phi (**Table 2**). The interaction PAR × Pi × Phi was without a significant influence.

Regarding PAR × Pi interaction, both levels of Pi at higher PAR significantly increased the concentrations of 2-hydroxybut-3-enyl-, 4-methoxyindol-3-ylmethyl-GL and total indole-GLs (**Table 2**).

All three Phi levels combined with higher PAR significantly increased the concentrations of but-3-enyl-GL. The concentration of 5-methylsulfinylpentyl-GL reduced as the Phi did, independent of the PAR level. Nevertheless, such decrease was more evident in plants exposed to higher PAR (55.7%) in comparison to that observed in plants exposed to

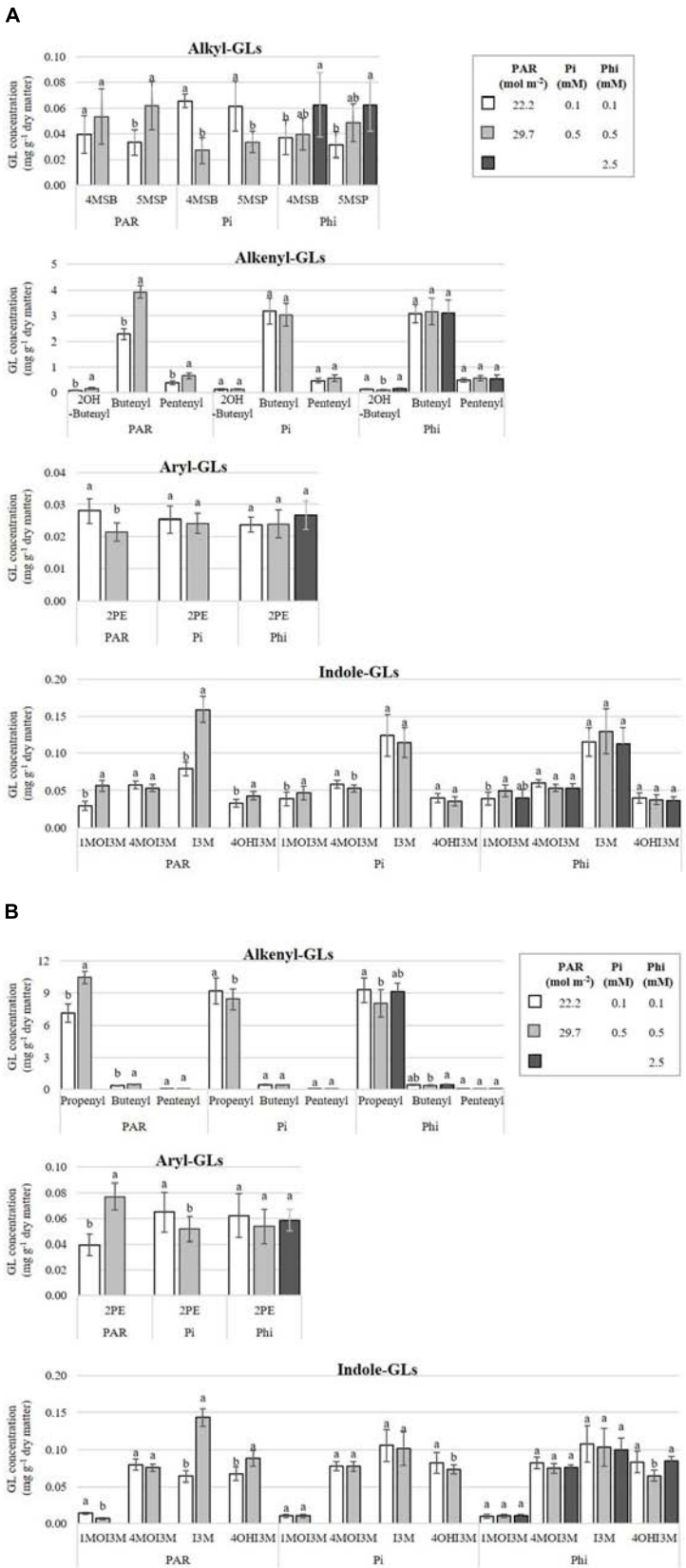


FIGURE 7 | Continued

FIGURE 7 | Influence of mean daily photosynthetically active radiation (PAR), phosphate (Pi), and phosphite (Phi) on glucosinolate (GL) concentrations. Bars ± SD with different letters in each subfigure indicate significant differences at $P \leq 0.05$ (Tukey's test). **(A)** *B. campestris* cv. Mibuna Early. **(B)** *B. juncea* cv. Red Giant. 4MSB, 4-methylsulfinylbutyl-GL; 5MSP, 5-methylsulfinylpentyl-GL; 2OH-Butenyl, 2-hydroxybut-3-enyl-GL; Butenyl, but-3-enyl-GL; Propenyl, prop-2-enyl-GL; Pentenyl, pent-4-enyl-GL; 2PE, 2-phenylethyl-GL; 1MOI3M, 1-methoxyindol-3-ylmethyl-GL; 4MOI3M, 4-methoxyindol-3-ylmethyl-GL; I3M, indol-3-ylmethyl-GL; 4OHI3M, 4-hydroxyindol-3-ylmethyl-GL.

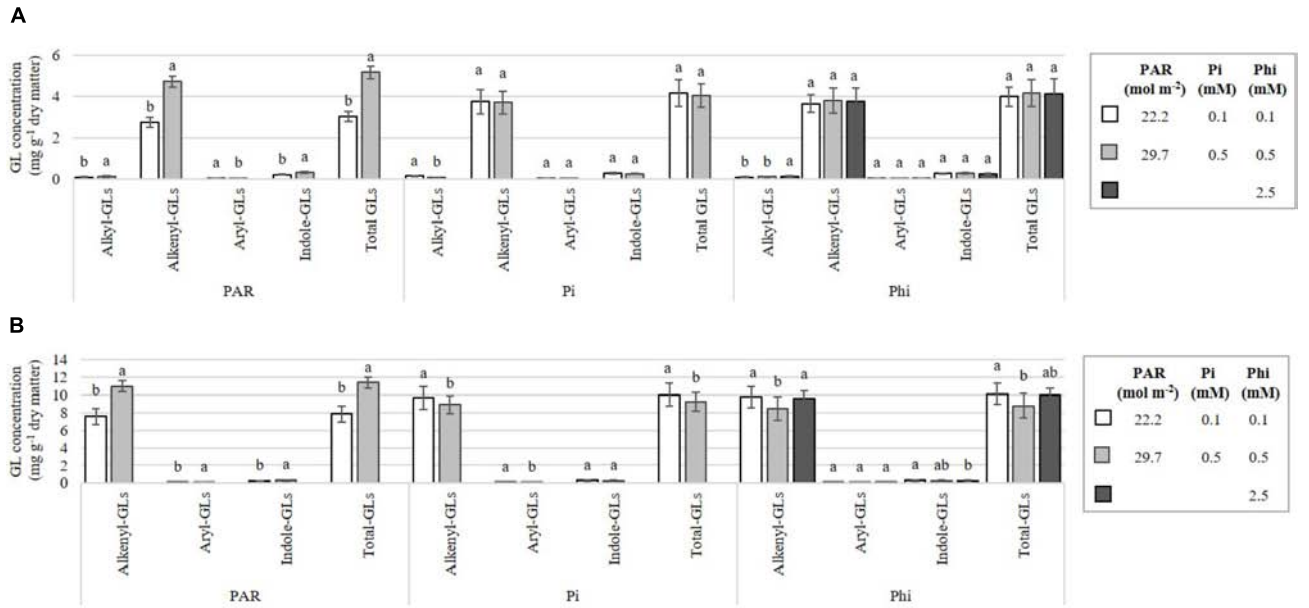


FIGURE 8 | Influence of mean daily photosynthetically active radiation (PAR), phosphate (Pi), and phosphite (Phi) on the alkyl-, alkenyl-, aryl-, indole-, and total glucosinolate (GL) concentrations. Bars ± SD with different letters indicate significant differences at $P \leq 0.05$ (Tukey's test). **(A)** *B. campestris* cv. Mibuna Early. **(B)** *B. juncea* cv. Red Giant.

lower PAR (36.1%). Concentration of 2-hydroxybut-3-enyl-GL with increasing Phi in the nutrient solution at lower PAR, whereas it increased at higher PAR (**Table 2**).

The Pi × Phi interaction significantly affected the concentrations of 4-methylsulfinylbutyl-GL and total alkyl-GLs (**Table 2**). In both cases, the highest levels were observed in Pi-deficient plants in combination with the high Phi concentration.

In *Brassica juncea*, all interactions between factors were significant at least for one GL analyzed (**Tables 3**, **4**).

Regarding the PAR × Phi interaction, the lower level of PAR combined with the medium Phi significantly reduced the concentrations of prop-2-enyl-GL and alkenyl-GLs, in comparison to the concentrations of those GLs found in plants exposed to high Phi. In plants exposed to higher PAR and high Phi the concentration of 2-phenylethyl-GL was significantly reduced in comparison to plants treated with low Phi. Furthermore, plants exposed to lower PAR displayed lower concentrations of 2-phenylethyl-GL, independent of the Phi concentrations tested (**Table 3**).

Significant effects of the PAR × Pi × Phi interaction on GL concentrations are presented in **Table 4**. The highest concentrations of indol-3-ylmethyl-GL, total indole-, and total-GLs were recorded in plants receiving higher PAR, independent of the Pi and Phi supply levels. However, at lower PAR, higher Phi and deficient Pi, similar concentrations of 4-hydroxyindol-3-ylmethyl- and total-GLs were reached.

DISCUSSION

Light affects photosynthesis, phototropism and morphogenetic processes, and consequently, it has a pivotal role in plant metabolism, growth and development. Importantly, light quality and intensity, direction and duration impact plant primary and secondary metabolism (Debiasi et al., 2003). Indeed, the intensity of photosynthetically active radiation (PAR) during the day determines the rate of mobilization of organic compounds, while its amount impacts the extent of CO_2 absorption at night (Diez et al., 2017). Carbon fixation and biosynthesis of primary and secondary metabolites are crucial for cell expansion and division, which are controlled at the molecular level. Although in *B. juncea* it has been reported that the expression of the gene *BjAPY2* (involved in the expansion of edible stems) was higher under short-day photoperiod (8 h/16 h) than under long-day photoperiod (16 h/8 h) (Cao et al., 2015), this species was neither affected by the study factors, nor by their interactions on growth characteristics evaluated under our experimental conditions. In *B. campestris*, only PAR influenced leaf number and dry matter (**Figure 1**).

TABLE 2 | Mean daily photosynthetically active radiation (PAR), phosphate (Pi), and phosphite (Phi) affecting glucosinolate (GL) concentrations in *B. campestris* cv. Mibuna Early.

PAR × Pi interaction				
PAR (mol m^{-2})	**Pi (mM)**	**GL concentration (mg g^{-1} dry matter)**		
		2OH-Butenyl	**4MOI3M**	**Total indole-GLs**
22.2	0.1	0.0777 ± 0.0081b	0.0345 ± 0.0032b	0.2059 ± 0.0084b
	0.5	0.0616 ± 0.0047b	0.0228 ± 0.0021b	0.1904 ± 0.0235b
29.7	0.1	0.1416 ± 0.0093a	0.0584 ± 0.0043a	0.2927 ± 0.0240a
	0.5	0.1715 ± 0.0158a	0.0540 ± 0.0034a	0.3312 ± 0.0202a
PAR × Phi interaction				
PAR (mol m^{-2})	**Phi (mM)**	**GL concentration (mg g^{-1} dry matter)**		
		5MSP	**2OH-Butenyl**	**Butenyl**
22.2	0.1	0.023 ± 0.004d	0.085 ± 0.018cd	2.465 ± 0.201b
	0.5	0.039 ± 0.015bc	0.059 ± 0.010d	2.189 ± 0.187b
	2.5	0.036 ± 0.008bc	0.064 ± 0.010d	2.155 ± 0.241b
29.7	0.1	0.039 ± 0.013bc	0.148 ± 0.017b	3.651 ± 0.198a
	0.5	0.058 ± 0.014ab	0.117 ± 0.011bc	4.116 ± 0.208a
	2.5	0.088 ± 0.021a	0.205 ± 0.027a	4.010 ± 0.246a
Pi × Phi interaction				
Pi (mM)	**Phi (mM)**	**GL concentration (mg g^{-1} dry matter)**		
		4MSB	**Total alkyl-GLs**	
0.1	0.1	0.0556 ± 0.0137b	0.0924 ± 0.0251bc	
	0.5	0.0429 ± 0.0095b	0.1041 ± 0.0257b	
	2.5	0.0984 ± 0.0240a	0.1841 ± 0.0381a	
0.5	0.1	0.0183 ± 0.0040b	0.0438 ± 0.0059c	
	0.5	0.0359 ± 0.0156b	0.0715 ± 0.0201bc	
	2.5	0.0264 ± 0.0052b	0.0649 ± 0.0121bc	

Means ± SD with different letters in each column and analyzed variable indicate significant differences at $P \leq 0.05$ (Tukey's test). 2OH-Butenyl, 2-hydroxybut-3-enyl-GL; 4MOI3M, 4-methoxyindol-3-ylmethyl-GL; 5MSP, 5-methylsulfinylpentyl-GL; Butenyl, but-3-enyl-GL; 4MSB, 4-methylsulfinylbutyl-GL.

TABLE 3 | Mean daily photosynthetically active radiation (PAR) and phosphite (Phi) affecting glucosinolate (GL) concentrations in *B. juncea* cv. Red Giant.

PAR × Phi interaction				
		GL concentration (mg g^{-1} dry matter)		
PAR (mol m^{-2})	**Phi (mM)**	**Propenyl**	**Alkenyl-GLs**	**2PE**
22.2	0.1	7.609 ± 0.819bc	8.018 ± 0.870bc	0.0363 ± 0.005de
	0.5	5.824 ± 0.550c	6.148 ± 0.583c	0.0291 ± 0.002e
	2.5	8.105 ± 0.770b	8.558 ± 0.803b	0.0527 ± 0.011cd
29.7	0.1	10.998 ± 0.673a	11.502 ± 0.690a	0.0880 ± 0.015a
	0.5	10.300 ± 0.586a	10.797 ± 0.607a	0.0787 ± 0.004ab
	2.5	10.128 ± 0.425a	10.649 ± 0.444a	0.0646 ± 0.005bc

Means ± SD with different letters in the column indicate significant differences at $P \leq 0.05$ (Tukey's test). Propenyl, prop-2-enyl-GL; 2PE, 2-phenylethyl-GL.

In kale (*B. oleracea* var. *sabellica*), Neugart et al. (2016) reported a higher number of genes regulated by light than by temperature. Those genes were mainly related to protein degradation (in response to light), phytohormone metabolism (in response to temperature), and secondary metabolism (in response to both treatments). The expression patterns of genes involved in the biosynthesis of flavonoids were correlated to the structure-dependent response of those metabolites to alterations in either light or temperature. Under our experimental conditions, differential effects of PAR on the synthesis of flavonoids were observed between the two *Brassica* species evaluated. In general, the biosynthesis of flavonoids (quercetin, kaempferol, isorharmnetin and total) in *B. campestris* was associated with a higher PAR level evaluated (**Figure 2**). Coincidentally, in kale (*B. oleracea* var. *sabellica*), the highest light intensity evaluated (14.4 mol m^{-2} d^{-1}) increased the foliar concentration of flavonols, including quercetin and kaempferol, in comparison to the means showed by plants exposed to lower light intensity (3.6 mol m^{-2} d^{-1}) (Neugart et al., 2016). Conversely, in spiked pepper (*Piper aduncum*), the lowest concentration of flavonoids was recorded in plants grown under 100% natural irradiance (value of 15.4 W m^{-2} seems to be erroneously given), in comparison to those grown at 50 and 70% natural irradiance. These differential responses observed among plant genotypes suggest the existence of different mechanisms developed by plants to protect themselves against irradiation (Ventorim et al., 2014). Indeed, in Chinese cabbage (*B. campestris* ssp. *Chinensis Makino*), the antioxidant activity of guaiacol peroxidase, catalase, and superoxide dismutase transiently increased in treatments with 75, 50, and 25% of the normal light intensity, especially 5 days after treatment (Zhu et al., 2017). Thus, although it has been documented that external factors, including light intensity, have an influence on the composition of secondary metabolites in plants, the main determinant of the metabolic profiles in plant tissues is the genotype (Zoratti et al., 2014). Among different rice genotypes remarkable variations in PAR transmission percentage with respect to variation in canopy shape were first observed after an early heading stage and continued thereafter (Yusoff and Zainol, 1989). Moreover, interactions among genotypes and N-management practices produced different PAR interception between two maize genotypes studied (Ghosh et al., 2017). Additionally, PAR absorption also differed among coffee genotypes (Mejía-Montoya et al., 2013). Indeed, PAR induced phytochemical changes in two *Brassica* species, which were different according to the genotypes tested (Fallovo et al., 2009, 2011). Coincidentally, Ghasemzadeh et al. (2010) as well as Pazuki et al. (2017) reported similar results. Taken together, our findings are in full agreement with those previously reported.

Various environmental conditions including N and P deficiencies bring about increases in flavonoid concentrations (Stewart et al., 2001). This response is in full agreement with our findings since the concentration of quercetin and isorhamnetin in *B. campestris* and of quercetin and cyanidin in *B. juncea* increased in Pi-deficient plants (**Figure 3**). It is well known that Phi suppresses the coordinated expression of genes under phosphate starvation, leading to negative effects on plant growth

TABLE 4 | Mean daily photosynthetically active radiation (PAR), phosphate (Pi), and phosphite (Phi) affecting glucosinolate (GL) concentrations in *B. juncea* cv. Red Giant.

PAR (mol m^{-2})	Pi (mM)	Phi (mM)	GL concentration (mg g^{-1} dry matter)			
			I3M	4OHI3M	Total indole-GLs	Total-GLs
22.2	0.1	0.1	0.060 ± 0.007d	0.057 ± 0.010cd	0.218 ± 0.026cd	8.221 ± 1.080bcd
		0.5	0.067 ± 0.009d	0.048 ± 0.003d	0.202 ± 0.020d	6.390 ± 0.886d
		2.5	0.081 ± 0.006cd	0.093 ± 0.004ab	0.262 ± 0.009bcd	10.018 ± 0.789abc
	0.5	0.1	0.063 ± 0.006d	0.079 ± 0.007bcd	0.247 ± 0.023bcd	8.352 ± 0.837bcd
		0.5	0.047 ± 0.006d	0.055 ± 0.002cd	0.193 ± 0.013d	6.358 ± 0.213 d
		2.5	0.063 ± 0.001d	0.070 ± 0.003bcd	0.221 ± 0.005cd	7.686 ± 0.230cd
29.7	0.1	0.1	0.153 ± 0.015ab	0.119 ± 0.016a	0.356 ± 0.023a	12.819 ± 0.756a
		0.5	0.161 ± 0.012a	0.080 ± 0.007bcd	0.326 ± 0.022ab	11.505 ± 0.762ab
		2.5	0.112 ± 0.003bc	0.093 ± 0.005ab	0.288 ± 0.015abc	11.120 ± 0.603ab
	0.5	0.1	0.155 ± 0.005a	0.078 ± 0.007bcd	0.309 ± 0.002ab	11.032 ± 0.323ab
		0.5	0.137 ± 0.010ab	0.074 ± 0.003bcd	0.288 ± 0.019abc	10.860 ± 0.525abc
		2.5	0.143 ± 0.001ab	0.083 ± 0.02bc	0.313 ± 0.005ab	10.907 ± 0.333abc

Means ± standard deviation with different letters in the column indicate significant differences at $P \leq 0.05$ (Tukey's test). I3M, indol-3-ylmethyl-GL; 4OHI3M, 4-hydroxyindol-3-ylmethyl-GL.

and metabolism (McDonald et al., 2001; Varadarajan et al., 2002). Under our experimental conditions, however, Phi did not impair the concentration of flavonoids in tissues of either species evaluated.

Flavonoid biosynthesis was stimulated at increasing Phi concentration in the nutrient solution only at higher PAR (**Table 1**). In potato (*Solanum tuberosum*) the expression levels of *F3H*, a gene involved in flavonoid synthesis, increased in UV-B-stressed plants only when pre-treated with potassium Phi, while Phi may prevent oxidative damages caused by UV-B light by increasing the enzymatic activity (Oyarburo et al., 2015). Phi has been classified as an emergent biostimulant in horticulture. As such, Phi may display hormetic effects in plants, which means that at low doses it induces a beneficial effect and at high doses it produces a toxic effect (Trejo-Téllez and Gómez-Merino, 2018). At the physiological level, hormesis can be translated as an adaptive response of an organism to a low level of such factor, accompanied by overcompensation, when the homeostasis readjustment has been interrupted (Calabrese and Blain, 2009; Vargas-Hernández et al., 2017). This allows the organism to acclimate to its new environment. Indeed, the level of eustress (beneficial stress) or distress (harmful stress) toward the same factor (e.g., a biostimulant such as Phi) is not always the same due to the process of adaptation of the plants, which must be taken into account when establishing a strict difference between low dose and high dose of a hormetic factor. In order Phi to induce eustress, plants must be established in the presence of sufficient Pi. Interestingly, under our experimental conditions, fluctuations between Pi and Phi did not influence growth parameters in any *Brassica* species evaluated (**Supplementary Materials S2, S3**). Hence, one can assume that the levels of Pi and Phi caused eustress stimulating secondary metabolite synthesis as observed. Apart from its effect on plant metabolism, Phi has been proved to enhance important traits including plant growth and development, nutrition efficiency, abiotic stress tolerance, yield and crop quality in the presence of sufficient Pi (Estrada-Ortiz et al., 2011, 2012; Rossall et al., 2016).

Previously, Fallovo et al. (2009, 2011) reported that N supply and PAR differentially affect phytochemical composition of *Brassica* species. For instance, when N was supplied as 100% NH_4^+ under medium PAR (i.e., 6.8 mol m^{-2} day^{-1}), the highest concentration of GLs as well as high levels of carotenoids in the leaves of both *Brassica* species were observed. However, the 100% NH_4^+ supply under low (5.0 mol · m^{-2} · day^{-1}) and medium (6.8 mol · m^{-2} · day^{-1}) PAR levels resulted in low concentrations of flavonoids. Our results are in accordance to previously reported studies, and importantly, they are supported by strict statistical analyses and mean comparisons.

The light activation of nitrate reductase occurs at both the transcriptional and posttranslational levels (Lillo and Appenroth, 2001). Since nitrate reductase catalyzes the reduction of nitrate into nitrite, a higher activity of this enzyme is expected to lower the levels of nitrate in plant tissues. While light renders nitrate reductase active, darkness results in inactivation of this enzyme (Lillo et al., 2004). Under our experimental conditions, PAR significantly affected the foliar nitrate concentrations in *B. juncea*; when PAR level increased, the nitrate concentrations in plant tissues decreased (**Figure 4A**), which indicates that a higher PAR induced a stronger activity of nitrate reductase. According to Fallovo et al. (2009), in *B. rapa* subsp. *nipposinica* var. *chinoleifera* and *B. juncea* exposed to three different PAR treatments (5.0 mol m^{-2} d^{-1}, 6.8 mol m^{-2} d^{-1} and 9.0 mol m^{-2} d^{-1}), low and high PAR levels increased the nitrate concentration in leaves of both crops compared to medium PAR level.

Nitrogen can increase P uptake in plants leading to a positive interaction between N and P nutrition (Fageria, 2001). In tomato (*S. lycopersicum*), N concentration in plant tissues decreased with increasing P limitation induced by the addition of Phi (de Groot et al., 2003). It is well documented that Phi is able to disrupt Pi-starvation responses in plants (Ticconi et al., 2001; Varadarajan et al., 2002), which may explain the results observed in tomato. Indeed, Phi prevents the activation of many genes involved in Pi-starvation responses thus altering P nutrition.

According to Danova-Alt et al. (2008), Phi inhibits Pi uptake in a competitive manner and induces a range of physiological and developmental responses by altering the homeostasis of Pi (Kobayashi et al., 2006; Berkowitz et al., 2013). In turn, Phi uptake is strongly and competitively inhibited in the presence of Pi (Pratt et al., 2009; Jost et al., 2015). Under our experimental conditions, *B. campestris* plants exposed to medium Phi level only exhibited negative effects on nitrate concentration, in comparison to plants exposed to low Phi level. Nonetheless, there were no significant differences between plants exposed to low and high Phi concentrations (**Figure 4B**). Conversely, in oat (*Avena sativa*), Phi did not impair N status, though it did reduce plant growth as well as magnesium and sulfur nutrition in a more pronounced manner than Pi-deficiency, which suggests toxic effects of Phi itself (Zambrosi, 2016).

In *B. campestris*, the PAR × Pi interaction showed that PAR significantly affected the concentrations of nitrate in plant tissues, independent of the Pi level supplied in the nutrient solution (**Figure 5**), which confirms the direct effect of PAR on the induction of the activity of the nitrate reductase enzyme (Gómez et al., 1998).

In apple (*Malus domestica*) trees, the supply of high N concentrations reduced flavonoid production in leaves, which was attributed to a decrease the enzymatic activity of phenylalanine ammonia lyase (PAL) when N availability increases, thus causing the decrease in flavonoid concentration. PAL is the first enzyme of the phenylpropanoid pathway, which provides one of the precursors for flavonoid formation and is responsible for the conversion of phenylalanine to cinnamic acid (Strissel et al., 2005). Likewise, in *B. campestris*, high nitrate concentrations in leaves were associated with a decrease in the flavonoid concentration, though the coefficient of determination was too low to certainly attribute the decrease in flavonoids to the nitrate present in leaves. In *B. juncea*, flavonoids were not affected by the increase in leaf nitrate concentration because the regression analyses did not render significant results for the relationship (**Figure 6**).

We observed a greater diversity of GLs in *B. campestris* in comparison to *B. juncea* (**Figure 7**). In total, two alkyl-GLs (4-methylsulfinylbutyl- and 5-methylsulfinylpentyl-GL), three alkenyl-GLs (but-3-enyl-, pent-4-enyl-, and 2-hydroxybut-3-enyl-GL), an aryl-GL (2-phenylethyl-GL) and four indole-GLs (indol-3-ylmethyl-, 4-hydroxyindol-3-ylmethyl-, 4-methoxyindol-3-ylmethyl-, and 1-methoxyindol-3-ylmethyl-GL) were identified in the former species. Nonetheless, *B. juncea* displayed 50% higher concentrations of GLs than *B. campestris* did.

Alkenyl-GLs (also known as aliphatic GLs) belong to the most abundant group of GLs found in *B. juncea* (**Figure 8B**). Among this group, prop-2-enyl-GL was found in the greatest concentration (**Figure 7B**). Coincidentally, Fallovo et al. (2011) and Tong et al. (2014) also reported that 90% of total GLs identified in plant tissues corresponded to alkenyl-GLs. Under our experimental conditions, the high PAR level rendered nearly double the concentrations of GLs reported by Fallovo et al. (2011) in *B. juncea*, with PAR levels of 5.0, 6.8, and 9.0 mol m^{-2} (**Figures 7B**, **8B**).

In *B. campestris*, the most abundant GL was but-3-enyl-GL, which belongs to the group of alkenyl-GLs, followed by the indole-GLs, alkyl-GLs and finally the aryl-GL, 2-phenylethyl-GL (**Figures 7A**, **8B**). In several subspecies belonging to *B. campestris*, but-3-enyl-GL (gluconapin) has been reported as the most abundant aliphatic GL (Chen et al., 2008), which is in full agreement with our results. Similarly, in *B. campestris* subsp. *pekinensis*, Verkerk et al. (2009) reported that pent-4-enyl-GL (glucobrassicanapin) is the most abundant of the aliphatic GLs.

In both species, PAR significantly affected GL concentrations (**Table 2**). Both in *B. campestris* and *B. juncea*, the higher PAR level evaluated increased the concentrations of individual (**Figure 7**) and total GLs (**Figure 8**). Low PAR levels reduce the GL concentrations due to a decrease in the enzyme flavin-containing monooxygenase, which catalyzes the formation of aliphatic aldoxime, a key compound in the formation of aliphatic GLs (Wallsgrove and Bennet, 1995). In canola (*B. napus*), it has also been reported that reduced PAR results in a decrease in the GL concentration (Wallsgrove and Bennet, 1995). Moreover, in *Arabidopsis thaliana*, the levels of GLs and glutathione were found to be higher during the day than during the night, which coincides with the variation of sulfur uptake as well as the activity of the key enzyme of the sulfur assimilation pathway, adenosine 5′-phosphosulfate reductase (APR) (Huseby et al., 2013). Similarly, broccoli (*B. oleracea*) sprouts grown in the light synthesized 33% more GLs in comparison to sprouts grown in the darkness (Pérez-Balibrea et al., 2008), which further demonstrates that light stimulates GLs biosynthesis in *Brassica* species. Coincidentally, during seedling development of Chinese cabbage (*B. rapa* subsp. *pekinensis*), transcription levels of almost all transcription factors involved in the biosynthesis of GLs (i.e., *Dof1.1*, *IQD1-1*, *MYB28*, *MYB29*, *MYB34*, *MYB51*, and *MYB122*, and their isoforms) under light conditions were higher than under dark conditions, while total GLs contents under light conditions were also higher, which further demonstrates that light affects the levels of GLs (Kim et al., 2014). Conversely, in cabbage (*B. oleracea* var. *capitata*), total and individual GLs in the roots and in the aerial part showed the highest concentrations in the dark cycle, at 02:00 h and 22:00 h, respectively, while the lowest levels were during the light cycle, mainly at 18:00 h. Regardless of the link that seems to exist between light and the biosynthesis of GLs, their total content of GLs often fluctuates more than the gene expression, and elevated levels of GLs can be detected during the dark period when the genes have low expression levels (Rosa, 1997; Klein et al., 2006; Schuster et al., 2006; Huseby et al., 2013).

Phosphate concentration in the nutrient solution significantly affected alkyl-GL concentrations in *B. campestris*. Indeed, in Pi-deficient plants those GLs displayed higher concentrations as compared with Pi-sufficient plants (**Figures 7A**, **8A**). This trend was also observed in *B. juncea* when significant effects of Pi on GL biosynthesis were detected (**Figures 7B**, **8B**). Coincidentally, in rocket salad (*Eruca sativa*), Chun et al. (2017) found that applications of N and P at low concentrations, or higher concentrations of potassium enhanced the synthesis of total GLs; in particular 5 and 2 mM N and P possessed much higher levels of several types of aliphatic GLs than

other nutrient concentrations tested. On the contrary, in yellow mustard (*Sinapis alba*) and oilseed radish (*Raphanus sativa*), P effects on GLs producing ionic or isothiocyanates were relatively insignificant (Brown et al., 2008).

Under our experimental conditions, high levels of Phi increased alkyl-GLs in *B. campestris* (**Figures 7A**, **8A**), while in *B. juncea* the highest concentration of Phi tested enhanced the concentration of but-3-enyl-GL (alkenyl-GL) (**Figure 7B**). This confirms that Phi and similar biostimulants can be used to enhance bioactive compounds as GLs (Gómez-Merino and Trejo-Téllez, 2015, 2016), contributing to provide horticultural crops rich in bioactive compounds imparting health benefits for the consumer. Nowadays, nutraceutics are becoming more significant for human health (Singh et al., 2017).

Pi deficiency at a light intensity of 640 μmol m^{-2} s^{-1}, increase GL concentrations, particularly of aliphatic- and indole-GLs, while the concentrations of free amino acids was increased by supplying the plant with low Pi concentrations (Yang et al., 2009). Among the increased amino acids is methionine, which is a precursor of aliphatic-GLs; tyrosine and phenylalanine also increased and function as precursors of aromatic-GLs. Interestingly, Ciereszko et al. (2005) reported an additive effect between Pi deficiency and light/dark conditions in gene expression, enhancing UDPG pyrophosphorylase activity and sugars concentrations, especially in Pi-deficient plants. During the GL biosynthesis, the thiohydroximic acid is released from the *S*-alkylthiohydroximate by action of a cysteine (C-S) lyase; the thiohydroximic acid is glucosylated by the action of UDPG, thus forming desulfo-glucosinolates (Wittstock and Halkier, 2002). Consequently, the accumulation of GLs stimulated by Phi observed in *B. juncea* may be a response to Pi deficiency (**Table 3**).

Summarizing, as a biostimulant, Phi has been proved to enhance not only production and productivity of diverse crops, but also the quality of products. In peaches, Phi enhanced both sugar content and soluble solid content, while in raspberry, Phi improved fruit firmness (an invaluable commercial trait leading to premium pricing of the product) (Achary et al., 2017). Nonetheless, precise studies on the economic trade-offs are lacking. Importantly, no negative effects on taste and odor of plants and plant products have been found in response to the application of Phi at rates not higher than 4.0 L ha^{-1} (content of active ingredient: nominal 504 g L^{-1} phosphonic acid equivalents) (Evaluator, 2017). However, sensory profiles of Phi-treated Brassicas remain a daunting task.

CONCLUSION

It was observed in this study that Pi deficiency has a positive effect on the accumulation of some flavonoids and GLs, mainly under higher PAR; it was also observed that a lower PAR level tend to decrease flavonoid and GL concentrations. Since Phi is not metabolized by the plant, applying it in the nutrient solution tends to increase Pi deficiency; therefore, it favors the increase of some flavonoids and GLs as a possible defense mechanism for coping with stress. However, a balanced application of Pi and Phi to enhance flavonoid and GLs may be difficult because it conflicts with an adequate yield in horticultural production, if not properly scheduled to fulfill the Pi requirements of crop plants in order to positively stimulate physiological processes. Since a number of transcription factors and mRNAs have proved to be involved in the biosynthesis of GLs, their activity should be measured in future studies in order to analyze their activity in response to PAR, Pi, Phi and their interactions. Moreover, how such genes interact with other molecules (i.e., other genes, transcripts, proteins, or metabolites) to regulate the biosynthesis and degradation of flavonoids and nitrate in response to PAR, Pi, and Phi changes remains to be elucidated. New innovative techniques and their use in omics research (e.g., genomics, transcriptomics, metabolomics, proteomics, and interactomics) will be of paramount importance in achieving this goal and improving nutraceutic quality in *Brassica* species.

AUTHOR CONTRIBUTIONS

DS and LT-T designed the study. EE-O performed the experiments in greenhouse and the measurements in laboratory. LT-T carried out the statistical analyses and wrote the first draft of the manuscript. FG-M provided inputs for the study and edited the manuscript. AK and CB performed part of the analyses in laboratory and revised and edited the manuscript. All authors have given final approval for this version of the manuscript.

FUNDING

We are grateful to the German Academic Exchange Service (DAAD Germany, Project-lD 544462) and the National Council of Science and Technology (CONACYT Mexico, Project-ID 166022) for funding the bilateral project "Phosphite in leafy vegetables." The funders had no role in study design, data collection and analysis, decision to publish, or preparation of the manuscript.

SUPPLEMENTARY MATERIAL

The Supplementary Material for this article can be found online at: https://www.frontiersin.org/articles/10.3389/fpls.2019.00371/full#supplementary-material

REFERENCES

Achary, V. M. M., Ram, B., Manna, M., Datta, D., Bhatt, A., Reddy, M. K., et al. (2017). Phosphite: a novel P-fertilizer for weed management and pathogen control. *Plant Biotechnol. J.* 15, 1493–1508. doi: 10.1111/pbi.12803

Agati, G., and Tattini, M. (2010). Multiple functional roles of flavonoids in photoprotection. *New Phytol.* 186, 786–793. doi: 10.1111/j.1469-8137.2010.03269.x

Bariola, P. A., MacIntosh, G. C., and Green, P. J. (1999). Regulation of S-like ribonuclease levels in Arabidopsis. Antisense inhibition of RNS1 or RNS2

elevates anthocyanin accumulation. *Plant Physiol.* 119, 331–342. doi: 10.1104/pp.119.1.331

Berkowitz, O., Jost, R., Kollehn, D. O., Fenske, R., Finnegan, P. M., O'Brien, P. A., et al. (2013). Acclimation responses of *Arabidopsis thaliana* to sustained phosphite treatments. *J. Exp. Bot.* 64, 1731–1743. doi: 10.1093/jxb/ert037

Brown, B. D., Gibson, R. C., Geary, B., and Morra, M. J. (2008). Biofumigant biomass, nutrient content, and glucosinolate response to phosphorus. *J. Plant Nutr.* 31, 743–752. doi: 10.1080/01904160801926848

Calabrese, E. J., and Blain, R. B. (2009). Hormesis and plant biology. *Eviron. Pollut.* 157, 42–48. doi: 10.1016/j.envpol.2008.07.028

Cao, L., Liu, B., Li, J., Yu, N., Zou, X., and Chen, L. (2015). Light- and temperature-regulated BjAPY2 may have a role in stem expansion of *Brassica juncea*. *Func. Integr. Genomics* 15, 753–762. doi: 10.1007/s10142-015-0458-8

Chen, X., Zhu, Z., Gerendás, J., and Zimmermann, N. (2008). Glucosinolates in Brassica campestris vegetables: Chinese cabbage, purple cai-tai, choysum, pakchoi, and turnip. *HortScience* 43, 571–574. doi: 10.21273/HORTSCI.43.2.571

Chun, J. H., Kim, S., Arasu, M. A., Al-Dhabi, N. A., Chung, D. Y., and Kim, S. J. (2017). Combined effect of nitrogen, phosphorus and potassium fertilizers on the contents of glucosinolates in rocket salad (*Eruca sativa* Mill.). *Saudi J. Biol. Sci.* 24, 436–443. doi: 10.1016/j.sjbs.2015.08.012

Ciereszko, I., Johansson, H., and Kleczkowski, L. A. (2005). Interactive effects of phosphate deficiency, sucrose and light/dark conditions and gene expression of UDP-glucose pyrophosphorylase in Arabidopsis. *J. Plant Physiol.* 162, 343–353. doi: 10.1016/j.jplph.2004.08.003

Crozier, A., Clifford, M. N., and Ashihara, H. (2006). *Plant Secondary Metabolites: Occurrence, Structure and Role in the Human Diet*. Oxford: Blackwell Publishing, 372. doi: 10.1002/9780470988558

Danova-Alt, R., Dijkema, C., Waard, P. D. E., and Köch, M. (2008). Transport and compartmentation of phosphite in higher plant cells. Kinetic and nuclear magnetic resonance studies. *Plant Cell Environ.* 31, 1510–1521. doi: 10.1111/j.1365-3040.2008.01861.x

de Groot, C. C., Marcelis, L. F. M., van den Boogaard, R., Kaiser, W. M., and Lambers, H. (2003). Interaction of nitrogen and phosphorus nutrition in determining growth. *Plant Soil* 248, 257–268. doi: 10.1023/A:1022323215010

Debiasi, C., Zaffari, G. R., and Guerra, M. P. (2003). Effect of photosynthetically active radiation on the in vitro initial development of banana cultures. *Rev. Bras. Agroc.* 9, 175–176. doi: 10.18539/CAST.V9I2.518

Diez, C., Moreno, F., and Gantiva, E. (2017). Effects of light intensity on the morphology and CAM photosynthesis of *Vanilla planifolia* Andrews. *Rev. Fac. Nac. Agron. Medellín* 70, 8023–8033. doi: 10.15446/rfna.v70n1.61736

Estrada-Ortiz, E., Trejo-Téllez, L. I., Gómez-Merino, F. C., Núñez-Escobar, R., and Sandoval-Villa, M. (2011). Biochemical responses in strawberry plants supplying phosphorus in the form of phosphite. *Rev. Chapingo Ser. Hortic.* 17, 129–138. doi: 10.5154/r.rchsh.2011.17.024

Estrada-Ortiz, E., Trejo-Téllez, L. I., Gómez-Merino, F. C., Núñez-Escobar, R., and Sandoval-Villa, M. (2013). The effects of phosphite on strawberry yield and fruit quality. *J. Soil Sci. Plant Nutr.* 13, 612–620. doi: 10.4067/S0718-95162013005000049

Estrada-Ortiz, E., Trejo-Téllez, L. I., Gómez-Merino, F. C., Silva-Rojas, H. V., Castillo-González, A. M., and Avitia-García, E. (2016). Physiological responses of chard and lettuce to phosphite supply in nutrient solution. *J. Agric. Sci. Technol.* 18, 1079–1090.

Estrada-Ortiz, E., Trejo-Teéllez, L. I., Goómez-Merino, F. C., Nuúñez-Escobar, R., and Sandoval- Villa, M. (2012). Phosphite on growth and fruit quality in strawberry. *Acta Hortic.* 947, 277–282. doi: 10.17660/ActaHortic.2012.947.35

Evaluator, D. E. (2017). *Registration Report Part A. Risk Management. Product Code LBG-01F34 / Veriphos. Active substance Potassium phosphonates 755 g/L*. Available at: https://www.bvl.bund.de/SharedDocs/Downloads/04_Pflanzenschutzmittel/01_zulassungsberichte/027207-00-00.html

Fageria, V. D. (2001). Nutrient interactions in crop plants. *J. Plant Nutr.* 24, 1269–1290. doi: 10.1081/PLN-100106981

Fallovo, C., Colla, G., Schreiner, M., Krumbein, A., and Schwarz, D. (2009). Effect of nitrogen form and radiation on growth and mineral concentration of two Brassica species. *Sci. Hortic.* 123, 170–177. doi: 10.1016/j.scienta.2009.09.003

Fallovo, C., Schreiner, M., Schwarz, D., Colla, G., and Krumbein, A. (2011). Phytochemical changes induced by different nitrogen supply forms and radiation levels in two leafy Brassica species. *J. Agric. Food Chem.* 59, 4198–4207. doi: 10.1021/jf1048904

Ghasemzadeh, A., Jaafar, H. Z. E., and Rahmat, A. (2010). Synthesis of Phenolics and Flavonoids in ginger (Zingiber officinale Roscoe) and their effects on photosynthesis rate. *Int. J. Mol. Sci.* 11, 4539–4555. doi: 10.3390/ijms11114539

Ghosh, A. P., Dass, A., Krishnan, P., Kaur, R., and Rana, K. S. (2017). Assessment of photosynthetically active radiation, photosynthetic rate, biomass and yield of two maize varieties under varied planting dates and nitrogen application. *J. Environ. Biol.* 38, 683–688. doi: 10.22438/jeb/38/4/MS-222

Gómez, I., Pérez-Rodríguez, E., Viñegla, B., Figueroa, F. L., and Karsten, U. (1998). Effects of solar radiation on photosynthesis, UV-absorbing compounds and enzyme activities of the green alga Dasycladus vermicularis from southern Spain. *J. Photochem. Photobiol. B Biol.* 47, 46–57. doi: 10.1016/S1011-1344(98)00199-7

Gómez-Merino, F. C., and Trejo-Téllez, L. I. (2015). Biostimulant activity of phosphite in horticulture. *Sci. Hortic.* 196, 82–90. doi: 10.1016/j.scienta.2015.09.035

Gómez-Merino, F. C., and Trejo-Téllez, L. I. (2016). Conventional and novel uses of phosphite in horticulture: potentialities and challenges. *Italus Hortus* 23, 1–13.

Gould, K. S., and Lister, C. (2006). "Flavonoid functions in plants," in *Flavonoids: Chemistry, Biochemistry and Applications*, eds Ø Andersen and K. Markham (Boca Raton, FL: CRC Press), 397–441.

Herrera-Estrella, L., and López-Arredondo, D. (2016). Phosphorus: the underrated element for feeding the world. *Trends Plant Sci.* 21, 461–463. doi: 10.1016/j.tplants.2016.04.010

Huseby, S., Koprivova, A., Lee, B. R., Saha, S., Mithen, R., Wold, A. B., et al. (2013). Diurnal and light regulation of sulphur assimilation and glucosinolate biosynthesis in Arabidopsis. *J. Exp. Bot.* 64, 1039–1048. doi: 10.1093/jxb/ers378

Jost, R., Pharmawati, M., Lapis-Gaza, H. R., Rossig, C., Berkowitz, O., Lambers, H., et al. (2015). Differentiating phosphate-dependent and phosphate-independent systemic phosphate-starvation response networks in *Arabidopsis thaliana* through the application of phosphite. *J. Exp. Bot* 66, 2501–2514. doi: 10.1093/jxb/erv025

Kim, Y. B., Chun, J. H., Kim, H. R., Kim, S. J., Lim, Y. P., and Park, S. U. (2014). Variation of glucosinolate accumulation and gene expression of transcription factors at different stages of Chinese cabbage seedlings under light and dark conditions. *Nat. Prod. Commun.* 9, 533–537.

Klein, M., Reichelt, M., Gershenzo, J., and Papenbrock, J. (2006). The three desulfoglucosinolate sulfotransferase proteins in Arabidopsis have different substrate specificities and are differentially expressed. *FEBS J.* 273, 122–136. doi: 10.1111/j.1742-4658.2005.05048.x

Knekt, A., Kumpulainen, J., Järvinen, R., Rissanen, H., Heliövaara, M., Reunanen, A., et al. (2002). Flavonoid intake and risk of chronic diseases. *Am. J. Clin. Nutr.* 76, 560–568. doi: 10.1093/ajcn/76.3.560

Kobayashi, K., Masuda, T., Takamiya, K., and Ohta, H. (2006). Membrane lipid alteration during phosphate starvation is regulated by phosphate signaling and auxin/cytokinin cross-talk. *Plant J.* 47, 238–248. doi: 10.1111/j.1365-313X.2006.02778.x

Krumbein, A., Schonhof, I., and Schreiner, M. (2005). Composition and contents of phytochemicals (glucosinolates, carotenoids and chlorophylls) and ascorbic acid in selected Brassica species (*B. juncea, B. rapa subsp. nipposinica var. chinoleifera, B. rapa subsp. chinensis and B. rapa subsp. rapa*). *J. Appl. Bot. Food Qual.* 79, 168–174.

Lee, E. R., Kang, G. H., and Cho, S. G. (2007). Effect of flavonoids on human health: old subjects but new challenges. *Recent. Pat. Biotechnol.* 1, 139–150. doi: 10.2174/187220807780809445

Lillo, C., and Appenroth, K. J. (2001). Light regulation of nitrate reductase in higher plants: Which photoreceptors are involved? *Plant Biol.* 3, 455–465. doi: 10.1055/s-2001-17732

Lillo, C., Meyer, C., Lea, U. S., Provan, F., and Oltedal, S. (2004). Mechanism and importance of post-translational regulation of nitrate reductase. *J. Exp. Bot.* 55, 1275–1282. doi: 10.1093/jxb/erh132

López-Arredondo, D. L., Leyva-González, M. A., González-Morales, S. I., López-Bucio, J., and Herrera-Estrella, L. (2014). Phosphate nutrition: improving low-phosphate tolerance in crops. *Annu. Rev. Plant Biol.* 65, 95–123. doi: 10.1146/annurev-arplant-050213-035949

Lunde, C., Zygadlo, A., Simonsen, H. T., Nielsen, P. L., Blennow, A., and Haldrup, A. (2008). Sulfur starvation in rice: the effect on photosynthesis, carbohydrate metabolism, and oxidative stress protective pathways. *Physiol. Plant* 134, 508–521. doi: 10.1111/j.1399-3054.2008.01159.x

Malusà, E., Russo, M. A., Mozzetti, C., and Belligno, A. (2006). Modification of secondary metabolism and flavonoid biosynthesis under phosphate deficiency in bean roots. *J. Plant Nutr.* 29, 245–258. doi: 10.1080/01904160500474090

McDonald, A. E., Grant, B. R., and Plaxton, W. C. (2001). Phosphite (phosphorous acid): its relevance in the environment and agriculture and influence on plant phosphate starvation response. *J. Plant Nutr.* 24, 1505–1519. doi: 10.1081/PLN-100106017

Mejía-Montoya, J. W., Cartagena-Valenzuela, J. R., and Riaño-Herrera, N. M. (2013). Morphometric and productive characterization of nineteen genotypes from the Colombian Coffea collections. *Rev. Fac. Nac. Agron. Medellín* 66, 7021–7034.

Misson, J., Raghothama, K. G., Jain, A., Jouhet, J., Block, M. A., Bligny, R., et al. (2005). A genome-wide transcriptional analysis using *Arabidopsis thaliana* Affymetrix gene chips determined plant responses to phosphate deprivation. *Proc. Natl. Acad. Sci. U.S.A.* 102, 11934–11939. doi: 10.1073/pnas.0505266102

Neugart, S., Krumbein, A., and Zrenner, R. (2016). Influence of light and temperature on gene expression leading to accumulation of specific flavonol glycosides and hydroxycinnamic acid derivatives in kale (*Brassica oleracea* var. sabellica). *Front. Plant Sci.* 7:326. doi: 10.3389/fpls.2016.00326

O'Connor, S. E. (2015). Engineering of secondary metabolism. *Annu. Rev Genet.* 49, 71–94. doi: 10.1146/annurev-genet-120213-092053

Oyarburo, N. S., Machinandiarena, M. F., Feldman, M. L., Daleo, G. R., Andreu, A. B., and Oliveiri, F. P. (2015). Potassium phosphite increases tolerance to UV-B in potato. *Plant Physiol. Biochem.* 88, 1–8. doi: 10.1016/j.plaphy.2015.01.003

Pant, B. D., Pant, P., Erban, A., Huhman, D., Kopka, J., and Scheible, W. R. (2015). Identification of primary and secondary metabolites with phosphorus status-dependent abundance in Arabidopsis, and of the transcription factor PHR1 as a major regulator of metabolic changes during phosphorus limitation. *Plant Cell Environ.* 38, 172–187. doi: 10.1111/pce.12378

Pazuki, A., Aflaki, F., Pessarakli, M., Gurel, E., and Gurel, S. (2017). Plant responses to extended photosynthetically active radiation (EPAR). *Adv. Plants Agric. Res.* 7, 313–318. doi: 10.15406/apar.2017.07.00260

Peer, W. A., and Murphy, A. S. (2007). Flavonoids and auxin transport: modulators or regulators? *Trends Plant Sci.* 12, 556–563. doi: 10.1016/j.tplants.2007.10.003

Pérez-Balibrea, S., Moreno, D. A., and García-Viguera, C. (2008). Influence of light on health promoting phytochemicals of broccoli sprouts. *J. Sci. Food Agric.* 88, 904–910. doi: 10.1002/jsfa.3169

Plate, A., and Gallaher, D. (2006). Effects of indole-3-carbinol and phenethyl isothiocyanate on colon carcinogenesis induced by azoxymethane in rats. *Carcinogenesis* 27, 287–292. doi: 10.1093/carcin/bgi210

Pratt, J., Boisson, A. M., Gout, E., Bligny, R., Douce, R., and Aubert, S. (2009). Phosphate (Pi) starvation effect on the cytosolic Pi concentration and Pi exchanges across the tonoplast in plant cells. An *in vivo* 31P-NMR study using methyl-phosphonate as a Pi analogue. *Plant Physiol.* 151, 1646–1657. doi: 10.1104/pp.109.144626

Ramezani, M., Abdolmaleki, M. K., Shabani, S., and Dehestani, A. (2017). The role of potassium phosphite in chlorophyll fluorescence and photosynthetic parameters of downy mildew-challenged cucumber *Cucumis sativus* plants. *Arch. Phytopathol. Plant Prot.* 50, 927–940. doi: 10.1080/03235408.2017.1407470

Ratjen, A. M., and Gerendas, J. (2009). A critical assessment of the suitability of phosphite as a source of phosphorus. *J. Plant Nutr. Soil Sci.* 172, 821–828. doi: 10.1002/jpln.200800287

Roland, W. S., van Buren, L., Gruppen, H., Driesse, M., Gouka, R. J., Smit, G., et al. (2013). Bitter taste receptor activation by flavonoids and isoflavonoids: modeled structural requirements for activation of hTAS2R14 and hTAS2R39. *J. Agric. Food Chem.* 61, 10454–10466. doi: 10.1021/jf403387p

Rosa, E. A. S. (1997). Daily variation in glucosinolate concentrations in the leaves and roots of cabbage seedlings in two constant temperature regimes. *J. Sci. Food Agric.* 73, 364–368. doi: 10.1002/(SICI)1097-0010(199703)73:3<364::AID-JSFA742>3.0.CO;2-O

Rossall, S., Qing, C., Paneri, M., Bennett, M., and Swarup, R. (2016). A 'growing' role for phosphites in promoting plant growth and development. *Acta Hortic.* 1148, 61–68. doi: 10.17660/ActaHortic.2016.1148.7

Santamaria, P. (2005). Nitrate in vegetables: toxicity, content, intake and EC regulation. *Sci. Food Agric.* 86, 10–17. doi: 10.1002/jsfa.2351

SAS (2011). *SAS/STAT Users Guide. Version* 9.3. Cary, NC: SAS Institute Inc., 2340.

Schonhof, I., Blankenburg, D., Müller, S., and Krumbein, A. (2007). Sulfur and nitrogen supply influence growth, product appearance, and glucosinolate concentration of broccoli. *J. Plant Nutr. Soil Sci.* 170, 65–72. doi: 10.1002/jpln.200620639

Schuster, J., Knill, T., Reichelt, M., Gershenzon, J., and Binder, S. (2006). BRANCHED-CHAIN AMINOTRANSFERASE4 is part of the chain elongation pathway in the biosynthesis of methionine-derived glucosinolates in Arabidopsis. *Plant Cell* 18, 2664–2679. doi: 10.1105/tpc.105.039339

Singh, D., Sivalingam, P. N., Acharyya, P., and Meena, S. R. (2017). *Nutraceutical Values of Horticultural Crops and Products*. New Delhi: New India Publishing Agency, 274.

Stewart, A. J., Chapman, W., Jenkins, G. I., Graham, I., Martin, T., and Crozier, A. (2001). The effect of nitrogen and phosphorus deficiency on flavonol accumulation in plant tissues. *Plant Cell Environ.* 24, 1189–1197. doi: 10.1046/j.1365-3040.2001.00768.x

Stoin, D., Radu, F., and Dogaru, D. (2007). Researches regarding the isolation, purification and analysis of sinigrin glucosinolate from *Brassica nigra* and *Armoracia rusticana*. *Bull. USAMV Agric.* 63, 77–82.

Strissel, T., Halbwirth, H., Hoyer, U., Zistler, C., Stich, K., and Treutter, D. (2005). Growth-promoting nitrogen nutrition affects flavonoid biosynthesis in young apple (Malus domestica Borkh.) leaves. *Plant Biol.* 7, 677–685. doi: 10.1055/s-2005-872989

Thao, H. T. B., Yamakawa, T., and Shibata, K. (2009). Effect of phosphite-phosphate interaction on growth and quality of hydroponic lettuce (*Lecuca sativa*). *J. Plant Nutr. Soil Sci.* 172, 378–384. doi: 10.1002/jpln.200800192

Thao, H. T. B., Yamakawa, T., Shibata, K., Sarr, P. S., and Myint, A. K. (2008). Growth response of komatsuna (*Brassica rapa* var. peruviridis) to root and foliar applications of phosphite. *Plant Soil* 308, 1–10. doi: 10.1007/s11104-008-9598-0

Ticconi, C. A., Delatorre, C. A., and Abel, S. (2001). Attenuation of phosphate starvation responses by phosphite in Arabidopsis. *Plant Physiol.* 127, 963–972. doi: 10.1104/pp.010396

Tong, Y., Gabriel-Neumann, E., Ngwene, B., Krumbein, A., George, E., Platz, S., et al. (2014). Topsoil drying combined with increased sulfur supply leads to enhanced aliphatic glucosinolates in *Brassica juncea* leaves and roots. *Food Chem.* 152, 190–196. doi: 10.1016/j.foodchem.2013.11.099

Trejo-Téllez, L. I., and Gómez-Merino, F. C. (2018). "Phosphite as an inductor of adaptive responses to stress and stimulator of better plant performance," in *Biotic and Abiotic Stress Tolerance in Plants*, ed. S. Vats (Puducherry: Springer Nature), 203–238. doi: 10.1007/978-981-10-9029-5_8

Varadarajan, D. K., Karthikeyan, A. S., Matilda, P. D., and Raghothama, K. G. (2002). Phosphite, an analog of phosphate, suppresses the coordinated expression of genes under phosphate starvation. *Plant Physiol.* 129, 1232–1240. doi: 10.1104/pp.010835

Vargas-Hernández, M., Macías-Bobadilla, I., Guevara-González, R. G., Romero-Gómez, S. J., Rico-García, E., Ocampo-Velázquez, R. V., et al. (2017). Plant hormesis management with biostimulants of biotic origin in agriculture. *Front. Plant Sci.* 8:1762. doi: 10.3389/fpls.2017.01762

Ventorim, P. F., Caldeira, A. I. C., Ribeiro, J. P. M., Brasil, P. P. J. E., de Paula, A. R., and Alves, A. A. (2014). Growth and production of secondary compounds in monkey-pepper (*Piper aduncum* L.) leaves cultivated under altered ambient light. *Austr. J. Crop Sci.* 8, 1510–1516.

Verkerk, R., Schreiner, M., Krumbein, A., Ciska, E., Holst, B., Rowland, I., et al. (2009). Glucosinolates in Brassica vegetables: the influence of the food supply chain on intake, bioavailability and human health. *Mol. Nutr. Food Res.* 53, S219–S265. doi: 10.1002/mnfr.200800065

Wallsgrove, R. M., and Bennet, R. N. (1995). "The biosynthesis of glucosinolates in Brassicas," in *Amino Acids and their Derivates in Higher Plants*, ed. R. M. Wallsgrove (Cambridge: Cambridge University Press), 243–259. doi: 10.1017/CBO9780511721809.016

Williams, R. J., Spencer, J. P. E., and Rice-Evans, C. (2004). Flavonoids: antioxidants or signalling molecules? *Free Rad. Biol. Med.* 36, 838–849. doi: 10.1016/j.freeradbiomed.2004.01.001

Wittstock, U., and Halkier, B. A. (2002). Glucosinolate research in the Arabidopsis era. *Trends Plant Sci.* 7, 263–270. doi: 10.1016/S1360-1385(02)02273-2

Yang, J., Zhu, Z., and Gerendás, J. (2009). Interactive effects of phosphorus supply and light intensity on glucosinolates in pakchoi (*Brassica campestris* L. ssp.

chinensis var. communis). *Plant Soil* 323, 323–333. doi: 10.1007/s11104-009-9940-1

Yusoff, A. M., and Zainol, A. A. (1989). Interception of photosynthetically active radiation of some Malaysian rice genotypes. *MARDI Res. J.* 17, 178–185.

Zambrosi, F. C. B. (2016). Phosphite and phosphate have contrasting effects on nutrient status of plants. *J. Crop Improv.* 30, 421–432. doi: 10.1080/15427528.2016.1173611

Zambrosi, F. C. B., Mattos, J. D., and Syvertsen, J. P. (2011). Plant growth, leaf photosynthesis, and nutrient-use efficiency of citrus rootstocks decrease with phosphite supply. *J. Plant Nutr. Soil Sci.* 174, 487–495. doi: 10.1002/jpln.v174.3

Zhu, H., Li, X., Zhai, W., Liu, Y., Gao, Q., Liu, J., et al. (2017). Effects of low light on photosynthetic properties, antioxidant enzyme activity, and anthocyanin accumulation in purple pak-choi (*Brassica campestris* ssp. Chinensis Makino). *PLoS One* 12:e0179305. doi: 10.1371/journal.pone.0179305

Zimmermann, N., Gerendás, J., and Krumbein, A. (2007). Identification of desulphoglucosinolates in Brassicaceae by LC/MS/MS: comparison of electrospray ionisation and atmospheric pressure chemical ionisation mass spectrometry. *Mol. Nutr. Food Res.* 51, 1537–1546. doi: 10.1002/mnfr.200700103

Zoratti, L., Karppinnen, K., Luengo-Escobar, A., Häggman, H., and Jaakola, L. (2014). Light-controlled flavonoid biosynthesis in fruits. *Front. Plant Sci.* 5:534. doi: 10.3389/fpls.2014.00534

Conflict of Interest Statement: The authors declare that the research was conducted in the absence of any commercial or financial relationships that could be construed as a potential conflict of interest.

frontiers in Plant Science

POLICY AND PRACTICE REVIEWS
published: 16 April 2019
doi: 10.3389/fpls.2019.00494

General Principles to Justify Plant Biostimulant Claims

*Manuele Ricci[1], Lorraine Tilbury[2], Bruno Daridon[3] and Kristen Sukalac[2]**

[1] Independent Researcher, Perugia, Italy, [2] European Biostimulants Industry Council, Prospero & Partners, Antwerp, Belgium, [3] Olmix Group, Bréhan, France

The forthcoming European Union (EU) Fertilizing Products Regulation[1] proposes a claim-based definition of plant biostimulants, stipulating that "plant biostimulant" means a product stimulating plant nutrition processes independently of the product's nutrient content, with the aim of improving one or more of the following characteristics of the plant: nutrient use efficiency, tolerance to abiotic stress, crop quality traits or availability of confined nutrients in the soil and rhizosphere. The future regulation also specifies that a plant biostimulant "shall have the effects that are claimed on the label for the plants specified thereon." This creates an onus for manufacturers to demonstrate to regulators and customers that product claims are justified. Consequently, the justification of the agronomic claim of a given plant biostimulant will be an important element to allow it to be placed on the EU market once this new European regulation is applied. In this article, members of the European Biostimulant Industry Council (EBIC) propose some general guiding principles to follow when justifying plant biostimulant claims, that are outlined in this article. These principles are expected to be incorporated into harmonized European standards that are being developed by the European Committee for Standardization (CEN) to support the implementation of the regulation.

Keywords: plant nutrition, plant biostimulant, agronomic claim, nutrient efficiency, abiotic stress, crop quality, yield, trial design

OPEN ACCESS

Edited by:
Giuseppe Colla,
Università degli Studi della Tuscia, Italy

Reviewed by:
Fabrizio Colosimo,
Ngalab, Spain
Antonio Pannico,
University of Naples Federico II, Italy

***Correspondence:**
Kristen Sukalac
kristen@prospero.ag

Specialty section:
This article was submitted to Crop and Product Physiology, a section of the journal Frontiers in Plant Science

Received: *03 January 2019*
Accepted: *01 April 2019*
Published: *16 April 2019*

Citation:
Ricci M, Tilbury L, Daridon B and Sukalac K (2019) General Principles to Justify Plant Biostimulant Claims. Front. Plant Sci. 10:494. doi: 10.3389/fpls.2019.00494

INTRODUCTION

The forthcoming EU regulation for fertilizing products covers six types of products (fertilizers, liming materials, soil improvers, growing media, inhibitors, and plant biostimulants) as well as combinations of them. The definition used for plant biostimulants is claims-based (European Commission, 2016; Council of the European Union, 2018), meaning that it is the function of the product, not what it contains that defines it as a plant biostimulant. For this reason, demonstrating that a product is indeed a *bona fide* biostimulant depends on a demonstration of its effect. However, this should not be confused with guaranteeing a specific level of efficacy. In no case should the placing of a biostimulant on the EU market be considered to guarantee effectiveness under all conditions, as many factors may influence performance of a biostimulant in the field.

The requirements for claims justification should be proportional to the task; manufacturers should provide enough data to be credible, without the process becoming needlessly burdensome.

[1] At the time of publication, an informal agreement on the new regulation had been reached, but formal validation was still underway. The European Parliament approved the text on 27 March 2019 (European Parliament, 2019), with the Council vote expected by June.

Sound scientific support of product claims is one of the central tenets of the European Biostimulant Industry Council's (EBIC) work, and EBIC has taken inspiration from how other sectors justify product claims. However, existing claim justification protocols for other product categories cannot be automatically applied to biostimulants in a copy/paste manner, for several reasons:

- the diversity of existing biostimulant products (including microbial and non-microbial products) and the subsequent wide range of possible claims;
- the dependence of biostimulant effects on multiple contextual factors, and
- the interactions among biostimulant components as well as between the biostimulant itself and other systemic elements (e.g., weather, soil microbiome, soil type, crop variety, etc.).

In order to promote high-quality data supporting biostimulant claims – whether for placing on the market or for commercial purposes – EBIC engages with key stakeholders in the co-development of guidelines for compiling robust data on biostimulants that favor consistent and reliable results.

European Biostimulant Industry Council has identified a need for guidelines to address two levels of producing scientific argumentation for claims:

(1) Generating data to support a biostimulant claim;
(2) Using data either to support placing a biostimulant on the EU market and/or to support commercial product claims.

How much data is required to support a claim? It depends on the claim. The narrower the claim, the less data should be required. Given the variety of possible effects, crops or crop groupings, and growing conditions, manufacturers need the flexibility to design studies that are adapted to the specific agronomic situation. Furthermore, it should be recognized that when it comes to products that improve the availability of nutrients (notably micro-organisms), soil types and conditions may be more relevant than crop types themselves. Design flexibility is therefore needed to accommodate such cases.

EBIC'S FUNCTIONAL DEFINITION OF BIOSTIMULANTS

European Biostimulant Industry Council uses a functional definition to describe biostimulants that was developed over the course of a year-long consultation process with stakeholders including researchers, regulators and related industry sectors.

"Plant biostimulant means a material which contains substance(s) and/or microorganisms whose function when applied to plants or the rhizosphere is to stimulate natural processes to benefit nutrient uptake, nutrient efficiency, tolerance to abiotic stress, and/or crop quality, independently of its nutrient content."

The definition was crafted to respond to several needs as outlined below:

. . . a material which contains substance(s) and/or microorganisms (including microalgae) (Chiaiese et al., 2018) – Because there are system effects when substances and/or micro-organisms are combined, biostimulants can only be accurately defined and evaluated at the level of the final formulation (notwithstanding incorporation into another product like a fertilizer or a growing medium). Internal surveying of EBIC's members reveals a trend toward complex multi-component products rather than products with only one (or one type) of biostimulant substance (European Biostimulants Industry Council [EBIC], unpublished). Indeed, it is precisely the synergistic effects among different types of biostimulants (microbial/non-microbial, substances of different origins, etc.) allow manufacturers to design and develop efficient plant biostimulant products with specific properties in terms of yield and especially nutritional and functional quality (Krouk, 2015; Rouphael and Colla, 2018).

. . . whose function – EBIC advocates for a functional use definition because this corresponds best to how products are placed on the market and the form in which farmers use them. This is in contrast with a *substance-based definition* that is based on the chemical or biological identity of the components (or of a single component considered in isolation from the others) or with a *mode-of-action definition* that is based on the mechanism through which the effect is obtained. The problem with definitions based on content or mode of action is that more than one effect may be related to an ingredient or a mode of action, and those effects may fall under different regulatory frameworks. *The functional use definition is intimately tied to the claim and use of the product, both of which can be verified through post-marketing surveillance*. Therefore, a functional use definition is the most practical because it provides the information needed by final users and provides a basis for controlling appropriate marketing and sales.

. . . when applied to plants or the rhizosphere –Foliar applications are used for some biostimulants, but others are applied directly to the soil (or other growing medium) or as a seed treatment.

. . . to stimulate natural processes – Many of the processes that biostimulants influence are inside the plant, but others occur in the soil (or other growing medium) around the plant. Soil processes may be particularly relevant for microbial products, lixiviates, humic acids, organic matter derivates, etc. which are often applied to soils rather than for other biostimulants that are applied through foliar application.

. . . to benefit nutrient uptake, nutrient efficiency – Nutrient uptake is related to, but distinct from, nutrient efficiency. The latter covers how well the plant uses a nutrient once it has been made available. The former can include the effects of biostimulants that make nutrients more available, for example through biological fixation of nitrogen or solubilization of phosphorus.

. . . tolerance to abiotic stress – Because biostimulants affect plants' general well-being, it makes them more resilient

to harsh growing conditions. Specific products may also provide the plant with specific tolerance against an abiotic stress, such as drought, salinity or extreme temperatures.

. . . and/or crop quality – Like all products related to plant nutrition and soil fertility, biostimulants can directly influence crop quality. They do this in many ways, for example, by increasing the plant's access to essential nutrients or reducing the energy used by the crop in times of stress. These effects generally also have a positive influence on yield.

. . . independently of its nutrient content – Biostimulants may contain mineral elements found in fertilizers but are applied at such low doses that the benefit cannot be attributed to a fertilizer effect, and indeed trials can demonstrate that the nutrients present are not the cause of the observed effect.

Although not part of the product definition, the functions provided by biostimulants have a role to play in "agro-ecology," i.e., the application of ecological principles to agriculture and the food production system (**Table 1**; IFOAM EU Group et al., 2012).

CLAIM JUSTIFICATION SHOULD FOCUS ON THE EU DEFINITION OF BIOSTIMULANTS

With a view to placing products on the European market, all claims should be demonstrated regarding the agreed EU definition of a biostimulant, per one of the four categories indicated in the forthcoming EU Fertilizing Products Regulation (European Commission, 2016; Council of the European Union, 2018): improving nutrient use efficiency, tolerance to abiotic stress, crop quality traits or availability of confined nutrients in the soil and rhizosphere.

Because each of these categories of claims is quite broad, a more precise description of claims as illustrated in **Table 2** allows for specific measurements to be defined and conducted.

Furthermore, effects are often translated into claims that have value for farmers, such as increased yield (which is not specifically mentioned in the forthcoming EU Fertilizing Products Regulation but could be the result of the use of any of the products it covers) and improved crop quality (which is specifically mentioned as an effect of biostimulants). Although yield gains can be traced back to one of the underlying biostimulant claims defined in the regulation, EBIC has listed them as a separate category of claim, to ensure that they are measured and reported in appropriate empirical terms.

TABLE 1 | Agro-ecological principles and the role played by biostimulants.

Increase biodiversity
By improving soil micro-organism quality/quantity
Reinforce biological regulation and interactions
By reinforcing plant–micro–organism interactions
– symbiotic exchanges i.e., *mycorrhizae*
– symbiotic exchanges i.e., *rhizobiaceae/fava*
– secretions mimicking plant hormones (i.e., *trichoderma*)
By regulating plant physiological processes
– e.g., growth, metabolism, or plant development
Improve biogeochemical cycles
– improve absorption of nutritional elements
– improve bioavailability of nutritional elements in the soil
– stimulate degradation of organic matter

TABLE 2 | Categories of biostimulant claims and examples of sub-claims.

Category of claims in the forthcoming fertilizing products regulation	**Examples of sub-classes**
Nutrition efficiency improvement	Phosphorus acquisition improvement, yield increase
Abiotic stress tolerance	Salt stress, drought stress
Crop quality improvement	Potatoes size increase, sugar content increase, storage duration improvement as the result of improved quality (e.g., firmness, for ex), processing improvement
Derived claims	
Yield improvements	Yield increase or yield security

TYPE OF INFORMATION THAT CAN SUPPORT A CLAIM

Various types of data and empirical evidence can support claim justification. While not strictly speaking hierarchical, it makes sense to begin with published literature and existing data and then to complement that information as needed with new experimental data from controlled conditions and field trials (Rouphael et al., 2018).

Data generated under controlled conditions (glasshouse, growth chambers, phenotyping, etc.) from outside the European Union should be admissible if the climatic conditions tested could conceivably apply within the EU and:

- if it is from a manufacturer's own GEP/GLP-certified facility;
- if the independent research partner (contract facility, university, etc.) that generated the data is considered reputable, or
- if the manufacturer can otherwise demonstrate that the quality of the methodology and the data obtained are substantially equivalent to what would be achieved by a GEP/GLP facility.

Use of Published Literature and Existing Data

As mentioned in the beginning of the Section "Type of Information That Can Support a Claim" of this publication, peer-reviewed scientific literature can support a claim. Literature can be used to describe the mode of action of the product, the biology of the microorganisms used, or any preliminary studies described in relevant published papers supporting the basis of the proposed claim. Scientific literature can be used

to support a claim if it is of acceptable quality, for example, evaluated per criteria outlined in Klimisch et al. (1997) and used to determine which literature can be acceptable for data requirements under EU chemical legislation (REACH). At the same time, the synergistic or emergent effects that result from the combination of substances within a product (Krouk, 2015; Rouphael and Colla, 2018) mean that it is unlikely that literature alone will be enough to fully justify a claim.

Existing scientific information and existing field trial data should be evaluated per the criteria for relevance, reliability, and adequacy defined by the European Chemicals Agency (ECHA) for the implementation of the EU's chemicals regulation (REACH) (European Chemicals Agency [ECHA], 2011).

Experimental Data

Biostimulant claims can be supported by experimental data generated under controlled conditions (laboratory, greenhouse, growth chamber, phenotyping, etc.) and/or in the field (field trials). Additional data from carefully designed small-scale laboratory and growth chamber studies will often form a vital component of the overall claim justification package provided. If field data are used, at least some EU data must be included. Field data from outside the EU may support EU data if both are generated under similar geo-climatic conditions (and those correspond to the intended context for product use). Guidelines exist for determining the comparability of geo-climatic conditions (European and Mediterranean Plant Protection Organization [EPPO], 2014).

Field trials provide essential information about biostimulant effects under real-world conditions. However, for some claims, such as salt stress or cold stress, it is difficult to artificially create the appropriate field conditions. In such cases, the focus of field trials would be more on the "holistic" benefits of the biostimulant in terms of yield/quality, while a specific biostimulant claim related to its mode of action could be demonstrated in controlled conditions. For example: one could demonstrate that phosphorous solubilization occurs in controlled greenhouse or laboratory conditions when the biostimulant product is used, and, demonstrate the overall general benefit to the farmer in terms of improved yield and/or quality when field testing the same product. The field trial would not be needed if the manufacturer only wanted to claim the phosphorus solubilization without mentioning the subsequent improved yield and/or quality. (Although the farmer doesn't care about the mode of action if it doesn't result in a tangible on-farm benefit.)

The net agricultural benefit after considering both the positive and negative effects of the biostimulant should be large enough to justify its use. The benefit from the use of a product should be appropriate to the agronomic setting in which the product will be used. A low level of benefit may be acceptable in some situations, for example:

- when a product will be used as a component of an Integrated Crop Management (ICM) program, or
- in specialized situations, such as organic farming, or where the product makes it possible to maintain the same level of yield or quality while decreasing nutrient applications.

Where the data indicate that there are significant inconsistencies in the performance of a product, the reasons for these inconsistencies should be explained. The instructions for use should enable the user to identify the conditions under which the product will provide optimal performance and any factors that may have an impact on effectiveness. Unexplained variations in product performance should not be a barrier to placing the product on the market, but the uncertainties of the product claim should be indicated on the product label in that case. Transparency is critical to allow farmers to make informed choices.

General Guidelines for Trials/Assays of Biostimulants

Use of a statistical program should be adapted to field trial software such as ARM.

Trial study plan

The trial study plan should cover the following topics:

- The aim of the trial series;
- Statistical analysis and trial design;
- Trial conditions;
- Design and lay-out of trials;
- Control data;
- Application of treatments, and
- Mode of assessment.

The aim of the trial series.

- Objective of the trial and basic information on the trial site. The objective of the trial should be specified, including:
    - The biostimulant effect(s) to be demonstrated
    - The inclusion of other variables in the trial (dose rates, application conditions)
- Whether the trial is for evaluation of a claim or another purpose (germination test, quality of the harvested product, effects on succeeding crops, etc.).
- The following basic information should be provided on the trial site:
    - Full address and geographical coordinates, if possible;
    - Crop and cultivar;
    - Any useful details on the site (e.g., exposure and slope).

Statistical analysis and trial design. The placing of a biostimulant on the market should never be considered to guarantee effectiveness under all conditions, as many factors may influence the performance of a biostimulant in the field. Many additional factors are relevant to biostimulant products when determining acceptable, beneficial, levels of action. These can include:

- Offering an approach compatible with ICM systems and/or organic farming;
- Greater compatibility with cultivation practices;
- Mitigating undesirable effects (on human beings, beneficial organisms, non-target organisms, other crops etc.) of the alternative production system;

In such cases, biostimulant manufacturers should ensure that users can be provided with accurate information on the likely performance of the product and advice on how best to use the product so that it will perform as effectively and consistently as possible.

Under controlled conditions (greenhouse or laboratory), the level of confidence compared to an untreated control can be set at 90% probability (minimum for agronomic production trials in controlled conditions), given the nature of biostimulant effects and their inherent variability (physiology and biology sensitive to pedo-climatic conditions, local microbiome biodiversity and crop genetics).

A minimum of three field trials in the EU should be performed to demonstrate the desired biostimulant claim. The observation of consistent "agronomically" positive data trends (i.e., not necessarily statistically significant) compared to untreated plots in field trials could be considered sufficient to justify a biostimulant claim.

Trials do not need to be over multiple seasons if there are enough trials in one season and different geo-climatic conditions pertinent to the environmental conditions and relevant to the agronomic conditions for which the product will be sold to justify the claim. The trials should be conducted in the EU.

Trial design should be done in a way that allows to discriminate between treated and untreated plots It is recommended to have enough replicates to ensure to reduce the variability of the data and increase the chance to see consistently differences with the untreated plots.

Where a biostimulant's performance is affected by temperature, soil type, crop, or other parameters, the trial design, execution, and subsequent user recommendations should take these factors into account. Furthermore, some claims may be better tested under controlled conditions (e.g., abiotic stresses may be difficult to induce in the field).

When designed accordingly, multiple claims may be demonstrated in a single trial.

Untreated plot trial results in terms of yield and quality should reflect normal agronomic expectations for local production.

The study plan should specify what is assessed, how it is assessed, when, and why.

It is recommended to evaluate at least one measurable indicator related to a benefit perceived by the farmer (i.e., impact on yield and/or quality). For example, nodulation is an early measurable parameter, but it is not sufficient on its own to confirm an effect on yield and/or quality of the crop. If an indicator of biostimulation is not assessed in the trials designed to justify a claim, then this indicator cannot be claimed on the label. For example, a claim to increase root growth is measured and demonstrated in an experimental trial, but the crop yield increase is not quantified in the trial. Consequently, the label can claim "increased root growth" but cannot claim "improved yield" because an increase in yield was not quantified in the claims justification data.

Phytotoxicity should be verified during trials. If no evidence of phytotoxicity is observed, then there is no need to conduct additional phytotoxicity trials. However, if signs of phytotoxicity are observed, then phytotoxicity data are needed. At a minimum, the manufacturer should provide recommendations on how to minimize phytotoxicity when the biostimulant is used.

Trials must be conducted by qualified personnel who will record, document, and archive the trial study plan, the results, the final report and all the supporting raw data. The Fertilizing Products Regulation specifies that manufacturers must keep such information at the disposal of national authorities for 5 years after the product has been placed on the market to facilitate market surveillance. Good Experimental Practice (GEP) for plant protection products call for such information to be archived for 10 years (European and Mediterranean Plant Protection Organization [EPPO], 2012); however, this may be influenced by the record-keeping requirements found in the EU's plant protection regulation (European Parliament and of the Council, 2009).

Data from GEP/GLP-certified facilities can be considered credible, even if they belong to the manufacturer. Nonetheless, it is desirable that manufacturers can demonstrate that at least some of the research was conducted with impartial and competent third parties. As much as possible, trials to support product claims should be conducted with an independent and competent partner, such as one of the following:

- National research agencies and extension officers;
- Institutes (including but not limited to universities and other institutes of higher learning and private research stations) and researchers with published research in agriculture and agronomy, and
- Certified private research centers (GLP/GEP or GLP/GEP-equivalent conditions).

Trial conditions. The relevant conditions of the plot and crop should be adequately described, for example:

- For an annual crop, sowing or planting date and density, row spacing;
- For a perennial crop, arrangement and spacing in rows or as single plants, pruning or training system, rootstock, canopy height, plant width, age, whether in production;
- For a glasshouse crop, arrangement within compartments, on benches, in soil-less culture, etc.;
- The cultivation practices of the crop could be described, such as tillage, fertilizer and irrigation regimes, and any other additional inputs;
- Information should be given on whether the crop was growing normally or was under stress at the time(s) of treatment [e.g., drought, frost, wind or effects of other overall chemical treatments, and/or effects of other pests (including diseases and weeds)];
- For a soil-applied product, the temperatures at the root zone level in topsoil should be recorded during at least the first month of the trial at 2-h intervals), and
- Soil characteristics should be described, i.e. the percentage of sand, clay, silt, and Organic Matter (O.M.), as well as the pH.

Design and lay-out of trials. The design and lay-out of the plots should be described, preferably with a plan, the number, size and

shape of plots, whether defined by plot dimensions on the ground or a certain lay-out of plants.

The type of experimental design should be indicated. The arrangements made for the untreated control (included, imbricated, and excluded) should be precisely indicated, together with details on any other control treatments.

Completely randomized blocks should be assured, while maintaining a scientific design that avoids any interference of experimental conditions between plots (for example, with regards to drought stress mitigation trials, the well-watered condition will have to be set up as a band reference beside the trial.

Enough replicates should be assured to obtain 12 degrees of freedom in the trial, so that a consistent difference between treated and untreated crops can be demonstrated.

Control data. Control data set can be completely untreated plot or an "omission" group i.e., the treatment regimen is the same with the exception of the biostimulant, which is absent from the "omission" plot.

Control object(s) selected for an improved nutrient uptake claim should include:

- Untreated
- The following additional control groups, when a biostimulant is included in a "support" nutrient-containing formulation:
    - option 1: the support formulation alone, if the support provides nutrient elements;
    - option 2: the biostimulant formulation alone (without the nutrient elements).
- Abiotic stress resistance claims should include the following control objects:
    - Stress condition object(s);
    - no-stress condition object(s);
    - characterization of the applied stress level.

Application of treatments. Precise information should be provided on the formulation, application method, concentration and amounts of the test product.

The justification of biostimulant claims should be done for the minimum recommended dose necessary to achieve the desired effect. However, for biostimulant products composed primarily of substances that occur naturally in the environment, this may be less important, unless the additional amount significantly increases existing background levels. Additionally, for some biostimulants (microbial biostimulants for example), the concept of a minimum effective dose may be more difficult to determine practically, and a range of doses may be more appropriate to justify the associated claim. In such cases, while an appropriate explanation for the proposed dose is required, providing field-generated data may not be necessary. Such explanations should refer to the mode of action, and to the biology of the microorganisms. One may also include any preliminary studies (including relevant published peer-reviewed papers) that provide the basis for the proposed concentration in the formulation and/or applied dose.

While manufacturers should always seek to justify the recommended dose, the lack of precise or conventional dose justification data should not preclude the placing of the biostimulant on the market. However, in such cases, the manufacturer should explain why such a dose may not be appropriate. Information demonstrating the minimum level required to provide a beneficial effect (as determined for effectiveness, in either laboratory or field studies) may suffice.

- Test and reference products: the products included in the trial (test and reference) should be specified, giving the common name or other specified standard (if available), and the exact name or other designation of each formulated product.
- Mode of application: The information provided should be sufficient to establish that good agricultural practice is being followed, for example:
    - The application method and equipment used;
    - Any significant deviations from the intended dosage;
    - The operating conditions, insofar as they may affect claims (e.g., for sprays, pressure, nozzle type, spray quality and speed of travel of sprayer);
    - The number of applications made;
    - The date of each application (including year, preferably by dd-mm-yyyy);
    - The growth stage of the crop at the time of each application (see BBCH Growth Stage Keys Meier et al., 2009);
    - The doses used (cc-g/hL or L-Kg/ha), and the spray volumes (L/ha).
- Meteorological data. The following meteorological data should be recorded:
    - Observations by the experimenter near the date of application of meteorological data that may affect the outcome of the trial. These depend on the judgment of the experimenter and need not be given at the same level of detail as on the day of application.
    - Observations made by the experimenter on the day of application, including certain standard data that should always be provided for the application day (temperature, humidity and wind); if rain occurred within 24 h of the foliar application of the product, it should be recorded as "yes/no" (rain fastness)
- Edaphic data should also be recorded during the trial.

Mode of assessment.

- Type, time and frequency of assessment
- The type and date of each assessment
- The methods used should be described. Any assessment scales used should be specified.
- Direct effects on the crop. The presence or absence of phytotoxic effects should be noted for each plot,

TABLE 3 | Proposed crop groupings to justify biostimulant claims.

• Cereals (wheat, barley, oat, rice, minor grains) and corn
• Pulses and oilseeds • Tree fruit, nuts, and olive • Grape (wine and table) • Other soft fruit and vegetables (all leafy, fruiting and root vegetables, and leguminosae) • All others [loam (turf), ornamentals, and mushrooms, etc.]

with an accurate description of any symptoms, for example: modifications in the development cycle, thinning, modifications in color, necrosis, deformations, effects on the quantity and quality of the yield.

- Yield and quality should, when specified, be recorded, taking careful note of the specific parameters required in each crop.

The trial series report

The trial report should include:

- The aim of the trial series;
- The list of test and reference products, with doses and application times of frequencies;
- The assessment methods;
- Results including statistical analysis if any were conducted;

Crop groupings for the conduct of biostimulant field trials

When trials are conducted to justify biostimulant claims, the crop groupings outlined in **Table 3** are proposed.

European Biostimulant Industry Council suggests the guideline proposed in **Table 4** to help manufacturers determine the appropriate number of trials, depending on the nature of the claim and to prevent excessive requests for trials from reviewing authorities. Notwithstanding this guideline, manufacturers will need to adapt their trial regime to the specific claim being made, especially as several of the examples listed in **Table 4** may apply.

Where applicable, appropriate scientific literature may be substituted for one or more of the trials suggested.

CONCLUSION

The ability to demonstrate that a product is indeed a *bona fide* biostimulant will depend on a demonstration of its effect. However, this should not be confused with guaranteeing a specific level of efficacy. In no case should the placing of a biostimulant on the EU market be considered to guarantee effectiveness under all conditions, as many factors may influence performance of a biostimulant in the field. The requirements for claims justification should be proportional to the task; manufacturers should provide enough data to be credible, without the process becoming needlessly burdensome. The narrower the claim, the less data should be required. Given the variety of possible effects, crops or crop groupings, and growing conditions, manufacturers need the flexibility to design studies that are adapted to the specific agronomic situation. Furthermore, it should be recognized that, in the case of products that improve the availability of nutrients (notably micro-organisms), soil types and soil conditions can be more relevant than crop type itself when designing trials. Trials will become ever more crucial as the industry trends toward the development of complex, multi-component products. Demonstrating multiple effects, especially when they are synergistic or emergent, will provide additional challenges for developing appropriate trial designs.

The forthcoming EU regulation is based on the New Legislative Framework, which means that harmonized standards play an important role during the conformity assessment process; measures obtained through the application of these standards are presumed to be in conformity with essential requirements of products both for safety and quality, if the values are within any applicable target ranges. Such harmonized standards play a role in characterization, verifying contaminant levels, declared contents and product claims.

The European Committee for Standardization (CEN) has begun work on processes and methods that will become the basis of a set of harmonized European standards to justify biostimulant claims (CEN and CENELEC Work Programme, 2018) under the forthcoming EU Fertilizing Products Regulation. This work includes the standardization of denominations, biostimulant

TABLE 4 | Suggested number of trials based on the claim to be justified.

Claim that can credibly be made on this basis	Suggested number of trials
Effect claimed for *a specific crop* Example: Improves strawberry ripening	3 trials on the crop Example: Product is successfully demonstrated on strawberries in the field in a single location over 2 years or tested in the field and in a commercially equivalent growing environment the same year.
Effect can be claimed for *the entire crop group* Example: improves brix content or yield of larger grade fruit	3 trials each for 2 different crops or 2 trials for 3 each diff crops within a single group. Example: Product is successfully demonstrated on apples and pears in a single location over 2 years or in two different locations with different growing conditions in a single year.
Effect can be claimed *without being required to limit it to any specific crop grouping* Example: Helps crops tolerate drought stress in open-air commercially equivalent growing environments	3 trials each from 4 different groups. Example: Product is successfully demonstrated on cereals, apples, grapes, and peppers.

specifications, marking, test methods, verifying product claims and safety requirements. It will be a challenge to develop standards that allow for comparability of products (i.e., knowing that two different products truly address the same abiotic stress, for example) while accommodating the wide range of products, claims and contexts for use.

The guidelines developed by EBIC and outlined in this article can inform the drafting of relevant CEN standards on claims justification, as they have already benefitted from significant discussion among professionals involved in the testing of biostimulant product claims.

AUTHOR CONTRIBUTIONS

All authors listed have made a substantial, direct and intellectual contribution to the work, and approved it for publication.

FUNDING

The work was carried out in the context of the European Biostimulants Industry Council (EBIC), which is financed by its member companies through annual membership dues.

ACKNOWLEDGMENTS

We like to acknowledge the hours of work contributed by EBIC's members and member company representatives to elaborate the guidelines. We cannot list them all, but this has truly been a collegial effort. We also especially like to thank Carlos Repiso and Céline Durieu, who assumed the leadership of EBIC's Agriculture Committee while this article was going through peer review. Their contributions helped us to revise and improve the article.

REFERENCES

CEN and CENELEC Work Programme (2018). *CEN – European Committee for Standardization, December 2017*. Available at: https://www.cencenelec.eu/News/Publications/Publications/WorkProgramme-2018_UK_acces.pdf (accessed November 22, 2018).

Chiaiese, P., Corrado, G., Colla, G., Kyriacou, M. C., and Rouphael, Y. (2018). Renewable sources of plant biostimulation: microalgae as a sustainable means to improve crop performance. *Front. Plant Sci.* 9:1782. doi: 10.3389/fpls.2018.01782

Council of the European Union (2018). *Proposal for a Regulation of the European Parliament and of the Council Laying Down Rules on the Making Available on the Market of CE Marked Fertilizing Products and Amending Regulations (EC)No 1069/2009 and (EC) No 1107/2009-Analysis of the Final Compromise Text With a View to Agreement*. Available at: http://data.consilium.europa.eu/doc/document/ST-15103-2018-INIT/en/pdf (accessed December 20, 2018).

European Chemicals Agency [ECHA] (2011). *ECHA Guidance on Information Requirements and Chemical Safety, R.4 Evaluation of Available Information*. Available at: https://echa.europa.eu/documents/10162/13643/information_requirements_r4_en.pdf/d6395ad2-1596-4708-ba86-0136686d205e (accessed November 2, 2018).

European Commission (2016). *Proposal for a Regulation of the European Parliament and of the Council Laying Down Rules on the Making Available on the Market of CE Marked Fertilising Products and Amending Regulations (EC) No 1069/2009 and (EC) No 1107/2009 COM(2016) 0157 Final – 2016/0084 (COD)*. Brussels: European Commission.

European and Mediterranean Plant Protection Organization [EPPO] (2012). *EPPO Standard PP 1/181(4). Conduct and Reporting of Efficacy Evaluation Trials Including Good Experimental Practice*. Available at: https://pp1.eppo.int/standards/PP1-181-4 (accessed March 14, 2019).

European Parliament (2019). *Parliament Approves New Rules on Fertilisers and Cadmium Limits*. Available at: http://www.europarl.europa.eu/news/en/press-room/20190321IPR32144/parliament-approves-new-rules-on-fertilisers-and-cadmium-limits (accessed April 5, 2019).

European and Mediterranean Plant Protection Organization [EPPO] (2014). *EPPO Standard (PP 1/241 (2) Guidance on Comparable Climates*. Available at: https://pp1.eppo.int/standards/PP1-241-2 (accessed March 14, 2019).

European Parliament and of the Council (2009). *Regulation (EC) No 1107/2009 of the European Parliament and of the Council of 21 October 2009 Concerning the Placing of Plant Protection Products on the Market and Repealing Council Directives 79/117/EEC and 91/414/EEC. Official Journal of the European Union. L 309, 24.11.2009*. 1–50. Available at: http://data.europa.eu/eli/reg/2009/1107/oj (accessed March 19, 2019).

IFOAM EU Group, TP Organics, and ARC 2020 (2012). *What is Agro-Ecology? Agro-Ecological Innovation Project*. Available at: http://www.ensser.org/fileadmin/files/2012_IFOAM-EU-Group.pdf (accessed July 26, 2017).

Klimisch, H., Andreae, M., and Tillmann, U. (1997). A systematic approach for evaluating the quality of the experimental toxicological and ecotoxicological data. *Regul. Toxicol. Pharmacol.* 25, 1–5.

Krouk, G. (2015). How plants respond to a combination of signals: Are biostimulant effects triggered by a cocktail of molecules? in *Paper Presented at the 2nd World Congress on the Use of Biostimulants in Agriculture*, Florence.

Meier, U., Bleiholder, H., Buhr, L., Feller, C., Hacks, H., Hess, M., et al. (2009). The BBCH system to coding the phenological growth stages of plants-history and publications. *J. Kulturpflanzen* 61, 41–52.

Rouphael, Y., and Colla, G. (2018). Synergistic biostimulatory action: designing the next generation of plant biostimulants for sustainable agriculture. *Front. Plant Sci.* 9:1655. doi: 10.3389/fpls.2018.01655

Rouphael, Y., Spíchal, L., Panzarová, K., Casa, R., and Colla, G. (2018). High-throughput plant phenotyping for developing novel biostimulants: from lab to field or from field to lab? *Front. Plant Sci.* 9:1197. doi: 10.3389/fpls.2018.01197

Conflict of Interest Statement: At the time this article was drafted and submitted, MR was employed by Bayer CropScience S.r.l., a member of the European Biostimulant Industry Council (EBIC). BD was employed by Olmix Group, another EBIC member. KS is a Consulting Partner at Prospero & Partners, which is contracted to provide the EBIC secretariat. LT is an independent consultant working for Prospero under contract to support EBIC on technical issues.

frontiers in Plant Science

ORIGINAL RESEARCH
published: 03 May 2019
doi: 10.3389/fpls.2019.00493

A Combined Phenotypic and Metabolomic Approach for Elucidating the Biostimulant Action of a Plant-Derived Protein Hydrolysate on Tomato Grown Under Limited Water Availability

Kenny Paul[1†], Mirella Sorrentino[1†], Luigi Lucini[2], Youssef Rouphael[3], Mariateresa Cardarelli[4], Paolo Bonini[5], Maria Begoña Miras Moreno[2], Hélène Reynaud[6], Renaud Canaguier[7], Martin Trtílek[1], Klára Panzarová[1] and Giuseppe Colla[8,9]**

[1] Photon Systems Instruments, spol. s.r.o., Drásov, Czechia, [2] Department for Sustainable Food Process, Research Centre for Nutrigenomics and Proteomics, Università Cattolica del Sacro Cuore, Piacenza, Italy, [3] Department of Agricultural Sciences, University of Naples Federico II, Portici, Italy, [4] Consiglio per la Ricerca in Agricoltura e l'Analisi dell'Economia Agraria, Centro di Ricerca Orticoltura e Florovivaismo, Pontecagnano Faiano, Italy, [5] NGAlab, Tarragona, Spain, [6] Italpollina USA, Inc., Anderson, IN, United States, [7] Nixe, Valbonne, France, [8] Department of Agriculture and Forest Sciences, Tuscia University, Viterbo, Italy, [9] Arcadia Srl, Rivoli Veronese, Italy

Plant-derived protein hydrolysates (PHs) are an important category of biostimulants able to increase plant growth and crop yield especially under environmental stress conditions. PHs can be applied as foliar spray or soil drench. Foliar spray is generally applied to achieve a relatively short-term response, whereas soil drench is used when a long-term effect is desired. The aim of the study was to elucidate the biostimulant action of PH application method (foliar spray or substrate drench) on morpho-physiological traits and metabolic profile of tomato grown under limited water availability. An untreated control was also included. A high-throughput image-based phenotyping (HTP) approach was used to non-destructively monitor the crop response under limited water availability (40% of container capacity) in a controlled environment. Moreover, metabolic profile of leaves was determined at the end of the trial. Dry biomass of shoots at the end of the trial was significantly correlated with number of green pixels (R^2 = 0.90) and projected shoot area, respectively. Both drench and foliar treatments had a positive impact on the digital biomass compared to control while the photosynthetic performance of the plants was slightly influenced by treatments. Overall drench application under limited water availability more positively influenced biomass accumulation and metabolic profile than foliar application. Significantly higher transpiration use efficiency was observed with PH-drench applications indicating better stomatal conductance. The mass-spectrometry based metabolomic analysis allowed the identification of distinct biochemical signatures in PH-treated plants. Metabolomic changes involved a wide and organized range of biochemical processes that included, among others, phytohormones (notably a decrease in cytokinins and an accumulation of salicylates) and lipids (including

OPEN ACCESS

Edited by:
Angeles Calatayud, Instituto Valenciano de Investigaciones Agrarias, Spain

Reviewed by:
Serenella Nardi, University of Padua, Italy
Juan Parrado, Universidad de Sevilla, Spain

***Correspondence:**
Klára Panzarová
panzarova@psi.cz
Giuseppe Colla
giucolla@unitus.it

[†] *These authors have contributed equally to this work*

Specialty section:
This article was submitted to Crop and Product Physiology, a section of the journal Frontiers in Plant Science

Received: *28 January 2019*
Accepted: *01 April 2019*
Published: *03 May 2019*

Citation:
Paul K, Sorrentino M, Lucini L, Rouphael Y, Cardarelli M, Bonini P, Miras Moreno MB, Reynaud H, Canaguier R, Trtílek M, Panzarová K and Colla G (2019) A Combined Phenotypic and Metabolomic Approach for Elucidating the Biostimulant Action of a Plant-Derived Protein Hydrolysate on Tomato Grown Under Limited Water Availability. Front. Plant Sci. 10:493. doi: 10.3389/fpls.2019.00493

membrane lipids, sterols, and terpenes). From a general perspective, treated tomato plants exhibited an improved tolerance to reactive oxygen species (ROS)-mediated oxidative imbalance. Such capability to cope with oxidative stress might have resulted from a coordinated action of signaling compounds (salicylic acid and hydroxycinnamic amides), radical scavengers such as carotenoids and prenyl quinones, as well as a reduced biosynthesis of tetrapyrrole coproporphyrins.

Keywords: protein hydrolysates, high-throughput phenotyping, metabolomics, morpho-physiological traits, foliar spray, drench application

INTRODUCTION

Competition among agriculture, industry, and cities for limited water supplies is already constraining development efforts in many countries. As populations expand and economies grow, the competition for limited supplies will intensify and so will conflicts among water users. Agriculture is not only the world's largest water user in terms of volume; it is also a relatively low-value, low-efficiency, and highly subsidized water user (Rouphael et al., 2012).

These facts are forcing farmers to grow crops with diminishing water supplies. Limited water availability can affect morphological, physiological, biochemical, and molecular processes in plants, resulting in growth depression and yield reduction (Liu et al., 2014; Kumar et al., 2017). Under these conditions, the application of plant biostimulants can help crops to use water more efficiently by changing the root-to-shoot ratio, plant metabolism, and hormonal balance (Colla et al., 2017b; Rouphael and Colla, 2018).

Protein hydrolysates (PHs) represent an important category of plant biostimulants that have been extensively used for improving crop yield and quality especially under abiotic stress conditions such as limited water, salinity, and heavy metals (Ertani et al., 2009; Colla et al., 2015; du Jardin, 2015). PHs could directly stimulate carbon and nitrogen metabolism and could indirectly enhance nutrient availability of substrates and increase nutrient uptake as well as nutrient-use efficiency in plants (Haplern et al., 2015; Colla et al., 2017b; Rouphael et al., 2017). PHs can be applied by foliar spray or substrate drench, affecting molecular and physiological crop response in a different way (Lucini et al., 2015; Sestili et al., 2018). In a recent study, substrate drench applications of a plant-derived PH were more effective to improve plant growth and total N uptake than foliar sprays in tomato (Sestili et al., 2018). In the same study, the application method (drench or foliar) of the plant-derived PH affected the expression of genes encoding ammonium and nitrate transporters differently as well as seven enzymes involved in N metabolism of tomato (Sestili et al., 2018). Biostimulant activity of PH can be due to the direct effect of bioactive compounds (e.g., signaling peptides, free amino acids) on plant metabolism and to the indirect effect resulting from the PH-mediated enhancement of plant growth promoting microorganisms in plant microbiome (Luziatelli et al., 2019).

A successful evaluation of biostimulant activity of PHs requires an accurate measurement of morpho-physiological traits of plants over time. Use of advanced image-based automated phenotyping platforms offers opportunities to increase both the speed at which these measurements are collected and the accuracy of measurements (Povero et al., 2016). Dynamic screening of plants can be done for multiple morpho-physiological traits related to growth, yield, and performance throughout their development or onset, progression, and recovery from abiotic stress (Petrozza et al., 2014). Functional action and characterization of PHs in plants can be thus monitored with high precision and in high resolution in each phase of plant development and/or plant response to environmental conditions, depending on the target substance application or type of experimental layout (Rouphael et al., 2018b). Range of morpho-physiological traits can be monitored in a fully automated, high-resolution, and high-sensitivity manner. A key descriptive parameter in plant physiology, except for root analysis, is the shoot growth of the plants. Quantitative and qualitative dynamic assessment of growth performance by RGB imaging was used to characterize range of traits such as shoot biomass or yield (Li et al., 2014; Humplík et al., 2015b). Non-invasive monitoring of plant photosynthetic activity is also critical for understanding the physiological and metabolic condition, as well as its susceptibility to various stress conditions (Gorbe and Calatayud, 2012; Humplík et al., 2015a; Paul et al., 2016). Pulse-amplitude-modulation-based kinetic chlorophyll fluorescence imaging is a broadly applied technique used to understand the plant phenology in response to external stimuli or agents (Murchie and Lawson, 2013). In a high-throughput phenotyping setup, modern imaging systems were recently successfully used to monitor dynamically PSII parameters and electron flow dynamics at the whole plant level (Humplík et al., 2015b; Awlia et al., 2016; Tschiersch et al., 2017). Usage of automated photosynthetic phenotyping approaches helps us to screen and characterize PH real-time interaction throughout the grow regime. Water taken up by plants or plant water content is key for understanding the efficiency with which plants are able to regulate stomatal conductance and CO_2 fixation. Water content in plants is the result of the equilibrium between root water uptake and shoot transpiration (Berger et al., 2010). Thermoimaging has been used in high-throughput phenotyping platforms to monitor plant transpiration rate and transpiration use efficiency (TUE) (Kaňa and Vass, 2008; Paul et al., 2016).

In addition to dynamic screening of plant performance by automated plant phenotyping, metabolomics offers unique opportunities to understand the mode of action of PHs on crops and to identify biomarkers of biostimulant action. For instance, Lucini et al. (2015) identified several differentially expressed key

metabolites associated with osmotic adjustment, oxidative stress mitigation, and hormone network in PH-treated lettuce plants exposed to salt stress. Considering that tomato is among the most important crops grown in the world, an experimental trial was performed to evaluate the biostimulant activity of a plant-derived PH applied through foliar spray or substrate drench on tomato plants grown under limited water availability in a controlled environment. The research phases of the trial included (1) the use of a high-throughput phenotyping platform for evaluating the treatment effects on selected morpho-physiological traits of plants (e.g., digital biomass, kinetic chlorophyll fluorescence and leaf surface temperature) and (2) the use of mass-spectrometry (MS) based metabolomics for identifying distinct biochemical signatures in PH-treated plants (including hormones and secondary metabolites produced by plants in response to low water availability stress conditions).

MATERIALS AND METHODS

Plant Material and Growing Conditions

Seeds of tomato (*Solanum lycopersicum* L.–Hybrid F1 Chicco Rosso) were sown in trays with size of pots of 100 ml each containing a commercial peat-based substrate (Substrate 2, Klasmann-Deilmann GmbH, Germany) having the following characteristics: density, 160 kg m^3; total pore space, 85% v/v; total carbon, 55%; pH 5.5; N, 210 mg L^{-1}; P, 105 mg L^{-1}; K, 224 mg L^{-1}; and Mg, 100 mg L^{-1}; trace elements in chelated forms. Substrate was watered to water holding capacity. Trays with seeds were kept for 2 days at 4°C in the dark. Trays with seeds were placed in the controlled growth chamber (FS-WI, PSI, Czechia) at a 16-h day/8-h night regime, 22°C day/20°C night, 60% relative humidity, and with cool-white LED (250 μmol photons m^{-2} s^{-1}) and far-red LED (5.5 μmol photons m^{-2} s^{-1}) lighting.

Fertigation and Watering Protocol

Prior to plant transplanting into 3-L pots, trays were uniformly watered at 6, 7, 12, and 14 days after placement of trays in a controlled growth chamber. On day 7 and day 14, plants were fertigated with a solution containing 1.04 g L^{-1} calcium nitrate (15.5% N; 28% CaO), 0.04 g L^{-1} ammonium nitrate (34% N), 0.14 g L^{-1} monopotassium phosphate (52% P_2O_5, 34% K_2O), 0.18 g L^{-1} potassium sulfate (50% K_2O, 45% SO_3), 0.5 g L^{-1} magnesium sulfate (10% N, 16% MgO), and 0.5 ml L^{-1} FloraMicro (5% N, 1% K_2O, 5% Ca, 0.01% B, 0.001% Cu, 0.1% Fe, 0.05% Mn, 0.0008% Mo, and 0.015% Zn).

Twenty-day-old plants were selected with uniform growth characteristics and transplanted into 3-L pots (mixture of Substrate 2 Klasmann soil and river sand in 3:1 ratio was used). The pots were labeled with unique identification codes for each plant replicate and treatment. For determining the water content at container capacity, one set of substrate pots was dried for 3 days at 80°C and another set was saturated with water and left to drain for 1 day before weighing 100% water holding capacity (Awlia et al., 2016). Water content at container capacity was calculated as the difference between substrate weight at water holding capacity and dried substrate. On the day before transplantation, soil was prepared, and moisture content was adjusted to 60% of container capacity. Twenty-one-day-old plants were transplanted into the prepared substrate mixture with 60% of container capacity. Following the transplantation, plants were regularly watered to reference weight (40% of container capacity) defined as low water availability condition by using the automated watering and weighing unit of the PlantScreen™ Modular System (Photon Systems Instruments (PSI), Czechia).

Biostimulant Characteristics

Plant-derived PH biostimulant Trainer® was provided by Italpollina Company (Rivoli Veronese, Italy). The plant-derived PH Trainer® is a commercial PH obtained through enzymatic hydrolysis of proteins derived from legume seeds. Briefly, the seeds are ground, and the flour was dispersed in acidified water to extract the soluble compounds. Filtration and centrifugation are then used to separate the protein concentrate from the other organic compounds. Enzymatic hydrolysis is used to release the amino acids and peptides from protein concentrate. Insoluble residual compounds are separated from amino acids and peptides by centrifugation. The resulting PH is concentrated through water evaporation (Colantoni et al., 2017). The final product contains mostly peptides and amino acids and, with a less extent, soluble carbohydrates, mineral elements and phenolic compounds. Trainer® has a density of 1.21 kg L^{-1}, a dry matter of 46%, and a pH of 4.0. It contains 310 g kg^{-1} of free amino acids and soluble peptides (Rouphael et al., 2018a). The aminogram of the product (in g kg^{-1}) was as follows: Ala (12), Arg (19), Asp (33), Cys (4), Glu (54), Gly (13), His (8), Ile (12), Leu (24), Lys (19), Met (4), Phe (16), Pro (15), Ser (17), Thr (11), Trp (4), Tyr (13), and Val (16). The antioxidant activity of Trainer®, as measured by ferric-reducing antioxidant power (FRAP), was 41.9 mmol Fe^{2+} g^{-1} f.w., while the total phenolics and flavonoids, determined following the methods reported by Borgognone et al. (2014), were 8.93 mg of gallic acid equivalent per gram of f.w. product and 0.95 mg of quercetin equivalent per gram of f.w. product, respectively. The Trainer® content of soluble sugars was 90 g kg^{-1} f.w., and its elemental composition was as follows (g kg^{-1} f.w): N (50.0), P (0.9), K (41.1), Ca (10.9), Mg (0.5), Fe (0.024), Zn (0.010), Mn (0.001), B (0.005), and Cu (0.001) (Colla et al., 2017a). The Trainer® content of N–NO_3 and N–NH_4 was 3.13 and 6.00 μg g^{-1} f.w., respectively (Ceccarelli, 2018). No detectable phytohormones in Trainer® have been reported (Luziatelli et al., 2019).

Plant Identification and Biostimulant Treatments

Plants were randomly distributed into three groups with six biological replicates per group. Three groups each containing six plants were identified as follows: no application, foliar application, and drench application of PH. Each plant was labeled with a unique barcode identifier used for registration of the plants in the PlantScreen™ Modular System.

The PH was applied either as foliar spray or as substrate drench (**Supplementary Figure S1B**) as water solution containing a non-ionic surfactant Triton X-100 at 0.1%. A control group (no application) was sprayed with distilled water containing 0.1% Triton X-100. PH application was performed

twice: 5 days after transplanting (DAT) referred to as Treatment 1 (T1) and 12 DAT referred to as Treatment 2 (T2). For 24 h prior to and following spraying, humidity in the cultivation chamber was kept at 85% relative humidity. For foliar spray treatments, 2 ml of PH was diluted in 500 ml of distilled water with 0.1% Triton X-100, and 60 ml of solution was applied by homogeneous foliar spray over the entire plant surface per plant replica. Substrate of each pot was covered with aluminum foil during and upon spraying and was removed prior to the next phenotypical analysis in the PlantScreen^{TM} Modular System. For drenching treatment, 4 ml of biostimulant was diluted in 1,000 ml of 0.1% Triton, and 60 ml per plant replicate was applied by drenching. At both PH application times (T1 and T2), plants in control treatment and those foliarly sprayed with PH were irrigated with 60 ml of water each to avoid changes of substrate water status in comparison with plants treated by drench application of PH. Right after PH treatment, plants were taken back to fytoscope FS-WI.

High-Throughput Plant Phenotyping Protocol and Imaging Sensors

Plant phenotypic measurements were performed using the PlantScreen^{TM} Modular System installed in semi-controlled greenhouse environment conditions in the PSI Research Center (PSI, Drásov, Czechia). The platform was operated in closed imaging loop located in a climatized environment with temperature ranging between 21°C and 24°C. The platform is equipped with four robotic-assisted imaging units, an automatic height measuring light curtain unit, an acclimation tunnel, and a weighing and watering unit. Plants placed in individual transportation disks were transported by moving belt toward individual imaging units and watering and weighing stations.

Twenty-two-day-old plants were randomly distributed into three batches, each batch containing 12 plants. Plant imaging started with 22-day-old plants (1 DAT, day 1 of phenotyping) and continued for 15 days (15 DAT, day 15 of phenotyping). Plants were imaged using the following protocol. Briefly, plants were manually transferred from the climate-controlled growth chamber to the manual loading station of the PlantScreen^{TM} Modular System and were transported through the acclimation tunnel with automatic height measuring unit. Prior to the imaging, plants were dark-adapted in acclimation tunnel for 15 min. Each batch of plants was automatically phenotyped for around 30 min by using kinetic chlorophyll fluorescence imaging measurement for photosynthetic performance analysis; top view and multiple-angle side view Red Green Blue (RGB) imaging for morphological, growth, and color analysis; and finally a thermal imaging unit for plant surface temperature quantification (**Supplementary Figure S1A**). Following the imaging, plants were automatically transported to the watering and weighting unit for maintaining precise soil water holding capacity. After completion of the phenotyping protocol, plants were manually moved back to the climate-controlled growth chamber until the subsequent phenotyping day. We used the automatic timing function of the PlantScreen^{TM} Scheduler (PSI, Czechia) to schedule the initiation of the phenotyping protocol at the same time of the diurnal cycle (after 3 h of illumination in the climate-controlled growth chamber). The phenotyping data were acquired twice prior to biostimulant application in days 1 and 3 (pre-T measurements), three times post-first biostimulant application in days 6, 8, and 10 (post-T1 application), and twice post-second biostimulant application in days 13 and 15 (post-T2 application). The acquired images were automatically processed using Plant Data Analyzer (PSI, Czechia), and the raw data exported into CSV files were provided as input for further analysis.

Kinetic Chlorophyll Fluorescence Measurement

Kinetic chlorophyll fluorescence (ChlF) measurements were acquired using an enhanced version of the FluorCam FC-800MF pulse amplitude modulated (PAM) chlorophyll fluorometer (PSI, Czechia) with an imaging area in top view position of 800 × 800 mm, as described in Tschiersch et al. (2017). We assessed the photosynthetic performance in the plants by quantifying the rate of photosynthesis at different photon irradiances using the light curve protocol (Henley, 1993; Rascher et al., 2000). The measuring protocol described previously (Awlia et al., 2016) was optimized for the tomato plants from early to later developmental stage. For the light curve characterization, three actinic light irradiances (Lss1–170 μmol photons m^{-2} s^{-1}, Lss2–620 μmol photons m^{-2} s^{-1}, and Lss3–1,070 μmol photons m^{-2} s^{-1}) were used with a duration of 30 s in order to quantify the rate of photosynthesis.

From the fluorescence data, a range of parameters were extracted as described in detail by Awlia et al. (2016). Additionally, 1 - q_P was calculated, which reflects the proportion of PSII reaction centers that are closed (Maxwell and Johnson, 2000; Na et al., 2014).

Visible Red Green Blue Imaging

To assess digital biomass of the plants, RGB imaging was done from top view (RGB2) and side view from multiple angles (RGB1). The RGB imaging unit is a light-isolated box equipped with turning table with precise angle positioning and two RGB cameras (top and side) mounted on robotic arms, each supplemented with LED-based lighting source to ensure homogeneous illumination of the imaged object.

Projected shoot area (PSA) parameter, together with regularly determined weight of the plants, was used to estimate TUE. TUE was defined by the ratio of aboveground biomass produced per unit of water transpired and depends on the characteristics of the plants and on the environment where the plants grow (Al-Tamimi et al., 2016). TUE was estimated from transpiration defined by measures of water loss and growth from PSA by plant-specific pixel counts quantification.

Thermal Imaging

To assess leaf surface temperature of the plants, a thermal imaging unit based on side view imaging was used. The thermal imaging unit incorporated in the PlantScreen^{TM} System consists of a light-isolated box with one side view camera mounted

on a robotic arm, precise plant positioning, and a background heated wall with an integrated temperature sensor to increase contrast for the image processing step. The imaged area is 1,205 × 1,005 mm (height × width). To assess spatio-temporal variations in temperature over plant surface, we used FLIR A615 thermal camera with 45° lens and resolution 640 × 710 pixels, with high-speed infrared windowing option and <50 mK thermal sensitivity (FLIR Systems Inc., Boston, MA, United States). The thermal images were acquired in line scan mode with each image consisting of 710 pixels with a scanning speed of 50 Hz (lines per second). Thermal images were acquired in darkness. Image acquisition conditions, plant positioning, and camera settings were fixed throughout the experiment. Leaf surface temperature of each plant was automatically extracted with Plant Data Analyzer software (PSI, Czechia) by mask application, background subtraction, and pixel-by-pixel integration of values across the entire plant surface area. To minimize the influence of the environmental variability and the difference in the image acquisition timing among individual plants, the raw temperature of each plant (°C) was normalized by the actual background temperature and expressed as ΔT (°C).

Sample Harvest and Metabolomic Analysis

Plant material was harvested 19 DAT for metabolomic analysis by harvesting and combining the third and fourth fully expanded leaves from the top of each plant. Additionally, the final biomass of each plant was determined by measuring fresh weight and dry weight of the remaining shoot.

Plant samples were homogenized in pestle and mortar using liquid nitrogen, and then an aliquot (1.0 g) was extracted in 10 ml of 0.1% HCOOH in 80% aqueous methanol using an Ultra-Turrax (Ika T-25, Staufen, Germany) (Borgognone et al., 2016). The extracts were centrifuged (12,000 × *g*) and filtered into amber vials through a 0.22-μm cellulose membrane for analysis. Thereafter, metabolomic analysis was carried out through a ultra-high performance liquid chromatograph (UHPLC) coupled to a quadrupole-time-of-flight mass spectrometer (UHPLC/QTOF-MS). The metabolomic facility included a 1290 ultra-high-performance liquid chromatograph, a G6550 iFunnel Q-TOF mass spectrometer, and a JetStream Dual Electrospray ionization source (all from Agilent Technologies, Santa Clara, CA, United States). The untargeted analysis was carried out as previously described (Rouphael et al., 2016). Briefly, reverse-phase chromatography was carried out on an Agilent Zorbax Eclipse-plus C18 column (100 × 2.1 mm, 1.8 μm) and using a 34-min linear elution gradient (5% to 95% methanol in water, with a flow of 220 μL min^{-1} at 35°C). The mass spectrometric acquisition was done in SCAN (100–1,000 *m/z*) and positive polarity (Pretali et al., 2016).

Features deconvolution and post-acquisition processing were done in Agilent Profinder B.06. Mass and retention time alignment followed by a filter-by-frequency postprocessing filter were done to retain only those compounds that were present in >75% of replications within at least one treatment. Compound annotation was done using the "find-by-formula" algorithm, i.e., using monoisotopic accurate mass, isotope spacing, and isotope ratio, with a mass accuracy tolerance of <5 ppm. The database PlantCyc 12.5 (Plant Metabolic Network[1]) was used for annotation purposes. Based on the strategy adopted, identification was carried out according to Level 2 (putatively annotated compounds) of the COSMOS Metabolomics Standards Initiative[2]. The classification of differential compounds into biochemical classes was carried out following PubChem (NCBI[3]) and PlantCyc information.

Data Management and Statistical Analysis

For automatic image data processing, we used the data processing pipeline Plant Data Analyzer, which includes preprocessing, segmentation, feature extraction, and postprocessing of acquired images. Values for projected shoot area were calculated from images taken in the visible light spectrum and correspond to plant volume estimation. The plant volume was used as a proxy for the estimated biomass of the plants. Data were processed using MVApp application. Statistical differences between treatments and time points were determined by one-way analysis of variance (ANOVA) with *post hoc* Tukey's Honest Significant Difference (HSD) test (p-value < 0.05) performed using appropriate scripts in MVApp tool. Data are displayed as mean ± standard error of the six independent plants per treatment.

Elaboration of metabolomic data was carried out using Mass Profiler Professional B.12.06 as previously described (Salehi et al., 2018). Briefly, compounds' abundance was Log2 transformed and normalized at the 75th percentile and then baselined against the median. Unsupervised hierarchical cluster analysis was carried out using the fold-change-based heatmap, setting similarity measure as "Euclidean" and "Wards" linkage rule. Thereafter, the dataset was exported into SIMCA 13 (Umetrics, Malmö, Sweden), Pareto-scaled, and elaborated for Orthogonal Projections to Latent Structures Discriminant Analysis (OPLS-DA). This latter supervised statistic allowed the separation of variance into predictive and orthogonal (i.e., ascribable to technical and biological variation) components. Outliers were excluded using Hotelling's T2 and adopting 95 and 99% confidence limits, for suspect and strong outliers, respectively. Model cross-validation was done through CV-ANOVA (p < 0.01), and permutation testing (N = 300) was used to exclude overfitting. Model parameters (goodness-of-fit R^2Y and goodness-of-prediction Q^2Y) were also produced. Finally, Variable Importance in Projection (VIP) analysis was used to select the metabolites having the highest discrimination potential. A subsequent fold-change analysis and two-way ANOVA were finally performed from VIPs to identify extent and direction of the changes in accumulation related to the use of the biostimulants.

Chemical Similarity Enrichment Analysis (Barupal and Fiehn, 2017) was finally performed on VIP metabolites to critically highlight the chemical nature of the discriminant

[1]http://www.plantcyc.org; released April 2018

[2]http://cosmos-fp7.eu/msi.html

[3]https://pubchem.ncbi.nlm.nih.gov/

compounds, as previously described (Showalter et al., 2018). Such enrichment analysis is based on chemical similarities and used Tanimoto substructure chemical similarity coefficients to cluster metabolites into non-overlapping chemical groups. In our elaborations, OPLS-DA VIP scores were used instead of individual *p*-values, and the regulation (up- or down-accumulation) of discriminant metabolites was compared across treatments following chemical enrichment. The online web-app tool[4] was used for this analysis.

RESULTS

Advanced Simultaneous Analysis of Morpho-Physiological Traits

Integrative phenotyping facilities provide an opportunity to combine various methods of automated, simultaneous, non-destructive analyses for assessment of plant growth, morphology, and physiology. Here, we used the PlantScreen™ Modular System (PSI, Czechia) available in the PSI Research Center (Drásov, Czechia) for simultaneous analysis of multiple morpho-physiological traits in tomato plants treated with plant-derived PH biostimulant substances (**Supplementary Figure S1A**). Tomato plants were cultivated under control conditions and were phenotyped by using RGB imaging to capture plant growth dynamics, morphology and color, by chlorophyll fluorescence (ChlF) imaging to quantify photosynthetic performance and by thermal imaging to analyze leaf surface temperature prior to and following the PH treatment (**Figure 1**). Finally, an automated watering and weighing unit was used to maintain constant low water availability conditions in the tomato plants treated with PH by both drenching and spraying applications (**Supplementary Figure S1B**).

Visible Red Green Blue Imaging to Assess the Effect of Protein Hydrolysate on Plant Growth Dynamics

Visible RGB digital color imaging was used for the assessment of range of visual traits in control plants (no application) and plants treated with PH by either drenching (drench application) or spraying application (foliar application) (**Figures 1A,B**). RGB imaging was used to quantify the effect of the PH on growth status, biomass accumulation, and color of tomato plants cultivated under limited water availability conditions (**Figure 2A**). Simple image stacks acquired from top view and two side view images were used to extract and calculate shoot volume as a proxy of shoot digital biomass and quantify shoot color throughout the cultivation period. The morphological traits were assed dynamically and were used to calculate growth rates (**Figure 2B**).

The analysis of the growth-related above-mentioned traits revealed that tomato plants cultivated under low water availability conditions and treated with PH by either spraying or drenching grew better than control plants. The best-performing plants treated with PH were those where PH was applied as substrate drench. At the end of the phenotyping period, the digital shoot biomass was significantly increased (**Figure 2A** and **Supplementary Tables S1**–**S3**) as well as the height and width of the plants (**Supplementary Tables S4**, **S5**). In addition, the growth rate calculated over the entire phenotyping period was also strongly enhanced in drench treated plants compared to foliarly sprayed ones under limited water availability (**Figure 2B**), suggesting that overall growth performance of the plants was improved following the drenching application of PH. The image-based data could be further confirmed by destructive plant biomass assessment as both fresh and dry weights of the PH-treated plants harvested at the end of the experiment were increased (**Supplementary Figure S2A**). Measurements of projected shoot area obtained using HTP imaging approach were strongly correlated with fresh and dry weights of the plants, and there was no indication of any deviation from a linear relationship even at the highest biomasses measured in this experiment (**Supplementary Figures S2B,C**).

The variation in shoot color of the tomato plants over the phenotyping period was assessed by quantification of greenness hue abundance from the color-segmented RGB images (**Supplementary Figure S3**). The analysis algorithms were calibrated by using RGB images from all treatments and all measurements as described previously (Awlia et al., 2016). Some minor changes were observed in the analyzed green hues, but no clear trend could be observed except for the slight increase in darker green hues at the end of the phenotyping period for the drench application variant (**Supplementary Table S6**).

Mining the Biostimulant Action on Photosynthetic Performance

To assess the effect of PH application on photosynthetic performance of tomato plants under water-limiting conditions, chlorophyll fluorescence measurements were acquired using automated chlorophyll fluorescence imaging setup (**Figure 1C** and **Supplementary Figure S1**). The rate of photosynthesis at different photon irradiances was quantified using the light curve protocol reported by Henley (1993) and Rascher et al. (2000). From the measured fluorescence transient states, the basic ChlF parameters were derived (i.e., F_o, F_m, F_t, and F_v), which were used to calculate a range of parameters characterizing plant photosynthetic performance (i.e., F_v/F_m, NPQ, q_P, and ΦPSII) [for an overview, refer to Paul et al. (2011); Awlia et al. (2016); Tschiersch et al. (2017)]. In addition, photochemical quenching (1 - q_P) and photosynthetic electron transport rate (ETR) parameters were calculated, which refer to proportion of closed PSII reaction centers (Maxwell and Johnson, 2000) and ETR of photosystem II and indicate the efficiency of linear electron flow route in the photosynthetic machinery for producing energy-rich molecules adenosine triphosphate (ATP) and the reduced form of nicotinamide adenine dinucleotide phosphate (NADPH), respectively.

A few of the parameters were selected to dynamically characterize the photosynthetic function of PSII in the tomato plants prior to and after the biostimulant treatment under limited water availability (**Figure 3**): the maximum quantum yield of PSII photochemistry in the dark-adapted state (F_v/F_m),

[4]http://chemrich.fiehnlab.ucdavis.edu

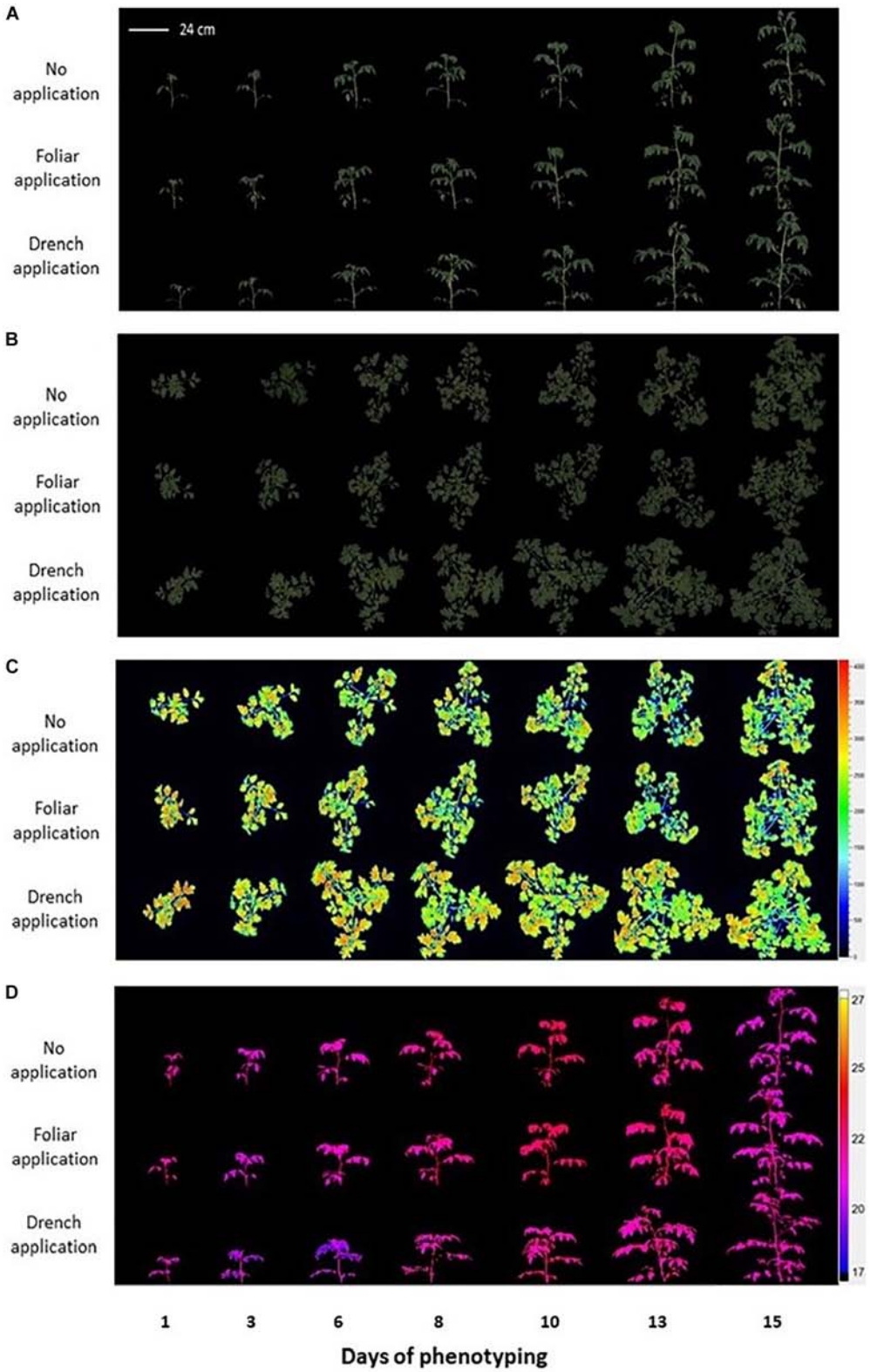


FIGURE 1 | Non-invasive image-based phenotypical analysis of protein hydrolysate treated and control tomato plants grown under water-limiting conditions by using the PlantScreen™ Modular System. **(A)** Color-segmented side view Red Green Blue (RGB) images of the tomato plants over the time of phenotyping period (D1–D15). **(B)** Color-segmented top view RGB images of the tomato plants. **(C)** False-color images of maximum fluorescence value (Fm) of tomato plants captured by kinetic chlorophyll fluorescence imaging. **(D)** False-color side view images of plant leaf surface temperature captured by thermal camera.

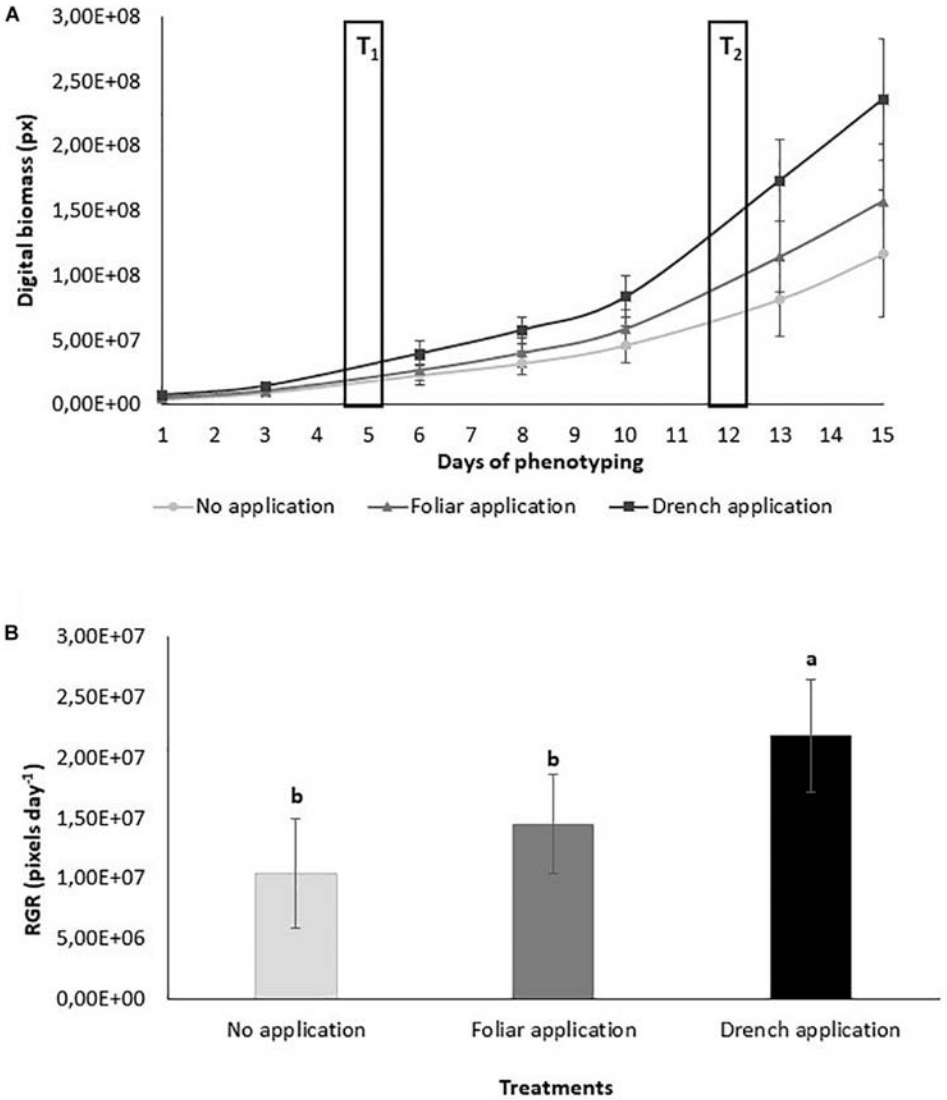


FIGURE 2 | Growth performance of protein hydrolysate treated and control tomato plants. **(A)** Digital biomass quantified over time of phenotyping period. Values represent the average of six biological replicates per treatment. Error bars represent standard deviation. T1 and T2 correspond to days of protein hydrolysate application by foliar spraying or substrate drench. **(B)** Comparison of relative growth rate for the different treatments quantified over phenotyping period following the protein hydrolysate treatments. Values represent the average of six biological replicates per treatment. Error bars represent standard deviation. Different letters indicate significant difference according to one-way ANOVA *post hoc* Tukey's test ($p < 0.05$).

the photochemical quenching coefficient that estimates the fraction of open PSII reaction centers (q_P), steady-state non-photochemical quenching (NPQ), and ETR correlating to the quantum yield of the CO_2 assimilation mechanisms and to the overall photosynthetic capacity of the plants (Genty et al., 1989). No significant changes of those parameters between the control and PH-treated plants (**Figure 3** and **Supplementary Table S7**) were recorded during the phenotyping period. However, minor dynamic changes in lower actinic irradiance of the 1 - q_P parameter were observed at the end of the phenotyping period on day 15 (**Supplementary Figure S4**). 1 - q_P was used as an indicator of the closed PSII reaction center and as an estimate of the relative PSII excitation pressure to which an organism is exposed (Maxwell and Johnson, 2000), suggesting that PH application induced a higher redox status than control treatment, resulting in slightly lowered ETRs (**Supplementary Figure S4**).

Thermal Infrared Imaging for Monitoring Shoot Temperature and Leaf Transpiration

Plant water status is determined by the equilibrium between root water uptake and shoot transpiration (Berger et al., 2010). Under limited water availability in tomato plants, triggering of shoot transpiration and root respiration has been carried out by

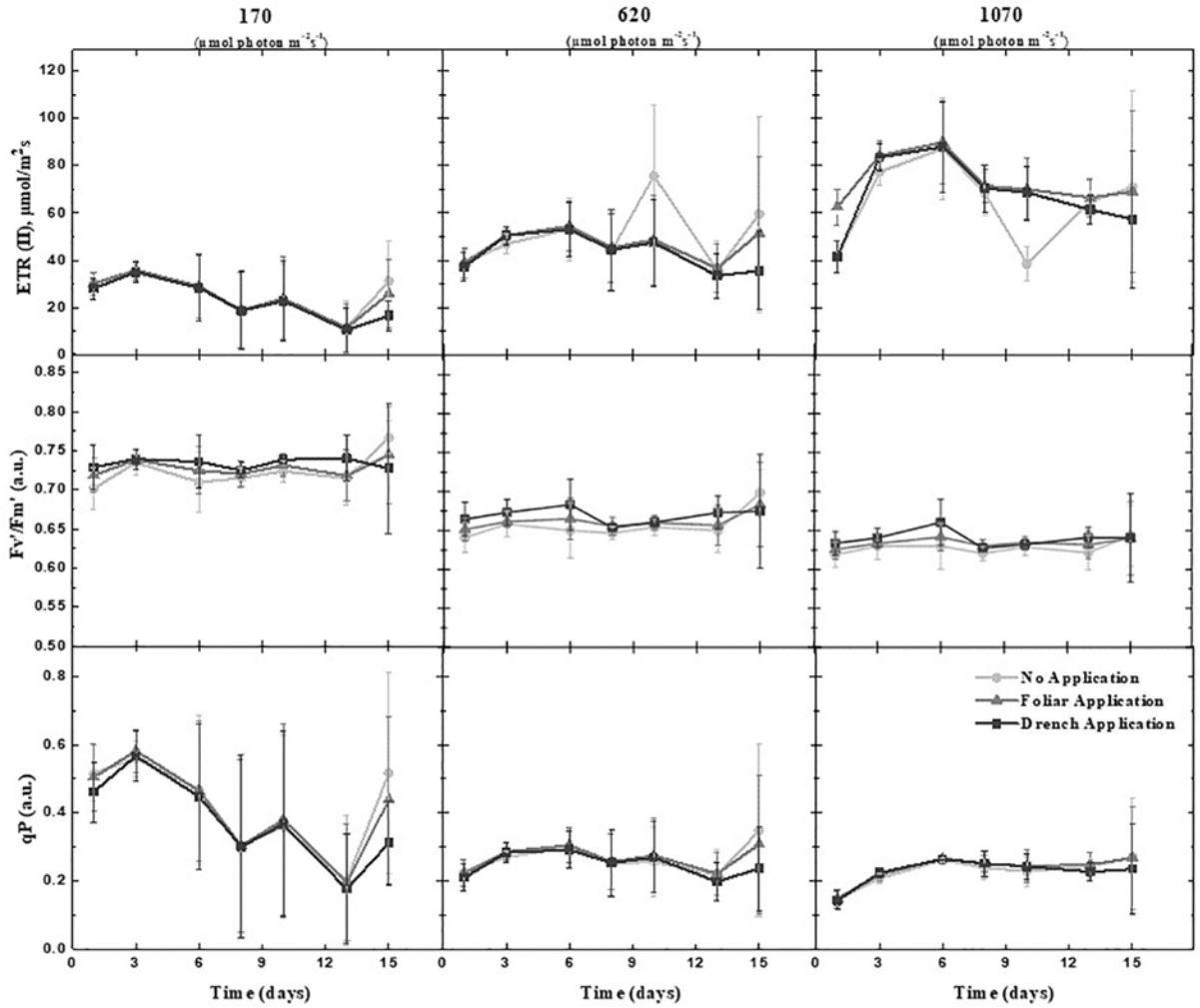


FIGURE 3 | Photosynthetic performance of the tomato plants treated or untreated with protein hydrolysate. Range of photosynthetic parameters were deduced from kinetic chlorophyll fluorescence imaging prior to and following the PH treatments. The photochemical quenching coefficient that estimates the fraction of open PSII reaction centers (q_P), maximum quantum yield of PSII photochemistry for the dark-adapted state (F_V'/F_M'), and electron transport rate (ETR) were measured using the light curve protocol. Data are mean of six independent plants per treatment. Measurements at three actinic photon irradiance intensities were acquired. Measurements were taken at 170, 620, and 1,070 µmol photons m^{-2} s^{-1}, respectively.

commercial PH provided to the plant by foliar and drenching application, respectively. Imaging thermography approach was used to measure the whole plant temperature in an automated manner, and the image data were utilized to assess the leaf transpiration of plants (**Figure 1D**).

To minimize the influence of the environmental variability and the difference in the image acquisition timing among individual plants, the raw temperature of each plant (°C) was normalized by the actual background temperature and expressed as ΔT (°C) (Paul et al., 2016). Experimental data showed that leaf surface temperature of the tomato plants was not influenced by PH treatment, and no difference compared to control plants was observed throughout the entire phenotyping period (**Supplementary Figure S5A**). In addition to leaf surface temperature we assessed TUE that increased in drenching PH-treated plants in comparison with foliar and control treatments (**Supplementary Figure S5B**).

A strong correlation was reported between plant transpiration rate and stomatal conductance (Berger et al., 2010). As stomatal conductance is the measure of the CO_2 entering or leaving the stomata of a leaf, higher TUE observed in PH-drench application might suggest that more CO_2 might get fixed and generate more organic matter, thereby increasing in biomass compared to other treatment methods.

Metabolomic Profiles

An untargeted UHPLC/QTOF-MS metabolomic analysis was carried out to elucidate the molecular mechanisms underlying the effect of PH application on leaves of tomato plants grown under limited water availability. Multivariate statistics from the metabolomic dataset pointed out similarities/dissimilarities among phytochemical profiles. The use of an untargeted profiling followed by annotation on the basis of a comprehensive database (namely, PlantCyc) produced over 1,900 compounds annotated,

overall. These compounds exhibited a large chemical diversity and included metabolites from a wide range of biochemical classes and metabolic processes.

The first step of interpretation was a hierarchical clustering, produced from the fold-change-based heatmap according to Euclidean distances. This unsupervised clustering approach allowed describing similarities/dissimilarities among treatments, as shown in **Figure 4**. As provided, two main clusters were generated–one comprising drench application and the other including foliar application and control. In this latter cluster, two distinct subclusters could be identified, thus indicating different metabolic profiles between foliar application

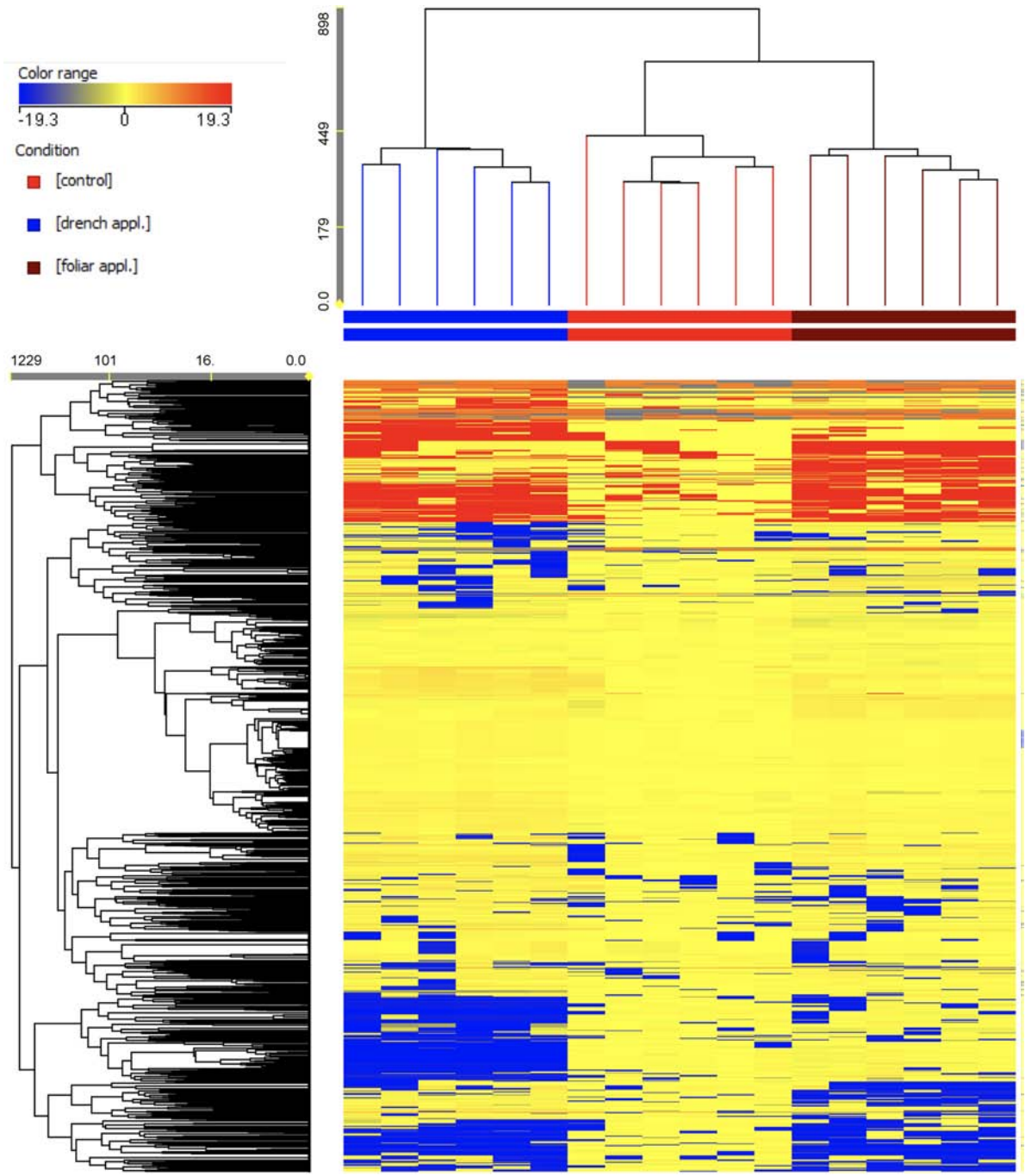


FIGURE 4 | Unsupervised hierarchical cluster analysis (Euclidean similarity; linkage rule: Ward's) carried out from metabolite profiles in tomato leaves from protein hydrolysate treated or untreated plants, as gained from UHPLC liquid chromatograph coupled to a quadrupole-time-of-flight mass spectrometer (UHPLC/QTOF-MS) untargeted metabolomics. Compound intensity was used to produce fold-change-based heat maps, based on which clustering was done.

of the biostimulant and control plants. Even though the application of PHs resulted in distinctive profiles in tomato under limited water availability, the naive (unsupervised) hierarchical clustering of metabolomic signatures suggested that the application method of the PH was an additional and relevant factor determining the actual difference in such phytochemical profiles.

A consistent outcome could be produced through the supervised OPLS-DA multivariate modeling. This analysis allowed separating predictive and orthogonal components (i.e., those components ascribable to technical and biological variation) of variance. Therefore, OPLS-DA effectively discriminated among the three groups into the score plot hyperspace. The OPLS-DA score plot (**Figure 5**) indicated a complete separation among control, foliar, and drench applications. The model parameters of the OPLS-DA regression were excellent, being $R^2Y = 0.99$ and $Q^2Y = 0.94$, respectively. The model was validated (CV-ANOVA $p = 2.47 \times 10^{-10}$) and overfitting could be excluded through permutation testing ($N = 100$). Validation through a misclassification table indicated a 100% model accuracy (Fisher's probability 3.5×10^{-7}). Furthermore, Hotelling's T2 allowed us to exclude suspect and strong outliers. Given the validated model outcomes, the variable selection method called VIP (Variable Importance in Projection) was used to identify compounds explaining the differences observed. The discriminating compounds having a VIP score >1.25 were exported and subjected to fold-change analysis to identify the trends of regulation altered by the treatments. Thereafter, one-way ANOVA (Tukey *post hoc*) was used to describe significance of the differences. The discriminant compounds, together with their VIP score, *P*, and fold-change values, were grouped into chemical classes to facilitate the discussion of results (**Table 1**).

Notably, relatively few biochemical classes included most of the discriminant metabolites. In more detail, lipids (including membrane lipids, sterols, carotenoids, and other terpenes) were the most represented class of compounds among VIP discriminants, followed by phytohormones, polyamine conjugates, prenyl quinones, and chlorophyll-related compounds. Among hormones, brassinosteroids, indole conjugates, salicylate, cytokinins, and two gibberellins were identified among discriminant compounds of treatments (**Table 1**). Furthermore, abietane diterpene resin acids, as well as pteridins and few other compounds, could be outlined by VIP analysis. Interestingly, two osmolytes (trehalose and glycine betaine) were identified among VIP discriminants (**Table 1**).

The following chemical enrichment analysis carried out in chemRICH highlighted sterols (cholestanes, cholestadienols, and hydroxycholesterols), carotenoids, unsaturated fatty acids and phosphatidic acids, terpenes, and coproporphyrins as the most represented chemical groups (**Supplementary Figure S6**). The analysis, carried out separately for each application method (foliar or drench as compared to control), represented differences in accumulation for the selected metabolites. Most of the classes reported exhibited a down-accumulation following biostimulants treatment, as compared to control, except for terpenes (foliar application treatment) and unsaturated fatty acids (drench application treatment).

DISCUSSION

The biostimulant effect on sink and source organs is clearly visible in this study. PH biostimulant directly enters sink areas like the roots through drenching application, while the same biostimulant, foliarly sprayed, directly enters the source region,

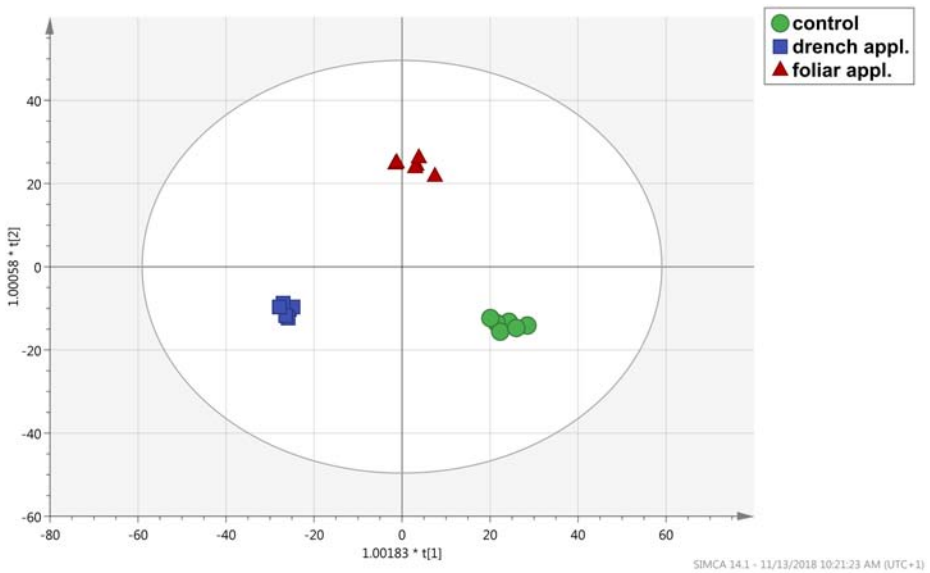


FIGURE 5 | Score plot of Orthogonal Projection to Latent Structures Discriminant Analysis (OPLS-DA) supervised analysis carried out from metabolite profiles in tomato leaves from protein hydrolysate treated or untreated plants, as gained from UHPLC/QTOF-MS untargeted metabolomics.

TABLE 1 | Metabolites discriminating biostimulant-treated tomato plants (foliar and drench application) from control; results were gained from UHPLC/QTOF-MS untargeted metabolomics followed by OPLS-DA supervised statistics.

Compound		VIP score	VIP SE	*p*-Value	Log FC (foliar appl. vs. control)		Log FC (drench appl. vs. control)	
Lipids	A 1-acyl-sn-glycero-3-phosphoethanolamine (n-C14:1)	1.42	0.21	1.41E-24	–17.65	Down	–17.38	Down
	(5Z)-(15S)-11-alpha;-hydroxy-9,15-dioxoprostanoate	1.41	0.27	1.41E-24	–19.81	Down	–19.55	Down
	1-Palmitoyl-2-vernoloyl-phosphatidylcholine	1.39	0.20	2.48E-02	0.18	Up	–8.64	Down
	1-18:1-2-*Trans*-16:1-phosphatidylglycerol	1.39	0.44	2.07E-05	–1.38	Down	0.05	Up
	Dipalmitoyl phosphatidate	1.36	0.37	9.07E-05	0.18	Up	0.38	Up
	Phytosphingosine 1-phosphate	1.36	0.31	6.43E-23	–0.38	Down	–21.52	Down
	Arachidoyl dodecanoate	1.36	0.28	NS	–	–	0.20	Up
	14-Oxolanosterol/4-alpha-formyl,4-beta,14-alpha-dimethyl-9-beta,19-cyclo-5-alpha-cholest-24-en-3-beta-ol	1.35	0.31	1.19E-03	0.13	Up	–15.58	Down
	All-*trans*-heptaprenyl diphosphate	1.33	0.50	3.09E-21	0.34	Up	18.12	Up
	Sphinganine 1-phosphate	1.33	0.36	9.11E-22	–0.37	Down	–21.38	Down
	4-Alpha-formyl-stigmasta-7,24(24^1)-dien-3-beta-ol	1.35	0.31	1.19E-03	0.13	Up	–15.58	Down
	Stearate	1.35	0.57	5.09E-03	13.73	Up	–2.40	Down
	9,10-Epoxy-18-hydroxystearate	1.35	0.55	NS	11.39	Up	10.34	Up
	(9Z)-12,13-Dihydroxyoctadeca-9-enoate	1.35	0.55	2.68E-02	11.39	Up	10.34	Up
	1-18:3-2-18:3-Monogalactosyldiacylglycerol	1.34	0.38	NS	–1.73	Down	–8.88	Down
	1-18:2-2-18:2-Monogalactosyldiacylglycerol	1.35	0.32	NS	–1.69	Down	–4.66	Down
	1-18:3-2-16:2-Monogalactosyldiacylglycerol	1.28	0.34	3.23E-02	–14.89	Down	–3.58	Down
	1-18:2-2-16:1-Phosphatidate	1.31	0.17	6.84E-05	–2.95	Down	–18.17	Down
	Vernoleate	1.38	0.33	4.67E-03	13.71	Up	12.14	Up
	(9R,10S)-Dihydroxystearate	1.34	0.15	NS	4.32	Up	–0.16	Down
	(9S,10S)-9,10-Dihydroxyoctadecanoate	1.34	0.15	NS	4.32	Up	–0.16	Down
	4-Hydroxybutanoate	1.37	0.32	1.01E-08	0.14	Up	3.28	Up
	9-*cis*-10′-apo-beta-carotenal	1.27	0.44	8.61E-04	–10.72	Down	–19.94	Down
	Farnesyl diphosphate	1.27	0.47	4.12E-05	0.63	Up	1.61	Up
	Epsilon, epsilon-carotene-3-diol/beta-carotene 15,15′ epoxide	1.31	0.42	1.57E-03	–17.52	Down	–17.62	Down
	All-*trans*-4,4′-diapolycopene	1.33	0.36	3.24E-12	0.05	Up	–7.17	Down
	Lutein	1.24	0.35	6.84E-05	3.42	Up	–15.52	Down
Resin acids	Palustradienal	1.51	0.37	0.00E+00	23.29	Up	4.07	Up
	Dehydroabietadiene	1.36	0.54	3.75E-04	1.31	Up	0.57	Up
	levopimaradiene/palustradiene/abieta-7,13-diene	1.39	0.35	1.57E-03	1.46	Up	0.22	Up
Triterpenes	Glycyrrhetinate/gypsogenin	1.39	0.22	3.24E-12	0.20	Up	–6.76	Down
	Betulinic aldehyde/ursolic aldehyde/11-oxo-beta-amyrin	1.35	0.31	1.19E-03	0.13	Up	–15.58	Down
Hormones	Gibberellin A98	1.36	0.24	9.07E-24	0.03	Up	–18.86	Down
	Indole-3-acetyl-phenylalanine	1.34	0.34	1.04E-21	–0.42	Down	–19.73	Down
	Indole-3-butyryl-glucose	1.34	0.35	3.97E-22	–0.28	Down	–20.55	Down
	A jasmonoyl-phenylalanine	1.33	0.32	1.59E-21	–0.42	Down	–20.51	Down
	Salicylate	1.29	0.57	NS	13.26	Up	18.76	Up
	Dihydrozeatin-7-N-glucose/dihydrozeatin-9-N-glucose	1.29	0.35	6.30E-05	–3.68	Down	–21.15	Down
	Isopentenyladenine-9-N-glucoside/isopentenyladenine-9-N-glucoside	1.29	0.37	6.30E-05	–3.45	Down	–19.71	Down
	Gibberellin A4/gibberellin A20	1.25	0.62	1.80E-03	0.77	Up	0.39	Up
	7-Oxateasterone	1.30	0.41	2.59E-21	–	–	–20.97	Down
	Cathasterone	1.25	0.66	8.05E-03	2.30	Up	–12.73	Down

(Continued)

TABLE 1 | Continued

Compound		VIP score	VIP SE	*p*-Value	Log FC (foliar appl. vs. control)		Log FC (drench appl. vs. control)	
Osmolytes	Alpha, alpha-trehalose	1.40	0.37	3.68E-02	14.23	Up	0.62	Up
	Glycine betaine	1.33	0.49	1.49E-02	–0.57	Down	–0.23	Down
Polyamines	Triferuloyl spermidine	1.28	0.18	NS	–2.29	Down	–9.75	Down
	Feruloylserotonin	1.34	0.35	1.96E-22	–0.15	Down	–19.56	Down
	Serotonin	1.29	0.41	3.35E-20	–0.30	Down	–18.65	Down
	p-Coumaroyltyramine	1.31	0.46	0.001	3.51	Up	–11.96	Down
	Sinapoyltyramine	1.34	0.18	0.001	18.77	Up	0.60	Up
Pteridins	2-Amino-6-carboxamido-7,8-dihydropteridin-4-one	1.31	0.47	1.97E-02	9.34	Up	10.70	Up
	5,10-Methylenetetrahydropteroyl mono-L-glutamate	1.25	0.25	6.51E-04	–6.33	Down	–18.05	Down
	10-Methyl-5,6,7,8-tetrahydropteroylglutamate	1.37	0.41	1.91E-22	–17.33	Down	–17.06	Down
Chlorophyll	Red chlorophyll catabolite	1.33	0.28	NS	6.73	Up	20.70	Up
	Coproporphyrinogen III	1.32	0.40	0.001	–0.66	Down	–0.87	Down
	Coproporphyrin III	1.34	0.42	0.001	–0.84	Down	–0.54	Down
	Pyropheophorbide *a*	1.31	0.32	NS	0.35	Up	0.83	Up
	Coproporphyrin I	1.26	0.72	0.001	–1.11	Down	–0.99	Down
Quinones	Phylloquinone	1.31	0.37	NS	–	–	–5.22	Down
	Demethylphylloquinol	1.35	0.31	1.19E-03	0.13	Up	–15.58	Down
	2-Heptyl-3-hydroxy-4(1H)-quinolone	1.35	0.41	NS	16.38	Up	22.27	Up
	3″-Hydroxy-geranylhydroquinone	1.34	0.66	1.17E-04	15.86	Up	0.60	Up
Others	(S)-Coclaurine	1.43	0.41	6.24E-05	2.20	Up	1.11	Up
	Coumarinic acid-beta-D-glucoside	1.46	0.17	3.36E-22	–19.86	Down	–0.78	Down
	3-Methoxy-4-hydroxy-5-hexaprenylbenzoate	1.40	0.16	7.52E-12	0.17	Up	–6.09	Down
	A 6-hydroxy-5-isopropenyl-2-methylhexanoate	1.39	0.25	6.70E-05	8.10	Up	7.56	Up
	Casbene	1.39	0.35	1.57E-03	1.46	Up	0.22	Up
	N,N-dihydroxy-L-isoleucine	1.36	0.21	6.93E-10	–0.17	Down	–2.53	Down
	Secologanin	1.36	0.35	8.83E-04	–0.99	Down	–0.38	Down
	Adenosine pentaphosphate	1.35	0.28	0.00E+00	16.57	Up	16.17	Up
	3-Hydroxy-16-methoxy-2,3-dihydrotabersonine	1.34	0.33	3.57E-22	–0.45	Down	–22.12	Down
	Thymidine	1.34	0.52	1.30E-19	–17.85	Down	–17.59	Down
	L-Valine	1.33	0.49	NS	–0.57	Down	–0.23	Down

Compounds are grouped into biochemical classes and are presented with their individual VIP score and standard error (SE), as well as p-Value (one-way ANOVA, Bonferroni multiple testing correction) and Log of fold-change values. NS, not significant ($p > 0.05$). Missing values denote fold-change values < 1.5. VIP, Variable Importance in Projection; UHPLC/QTOF-MS, UHPLC liquid chromatograph coupled to a quadrupole-time-of-flight mass spectrometer; OPLS-DA, Orthogonal Projections to Latent Structures Discriminant Analysis.

the shoot and leaves. This may be reflected in photosynthetic and physiologic functions differently. Regulation of stomatal function is an important mechanism in dealing with the adverse consequences of limited water availability. The typical response of plants to water limitation is stomatal closure, through which the amount of water loss through transpiration can be decreased. On the other hand, water stress-induced closing of stomata also limits CO_2 uptake; therefore, it decreases the efficiency of net photosynthesis. Drenched PH application affected the physiological and metabolic activity of plants. This could be due to enhanced stomatal conductance activity of drench application of PH through the sink region. Russell et al. (2006) reported that other biostimulant substances like humic fractions promoted stomatal opening in pea with a broad biphasic concentration dependence. The extent of opening was similar to that produced by auxin, and a component sensitive to inhibitors of calcium-independent phospholipase A2 was involved in signaling the response to humic fractions and auxin (Russell et al., 2006). Moreover, tomato plants drenched with PH obtained a more favorable balance between carbon gain and water loss as shown by the increase of TUE. The reduced CO_2 uptake imposed by limited water availability causes an imbalance between PSII activity and the following carbon assimilation via the Calvin cycle, thus increasing the excitation energy on PSII and inducing photodamage (Baker and Rosenqvist, 2004).

Furthermore, it is known that the water-related osmotic stress generates a secondary oxidative stress. Reactive oxygen species (ROS) are produced via incomplete reduction of oxygen ($O_2^{\bullet -}$) and are known as signaling molecules integrated with hormone signaling networks (Foyer, 2018). The specific application mode for the PH biostimulant imposed a wide variation of phytohormone profile. Two brassinosteroids (teasterone and cathasterone), a class of sterol-like hormones linked to several signaling networks including abiotic stress response, cell wall

development, and lignification, were detected. In more detail, brassinosteroids are reported to be involved in water stress resistance and osmotic stress-induced stomatal closure as well as to mediate ROS formation, jasmonate signaling, and abscisic acid (ABA) response (Lee et al., 2018; Lucini et al., 2018). ABA and cytokinins antagonistically regulate environmental stress responses in plants, and their integrated and coordinated action modulates drought stress response (Huang et al., 2018). Indeed, cytokinins were down-accumulated, following both foliar and drench application. In plants, cytokinin signaling involves a canonical two-component system that comprises histidine kinases and histidine phosphotransfer proteins. Considering that cytokinin signaling components have been shown to act as negative regulators of plant tolerance to limited water availability (Huang et al., 2018), the trend observed following biostimulant application might represent a significant contribution in water stress resistance. Salicylic acid is another phytohormone that plays a pivotal role in mediating water stress response via modulation of ROS production and redox state (La et al., 2019). Salicylic acid, together with jasmonate, has also been found to enhance water stress tolerance in plants (Li et al., 2018). The application of the PH biostimulant imposed a marked up-accumulation of salicylate, thus potentially modulating with ROS accumulation, ROS-mediated signaling, and tolerance to low water availability. Indeed, salicylate mediates redox balance with an antagonistic depression of ABA (La et al., 2019). Auxins are well-known phytohormones that promote root initiation and delay plant senescence (Li et al., 2018); interestingly, two conjugated forms (i.e., storage forms) of indoleacetic acid (IAA) were found down-accumulated following both PH treatments. The PH-mediated hydrolysis of IAA conjugates may have generated free IAA, leading to stimulation of stomatal opening in PH-treated plants. Besides affecting hormone profile, limited water availability conditions impair the consumption of reduction equivalents for CO_2 fixation, thus resulting in an oversupply of NADPH. Therefore, metabolic processes are expected to push toward the synthesis of highly reduced compounds (Radwan et al., 2017). With this regard, the increase in farnesyl diphosphate and triterpenes is not surprising. Consistently, Nasrollahi et al. (2014) reported a drought-induced accumulation of triterpenes.

Several other lipids, including membrane lipids and carotenoids, were modulated by biostimulant application under limited water availability conditions. Although a clear trend could not be outlined, membrane lipids are known to be altered under plant stress conditions and to play a role in plant adaptation to stress (Allakhverdiev et al., 2001; Lucini et al., 2015; Rouphael et al., 2016). These membrane components are involved in the production of signaling molecules, and they are regulated by plant signaling under abiotic stress (Hou et al., 2016). Indeed, lipid-dependent signaling cascades contribute to trigger plant adaptation processes (Hou et al., 2016).

In the current study, hydroxycinnamic amides (two tyramine derivatives, a serotonin, and a spermidine conjugate) were also induced by biostimulant application. This accumulation was observed for tyramine conjugates. It is interesting to note that biogenic amines and their hydroxycinnamic amides act in plants by interacting with phytohormone cross-talk together with mediating root growth and ROS signaling (Mukherjee, 2018). In particular, tyramine hydroxycinnamic amides are said to also stimulate wound healing and suberization processes (Voynikov et al., 2016). Nonetheless, exogenous polyamines are reported to alleviate the drought-induced detrimental effects as well as to alter auxins, zeatin, gibberellins, salicylic acid, and jasmonate (Li et al., 2018). Abietane diterpene resin acids were also stimulated by the treatment, particularly concerning palustric acid intermediates. These diterpenes are reported to function as antioxidants to protect membranes from oxidative stress (Munné-Bosch et al., 1999) and to display antibacterial and antifungal activity (Helfenstein et al., 2017).

An osmolyte, namely, the trehalose, was found to be up-accumulated following biostimulant treatment under water scarcity. Indeed, the accumulation of sugars, predominantly trehalose, is a known protection mechanism in plants experiencing abiotic stresses, since they contrast protein denaturation, scavenge free radicals, and stabilize biological membranes (Asaf et al., 2017; Farooq et al., 2018). Trehalose, in particular, is able to bind to the polar region of membranes to scavenge the ROS (Farooq et al., 2018).

The involvement of prenyl quinones, generally found up-accumulated, suggests the enrollment of both signaling and antioxidant functions under oxidative stress. The chloroplastic pool of these compounds is related to the oxidation by the cytochrome *b6f* complex as well as to other thylakoid electron transfer pathways. The modulation of such prenyl quinones has been related to their function as signaling molecules in chloroplast-to-nucleus signal transduction and is involved in plant acclimation to stress (Kruk et al., 2016). Finally, among others, intermediates (tetrapyrrole coproporphyrins) and catabolites (pheophorbide *a*) of chlorophyll biosynthetic pathway(s) were identified among VIP discriminants. The former were down-accumulated in treated plants, whereas an opposite trend could be observed for pheophorbide *a*. Ghandchi et al. (2016) reported that the degradation of chlorophyll to non-fluorescent pigments is a transcriptionally regulated intricate process that varies during the plant life cycle. These authors also suggested that the activity of the degrading enzyme pheophorbide *a* oxygenase (PAO) is altered by drought. Nonetheless, it is important to consider that chlorophyll intermediates play a pivotal role also in ROS signaling and production. Photoreduction of oxygen to the superoxide radical is related to a reduced electron transport in PSI and to a reaction linked to the photorespiratory cycle occurring in the peroxisome. This second process is enhanced under drought because of the limited availability of CO_2. Unlike mammals (where ROS are mainly produced in mitochondria), plants produce singlet oxygen mainly in thylakoids by chlorophyll and its tetrapyrrole intermediates in the presence of light. These compounds are partially hydrophobic and are therefore associated with the thylakoid membranes, which do not form pigment protein complexes. Considering that most carotenoids are located in the pigment–protein complexes, they are spatially far from tetrapyrroles and therefore they are poorly effective in quenching their triplet states (Tripathy and Oelmüller, 2012).

Therefore, coproporphyrins act as photosensitizers and their accumulation leads to light-dependent necrosis in plant (Hu et al., 1998; Ishikawa et al., 2001). On this basis, it can be postulated that the biostimulant-related down-accumulation of coproporphyrins under limited water availability can represent a key factor to mitigate ROS imbalance and to improve drought tolerance. Moreover, photosynthetic organisms can dissipate excess energy *via* non-photochemical quenching to avoid singlet oxygen formation; carotenoids play a crucial role in such non-photochemical quenching (Tripathy and Oelmüller, 2012). These findings suggest a complex and coordinated regulation of ROS under limited water availability involving both isoprenoid quinones and tetrapyrrole intermediates. Consistently, several carotenoids, as well as their epoxy- and diol-derivatives, were down-accumulated in biostimulant-treated tomato plants. These findings support and strengthen our previous evidence related to an improved capability of PH-treated tomato plants to cope with ROS-mediated oxidative stress.

Nonetheless, such biochemical reprogramming can be linked to the specific characteristics of PH biostimulants. In fact, it has been reported that peptides in PHs can activate signaling cascades in plant, including the elicitation of defense mechanisms against oxidative stress (Ertani et al., 2009; Percival, 2010; Storer et al., 2016; Lucini et al., 2018). Such cascade of events is typically hormone-mediated (Lucini et al., 2015, 2016, 2018). Some other components of PHs, such as free amino acids, might support the biostimulant activity we observed. A direct provision of glycine and proline might promote osmolyte accumulation, whereas tryptophan is a biosynthetic precursor of indoles and auxins in particular. The direct provision of antioxidant compounds could also be postulated, given the content of phenolics and peptides in the test product. Therefore, a coordinate action of different compounds might have induced the molecular alterations we observed via metabolomics. On the other hand, such classes of biologically active compounds are available to plants following application of PHs. Peptides could enter the leaves through the stoma following foliar application, rather than via ABC membrane transporters following drench application (Boursiac et al., 2013). However, smaller compounds can also use hydrophilic pores in leaves and other transporters in root. In fact, evidence indicated that hydrophilic solutes penetrate cuticles via a physically distinct pathway other than simple diffusion in the cuticle, and they are called "polar pores" (Fernandez and Eichert, 2009).

Therefore, although further investigation is advisable to better elucidate the complex mechanisms of interaction between biostimulants and plant, the modulation of the molecular signatures we observed can be connected to PH application.

CONCLUSION

Our findings indicate that PH application on tomato plants can be considered as a sustainable crop enhancement technology for agricultural productivity under water-limited conditions. Mining of variations in growth dynamics and physiological responses was clearly qualitatively and quantitatively phenotyped using high-throughput phenomic tools. Morpho-physiological data suggest that PH application, especially using the substrate drench method, can be recommended as a highly sustainable approach under less water available conditions. PH application in drenching mode causes plants to transpire more and increase stomatal conductance leading to a better TUE; however, light absorption parameters were unaffected by inducing higher redox status. The UHPLC-QTOF-MS metabolomic approach allowed the identification of the molecular bases of the improved water stress tolerance following biostimulant treatment. Our approach identified a distinct metabolic signature imposed by drench or foliar application of the PH under limited water availability in tomato, as highlighted by both unsupervised hierarchical clustering and supervised discriminant analysis. These outcomes supported and integrated phenomic outcomes, indicating the biochemical processes implicated in the enhanced tolerance to limited water availability following biostimulant application. In more detail, a wide and organized range of metabolic processes was involved in response of tomato plants to PH treatments. Phytohormone profile was significantly affected, even though the most represented among differential compounds were lipids (including membrane lipids, sterols, and terpenes). As a general overview, PH-treated tomato plants exhibited an improved tolerance to ROS-mediated oxidative imbalance. Such tolerance involved a coordinated action of salicylic acid, hydroxycinnamic amide signaling, carotenoids, and prenyl quinone radical scavenging, as well as reduced tetrapyrrole biosynthesis. Finally, further studies are advisable to understand if the biostimulant activity observed with foliar and drench applications of PH is related to changes of microbial community at the leaf or root level.

AUTHOR CONTRIBUTIONS

KeP wrote the first draft of the manuscript, followed the phenotyping measurements, and contributed to phenotype data interpretation. MS performed the big data analysis. LL, MM, and PB performed the metabolomics analysis, data interpretation, and wrote the metabolomic part. KlP, YR, MC, HR, RC, MT, and GC were involved in data analysis, data interpretation, and writing the manuscript. GC and KlP coordinated the whole project, provided the intellectual input, set up the experiments, and corrected the manuscript.

FUNDING

This work was supported by European Union's Horizon 2020 Research and Innovation Program under the Marie Skłodowska-Curie grant agreement no. 675006. This work was also supported by Italpollina Company (Rivoli Veronese, Italy).

ACKNOWLEDGMENTS

We thank Italpollina Company (Rivoli Veronese, Italy) for providing financial support for the trial. We also thank Jaromír

Pytela for helping with the preparation of plant material for the phenotyping experiments at the Photon Systems Instruments (PSI), Research Center (Drásov, Czechia), Zuzana Benedikty for useful discussions during the optimization phase of the phenotyping experiments at PSI, and Petr Polach for helping with raw images reprocessing at PSI.

SUPPLEMENTARY MATERIAL

The Supplementary Material for this article can be found online at: https://www.frontiersin.org/articles/10.3389/fpls.2019.00493/full#supplementary-material

FIGURE S1 | Schematic overview of plant handling and phenotyping protocol. **(A)** Plant phenotyping was carried out in the PlantScreen^{TM} Modular System installed in semi-controlled greenhouse environment conditions in the PSI Research Center. Tomato plants were transferred from a controlled environment to the phenotyping system and automated phenotyping protocol was initiated. Plants were regularly screened using a kinetic chlorophyll fluorescence imaging unit, a calibrated RGB camera for top and multiple-angle side projections, and a thermal imaging unit. A low irrigation level watering regime was maintained by regular weighing and watering (WW) of the plants by an automated WW unit. **(B)** Protein hydrolysate biostimulant application protocol. Tomato plants were treated with PHs either by spraying (foliar application) or by drenching (drench application). Following the PHs application, plants were transferred back to the control environment and were kept under high-humidity conditions for the following 24 h.

FIGURE S2 | Destructive biomass quantification and correlation with digital biomass. **(A)** Fresh and dry weight of tomato shoots harvested following the end of the phenotyping period (day 19). Values represent the average of six biological replicates per treatment. Error bars represent standard deviation. Different letters indicate significant difference according to one-way ANOVA *post hoc* Tukey's test ($p < 0.05$). **(B)** Correlation of digital shoot biomass (px) acquired on day 15 with fresh weight (g) of tomato plants harvested on day 19 of phenotyping period. **(C)** Correlation of digital shoot biomass (px) acquired on day 15 of phenotyping period with dry weight (g) of tomato plants harvested at the end of phenotyping period.

FIGURE S3 | Variation in shoot colors of tomato plants prior to and following the biostimulant treatment. Dynamic relative changes in greenness hue abundance over the phenotyping period in control tomato plants and plants treated with PH either by spraying or drenching. The six most representative color hues are shown in RGB color scale as percentage of the shoot area (pixel counts) of six biological replicates per treatment.

FIGURE S4 | Photosynthetic performance of the tomato plants. The photochemical quenching coefficient that estimates the fraction of closed PSII reaction centers (1 - q_P), steady-state non-photochemical quenching (NPQ), and electron transport rate (ETR) was measured using the light curve protocol. Data are mean of six independent plants per treatment. Measurements at three actinic photon irradiance intensities were acquired. Measurements were taken at 170, 620, and 1070 μmol photons m^{-2} s^{-1}, respectively.

FIGURE S5 | Leaf temperature quantification and estimation of transpiration use efficiency (TUE) in tomato plants prior to and following PH treatment. **(A)** Leaf temperature was quantified by thermal imaging. To minimize the influence of the environmental variability and the difference in the image acquisition timing among individual plants, raw temperature of each plant (°C) was normalized by the actual background temperature. Temperature of leaves of the plants was determined as the difference relative to the surrounding air temperature and was expressed as ΔT (°C). Air temperature data were obtained from a reference surface, which is in thermal equilibrium with air in the background of the plant. **(B)** TUE was estimated from transpiration and growth, measured by water loss and pixel counts over the whole experimental period, respectively. Values represent the average of six biological replicates per treatment. Error bars represent standard deviation. Different letters indicate significant difference according to one-way ANOVA *post hoc* Tukey's test ($p < 0.05$).

FIGURE S6 | Chemical Similarity Enrichment Analysis (ChemRICH) carried out from discriminant metabolites in biostimulant-treated tomato plants. Enrichment analysis is based on chemical similarities and uses Tanimoto substructure chemical similarity coefficients to cluster metabolites into non-overlapping chemical groups. Distinct analyses were performed for foliar **(A)** and drench application **(B)**.

TABLE S1 | Projected shoot area (PSA) of the tomato plants cultivated under limited irrigation and subjected to treatment by PH either by spraying or drenching. PSA values were extracted from multiple side view RGB images and are expressed as number of green pixels and represent the average of six biological replicates per treatment ± standard deviation. Within the same row and for the specified day different letters indicate significant difference according to one-way ANOVA *post hoc* Tukey's test ($p < 0.05$).

TABLE S2 | Projected shoot area (PSA) of the tomato plants cultivated under limited irrigation and subjected to treatment by PH either by spraying or drenching. PSA values were extracted from top view RGB images and are expressed as number of green pixels and represent the average of six biological replicates per treatment ± standard deviation. Within the same row and for the specified day different letters indicate significant difference according to one-way ANOVA *post hoc* Tukey's test ($p < 0.05$).

TABLE S3 | Digital biomass of tomato plants cultivated under limited irrigation and subjected to treatment by PH either by spraying or drenching. Values are expressed as number of green pixels and represent the average of six biological replicates per treatment ± standard deviation. Within the same row and for the specified day different letters indicate significant difference in digital biomass, according to one-way ANOVA *post hoc* Tukey's test ($p < 0.05$).

TABLE S4 | Width of the tomato plants extracted from multiple side view RGB images of the tomato plants cultivated under limited irrigation and subjected to treatment by PH either by spraying or drenching. Values are expressed as number of green pixels and represent the average of six biological replicates per treatment ± standard deviation. Within the same row and for the specified day different letters indicate significant difference according to one-way ANOVA *post hoc* Tukey's test ($p < 0.05$).

TABLE S5 | Height of the tomato plants extracted from multiple side view RGB images of the tomato plants cultivated under limited irrigation and subjected to treatment by PH either by spraying or drenching. Values are expressed as number of green pixels and represent the average of six biological replicates per treatment ± standard deviation. Within the same row and for the specified day different letters indicate significant difference according to one-way ANOVA *post hoc* Tukey's test ($p < 0.05$).

TABLE S6 | Variation in shoot colors of tomato plants cultivated under limited irrigation and subjected to treatment by PHs either by spraying or drenching. The values for 6 most representative color hues are shown as percentage of the shoot area (pixel counts). Values represent the average of six biological replicates per treatment ± standard deviation. Within the same row and for the specified day different letters indicate significant difference according to one-way ANOVA *post hoc* Tukey's test ($p < 0.05$).

TABLE S7 | Photosynthetic performance of tomato plants. Photosynthetic parameters deduced from kinetic chlorophyll fluorescence imaging on whole plant level in tomato plants cultivated under limited irrigation and subjected to treatment by PH either by spraying or drenching. Minimal fluorescence in dark-adapted state (F_0), maximum fluorescence in dark-adapted state (F_M), maximum quantum yield of PSII photochemistry for the light-adapted state (Fvt'/Fmt'), the photochemical quenching coefficient that estimates the fraction of open PSII reaction centers (qP), proportion of closed PSII reaction centers (1-qP), steady-state non-photochemical quenching (NPQ) and electron transport rate (ETR) were measured using the light curve protocol for tomato plants prior and upon two times of PHs treatments. Values represent the average of six biological replicates per treatment ± standard deviation. Within the same row and for the specified day different letters indicate significant difference according to one-way ANOVA *post hoc* Tukey's test ($p < 0.05$). Lss1, Lss2, and Lss3 represent actinic photon irradiance measurements taken at 170, 620, and 1070 μmol photons m^{-2} s^{-1} PAR values, respectively.

REFERENCES

Allakhverdiev, S. I., Kinoshita, M., Inaba, M., Suzuki, I., and Murata, N. (2001). Unsaturated fatty acids in membrane lipids protect the photosynthetic machinery against salt-induced damage in *Synechococcus*. *Plant Physiol.* 125, 1842–1853. doi: 10.1104/pp.125.4.1842

Al-Tamimi, N., Brien, C., Oakey, H., Berger, B., Saade, S., Ho, Y. S., et al. (2016). Salinity tolerance loci revealed in rice using high-throughput non-invasive phenotyping. *Nat. Commun.* 7:13342. doi: 10.1038/ncomms13342

Asaf, S., Khan, A. L., Khan, M. A., Imran, Q. M., Yun, B. W., and Lee, I. J. (2017). Osmoprotective functions conferred to soybean plants via inoculation with *Sphingomonas* sp. LK11 and exogenous trehalose. *Microbiol. Res.* 205, 135–145. doi: 10.1016/j.micres.2017.08.009

Awlia, M., Nigro, A., Fajkus, J., Schmoeckel, S. M., Negrão, S., Santelia, D., et al. (2016). High-throughput non-destructive phenotyping of traits that contribute to salinity tolerance in *Arabidopsis thaliana*. *Front. Plant Sci.* 7:1414. doi: 10.3389/fpls.2016.01414

Baker, N. R., and Rosenqvist, E. (2004). Applications of chlorophyll fluorescence can improve crop production strategies, an examination of future possibilities. *J. Exp. Bot.* 55, 1607–1621. doi: 10.1093/jxb/erh196

Barupal, D. K., and Fiehn, O. (2017). Chemical similarity enrichment analysis (ChemRICH) as alternative to biochemical pathway mapping for metabolomic datasets. *Sci. Rep.* 7:14567. doi: 10.1038/s41598-017-15231-w

Berger, B., Parent, B., and Tester, M. (2010). High-throughput shoot imaging to study drought responses. *J. Exp. Bot.* 61, 3519–3528. doi: 10.1093/jxb/erq201

Borgognone, D., Cardarelli, M., Rea, E., Lucini, L., and Colla, G. (2014). Salinity source-induced changes in yield, mineral composition, phenolic acids and ?avonoids in leaves of artichoke and cardoon grown in ?oating system. *J. Sci. Food Agric.* 94, 1231–1237. doi: 10.1002/jsfa.6403

Borgognone, D., Rouphael, Y., Cardarelli, M., Lucini, L., and Colla, G. (2016). Changes in biomass, mineral composition, and quality of cardoon in response to NO3−:Cl− ratio and nitrate deprivation from the nutrient solution. *Front. Plant Sci.* 7:978. doi: 10.3389/fpls.2016.00978

Boursiac, Y., Léran, S., Corratgé-Faillie, C., Gojon, A., Krouk, G., and Lacombe, B. (2013). ABA transport and transporters. *Trends Plant Sci.* 18, 325–333. doi: 10.1016/j.tplants.2013.01.007

Ceccarelli, A. V. (2018). *Valutazione Dell'attività Biostimolante Mediante Saggi Biologici in Ambiente Controllato*. Master's thesis, University of Tuscia, Viterbo.

Colantoni, A., Recchia, L., Bernabei, G., Cardarelli, M., Rouphael, Y., and Colla, G. (2017). Analyzing the environmental impact of chemically-produced protein hydrolysate from leather waste vs. enzymatically-produced protein hydrolysate from legume grains. *Agriculture* 7:62. doi: 10.3390/agriculture7080062

Colla, G., Cardarelli, M., Bonini, P., and Rouphael, Y. (2017a). Foliar applications of protein hydrolysate, plant and seaweed extracts increase yield but differentially modulate fruit quality of greenhouse tomato. *Hortscience* 52, 1214–1220. doi: 10.21273/HORTSCI12200-17

Colla, G., Hoagland, L., Ruzzi, M., Cardarelli, M., Bonini, P., Canaguier, R., et al. (2017b). Biostimulant action of protein hydrolysates: unravelling their effects on plant physiology and microbiome. *Front. Plant Sci.* 8:2202. doi: 10.3389/fpls.2017.02202

Colla, G., Nardi, S., Cardarelli, M., Ertani, A., Lucini, L., Canaguier, R., et al. (2015). Protein hydrolysates as biostimulants in horticulture. *Sci. Hortic.* 196, 28–38. doi: 10.1016/j.scienta.2015.08.037

du Jardin, P. (2015). Plant biostimulants: definition, concept, main categories and regulation. *Sci. Hortic.* 196, 3–14. doi: 10.1016/j.scienta.2015.09.021

Ertani, A., Cavani, L., Pizzeghello, D., Brandellero, E., Altissimo, A., Ciavatta, C., et al. (2009). Biostimulant activities of two protein hydrolysates on the growth and nitrogen metabolism in maize seedlings. *J. Plant. Nutr. Soil. Sci.* 172, 237–244. doi: 10.1002/jpln.200800174

Farooq, M., Ullah, A., Lee, D. J., Alghamdi, S. S., and Siddique, K. H. M. (2018). Desi chickpea genotypes tolerate drought stress better than kabuli types by modulating germination metabolism, trehalose accumulation, and carbon assimilation. *Plant Physiol. Biochem.* 126, 47–54. doi: 10.1016/j.plaphy.2018.02.020

Fernandez, V., and Eichert, T. (2009). Uptake of hydrophilic solutes through plant leaves: current state of knowledge and perspectives of foliar fertilization. *Crit. Rev. Plant Sci.* 28, 36–68. doi: 10.1080/07352680902743069

Foyer, C. H. (2018). Reactive oxygen species, oxidative signaling and the regulation of photosynthesis. *Environ. Exp. Bot.* 154, 134–142. doi: 10.1016/j.envexpbot.2018.05.003

Genty, B., Briantais, J. M., and Baker, N. R. (1989). The relationship between the quantum yield of photosynthetic electron transport and quenching of chlorophyll fluorescence. *Biochim. Biophys. Acta* 990, 87–92. doi: 10.1016/S0304-4165(89)80016-9

Ghandchi, F. P., Caetano-Anolles, G., Clough, S. J., and Ort, D. R. (2016). Investigating the control of chlorophyll degradation by genomic correlation mining. *PLoS One* 11:e0162327. doi: 10.1371/journal.pone.0162327

Gorbe, E., and Calatayud, A. (2012). Applications of chlorophyll fluorescence imaging technique in horticultural research: a review. *Sci. Hortic.* 138, 24–35. doi: 10.1016/j.scienta.2012.02.002

Haplern, M., Bar-Tal, A., Ofek, M., Minz, D., Muller, T., and Yermiyahu, U. (2015). The use of biostimulants for enhancing nutrient uptake. *Adv. Agron.* 130, 141–174. doi: 10.1016/bs.agron.2014.10.001

Helfenstein, A., Vahermo, M., Nawrot, D. A., Demirci, F., İşcan, G., Krogerus, S., et al. (2017). Antibacterial profiling of abietane-type diterpenoids. *Bioorg. Med. Chem.* 25, 132–137. doi: 10.1016/j.bmc.2016.10.019

Henley, W. J. (1993). Measurement and interpretation of photosynthetic light-response curves in algae in the context of photoinhibition and diel changes. *J. Phycol.* 29, 729–739. doi: 10.1111/j.0022-3646.1993.00729.x

Hou, Q., Ufer, G., and Bartels, D. (2016). Lipid signaling in plant responses to abiotic stress. *Plant Cell Environ.* 39, 1029–1048. doi: 10.1111/pce.12666

Hu, G., Yalpani, N., Briggs, S. P., and Johal, G. S. (1998). A porphyrin pathway impairment is responsible for the phenotype of a dominant disease lesion mimic mutant of maize. *Plant Cell* 10, 1095–1105. doi: 10.1105/tpc.10.7.1095

Huang, X., Hou, L., Meng, J., You, H., Li, Z., Gong, Z., et al. (2018). The antagonistic action of abscisic acid and cytokinin signaling mediates drought stress response in *Arabidopsis*. *Mol. Plant* 11, 970–982. doi: 10.1016/j.molp.2018.05.001

Humplík, J. F., Lazár, D., Fürst, T., Husičková, A., Hýbl, M., and Spíchal, L. (2015a). Automated integrative high-throughput phenotyping of plant shoots: a case study of the cold-tolerance of pea (*Pisum sativum* L.). *Plant Methods* 11:20. doi: 10.1186/s13007-015-0063-9

Humplík, J. F., Lazár, D., Husičková, A., and Spíchal, L. (2015b). Automated phenotyping of plant shoots using imaging methods for analysis of plant stress responses – a review. *Plant Methods* 11:29. doi: 10.1186/s13007-015-0072-8

Ishikawa, A., Okamoto, H., Iwasaki, Y., and Asahi, T. (2001). A deficiency of coproporphyrinogen III oxidase causes lesion formation in *Arabidopsis*. *Plant J.* 27, 89–99. doi: 10.1046/j.1365-313x.2001.01058.x

Kaňa, R., and Vass, I. (2008). Thermoimaging as a tool for studying light-induced heating of leaves: correlation of heat dissipation with the efficiency of photosystem II photochemistry and non-photochemical quenching. *Environ. Exp. Bot.* 64, 90–96. doi: 10.1016/j.envexpbot.2008.02.006

Kruk, J., Szymańska, R., Nowicka, B., and Dłużewska, J. (2016). Function of isoprenoid quinones and chromanols during oxidative stress in plants. *New Biotechnol.* 33(5 Pt B), 636–643. doi: 10.1016/j.nbt.2016.02.010

Kumar, P., Rouphael, Y., Cardarelli, M., and Colla, G. (2017). Vegetable grafting as a tool to improve drought resistance and water use efficiency. *Front. Plant Sci.* 8:1130. doi: 10.3389/fpls.2017.01130

La, V. H., Lee, B. R., Islam, M. T., Park, S. H., Jung, H., Bae, D. W., et al. (2019). Characterization of salicylic acid-mediated modulation of the drought stress responses: reactive oxygen species, proline, and redox state in *Brassica napus*. *Environ. Exp. Bot.* 157, 1–10. doi: 10.1016/j.envexpbot.2018.09.013

Lee, J., Shim, D., Moon, S., Kim, H., Bae, W., Kim, K., et al. (2018). Genome-wide transcriptomic analysis of BR-deficient micro-tom reveals correlations between drought stress tolerance and brassinosteroid signaling in tomato. *Plant Physiol. Biochem.* 127, 553–560. doi: 10.1016/j.plaphy.2018.04.031

Li, L., Gu, W., Li, J., Li, C., Xie, T., Qu, D., et al. (2018). Exogenously applied spermidine alleviates photosynthetic inhibition under drought stress in maize (*Zea mays* L.) seedlings associated with changes in endogenous polyamines and phytohormones. *Plant Physiol. Biochem.* 129, 35–55. doi: 10.1016/j.plaphy.2018.05.017

Li, L., Zhang, Q., and Huang, D. (2014). A review of imaging techniques for plant phenotyping. *Sensors* 14:20078. doi: 10.3390/s141120078

Liu, J., Li, J., Su, X., and Xia, Z. (2014). Grafting improves drought tolerance by regulating antioxidant enzyme activities and stress-responsive gene expression

in tobacco. *Environ. Exp. Bot.* 107, 173–179. doi: 10.1016/j.envexpbot.2014.06.012

Lucini, L., Borgognone, D., Rouphael, Y., Cardarelli, M., Bernardi, J., and Colla, G. (2016). Mild potassium chloride stress alters the mineral composition, hormone network, and phenolic profile in artichoke leaves. *Front. Plant Sci.* 7:948. doi: 10.3389/fpls.2016.00948

Lucini, L., Rouphael, Y., Cardarelli, M., Bonini, P., Baffi, C., and Colla, G. (2018). A vegetal biopolymer-based biostimulant promoted root growth in melon while triggering brassinosteroids and stress-related compounds. *Front. Plant Sci.* 9:472. doi: 10.3389/fpls.2018.00472

Lucini, L., Rouphael, Y., Cardarelli, M., Canaguier, R., Kumar, P., and Colla, G. (2015). The effect of a plant-derived protein hydrolysate on metabolic profiling and crop performance of lettuce grown under saline conditions. *Sci. Hortic.* 182, 124–133. doi: 10.1016/j.scienta.2014.11.022

Luziatelli, F., Ficca, A. G., Colla, G., Baldassarre Švecová, E., and Ruzzi, M. (2019). Foliar application of vegetal-derived bioactive compounds stimulates the growth of bene?cial bacteria and enhances microbiome biodiversity in lettuce. *Front. Plant Sci.* 10:60. doi: 10.3389/fpls.2019.00060

Maxwell, K., and Johnson, G. N. (2000). Chlorophyll fluorescence—a practical guide. *J. Exp. Bot.* 51345, 659–668. doi: 10.1093/jexbot/51.345.659

Mukherjee, S. (2018). Novel perspectives on the molecular crosstalk mechanisms of serotonin and melatonin in plants. *Plant Physiol. Biochem.* 132, 33–45. doi: 10.1016/j.plaphy.2018.08.031

Munné-Bosch, S., Schwarz, K., and Alegre, L. (1999). Response of abietane diterpenes to stress in *Rosmarinus officinalis* L.: new insights into the function of diterpenes in plants. *Free Rad. Res.* 31(Suppl.), S107–S112. doi: 10.1080/10715769900301391

Murchie, E. H., and Lawson, T. (2013). Chlorophyll fluorescence analysis: a guide to good practice and understanding some new applications. *J. Exp. Bot.* 64, 3983–3998. doi: 10.1093/jxb/ert208

Na, Y. W., Jeong, H. J., Lee, S. Y., Choi, H. G., Kim, S. H., and Rho, I. R. (2014). Chlorophyll fluorescence as a diagnostic tool for abiotic stress tolerance in wild and cultivated strawberry species. *Hort. Environ. Biotechnol.* 55, 280–286. doi: 10.1007/s13580-014-0006-9

Nasrollahi, V., Mirzaie-Asl, A., Piri, K., Nazeri, S., and Mehrabi, R. (2014). The effect of drought stress on the expression of key genes involved in the biosynthesis of triterpenoid saponins in liquorice (*Glycyrrhiza glabra*). *Phytochemistry* 103, 32–37. doi: 10.1016/j.phytochem.2014.03.004

Paul, K., Deaìk, Z., Csôsz, M., Purnhauser, L., and Vass, I. (2011). Characterization and early detection of tan spot disease in wheat in vivo with chlorophyll fluorescence imaging. *Acta Biol. Szeged.* 55, 87–90. doi: 10.13140/2.1.3021.6320

Paul, K., Pauk, J., Deaìk, Z., Sass, L., and Vass, I. (2016). Contrasting response of biomass and grain yield to severe drought in cappelle desprez and plainsman V wheat cultivars. *PeerJ* 4:e1708. doi: 10.7717/peerj.1708

Percival, G. C. (2010). Effect of systemic inducing resistance and biostimulant materials on apple scab using a detached leaf bioassay. *Arb. Urb. Forest.* 36, 41–46.

Petrozza, A., Santaniello, A., Summerer, S., Di Tommaso, G., Di Tommaso, D., Paparelli, E., et al. (2014). Physiological responses to megafol® treatments in tomato plants under drought stress: a phenomic and molecular approach. *Sci. Hortic.* 174, 185–192. doi: 10.1016/j.scienta.2014.05.023

Povero, G., Mejia, J. F., Di Tommaso, D., Piaggesi, A., and Warrior, P. (2016). A systematic approach to discover and characterize natural plant biostimulants. *Front. Plant Sci.* 7:435. doi: 10.3389/fpls.2016.00435

Pretali, L., Bernardo, L., Butterfield, T. S., Trevisan, M., and Lucini, L. (2016). Botanical and biological pesticides elicit a similar induced systemic response in tomato (*Solanum lycopersicum*) secondary metabolism. *Phytochemistry* 130, 56–63. doi: 10.1016/j.phytochem.2016.04.002

Radwan, A., Kleinwächter, M., and Selmar, D. (2017). Impact of drought stress on specialised metabolism: biosynthesis and the expression of monoterpene synthases in sage (*Salvia officinalis*). *Phytochemistry* 141, 20–26. doi: 10.1016/j.phytochem.2017.05.005

Rascher, U., Liebig, M., and Lüttge, U. (2000). Evaluation of instant light-response curves of chlorophyll fluorescence parameters obtained with a portable chlorophyll fluorometer on site in the field. *Plant Cell Environ.* 23, 1397–1405. doi: 10.1046/j.1365-3040.2000.00650.x

Rouphael, Y., Cardarelli, M., Schwarz, D., Franken, P., and Colla, G. (2012). "Effects of drought on nutrient uptake and assimilation in vegetable crops," in *Plant Responses to Drought Stress: From Morphological to Molecular Features*, ed. R. Aroca (Berlin: Springer-Verlag).

Rouphael, Y., and Colla, G. (2018). Synergistic biostimulatory action: designing the next generation of plant biostimulants for sustainable agriculture. *Front. Plant Sci.* 9:1655. doi: 10.3389/fpls.2018.01655

Rouphael, Y., Colla, G., Bernardo, L., Kane, D., Trevisan, M., and Lucini, L. (2016). Zinc excess triggered polyamines accumulation in lettuce root metabolome, as compared to osmotic stress under high salinity. *Front. Plant Sci.* 7:842. doi: 10.3389/fpls.2016.00842

Rouphael, Y., Colla, G., Giordano, M., El-Nakhel, C., Kyriacou, M. C., and De Pascale, S. (2017). Foliar applications of a legume-derived protein hydrolysate elicit dose-dependent increases of growth, leaf mineral composition, yield and fruit quality in two greenhouse tomato cultivars. *Sci. Hortic.* 226, 353–360. doi: 10.1016/j.scienta.2017.09.007

Rouphael, Y., Giordano, M., Cardarelli, M., Cozzolino, E., Mori, M., Kyriacou, M. C., et al. (2018a). Plant- and seaweed-based extracts increase yield but differentially modulate nutritional quality of greenhouse spinach through biostimulant action. *Agronomy* 8:126. doi: 10.3390/agronomy8070126

Rouphael, Y., Spìchal, L., Panzarova, K., Casa, R., and Colla, G. (2018b). High-throughput plant phenotyping for developing novel biostimulants: from lab to field or from field to lab? *Front. Plant Sci.* 9:1197. doi: 10.3389/fpls.2018.01197

Russell, L., Stokes, A. R., Macdonald, H., Muscolo, A., and Nardi, S. (2006). Stomatal responses to humic substances and auxin are sensitive to inhibitors of phospholipase A2. *Plant Soil* 283, 175–185. doi: 10.1007/s11104-006-0011-6

Salehi, H., Chehregani, A., Lucini, L., Majd, A., and Gholami, M. (2018). Morphological, proteomic and metabolomic insight into the effect of cerium dioxide nanoparticles to *Phaseolus vulgaris* L. under soil or foliar application. *Sci. Tot. Environ.* 616, 1540–1551. doi: 10.1016/j.scitotenv.2017.10.159

Sestili, F., Rouphael, Y., Cardarelli, M., Pucci, A., Bonini, P., Canaguier, R., et al. (2018). Protein hydrolysate stimulates growth and N uptake in tomato coupled with N-dependent gene expression involved in N assimilation. *Front. Plant Sci.* 9:1233. doi: 10.3389/fpls.2018.01233

Showalter, M. R., Nonnecke, E. B., Linderholm, A. L., Cajka, T., Sa, M. R., Lönnerdal, B., et al. (2018). Obesogenic diets alter metabolism in mice. *PLoS One* 13:e0190632. doi: 10.1371/journal.pone.0190632

Storer, K., Kendall, S., White, C., Roques, S., and Berry, P. (2016). *A Review of the Function, Efficacy and Value of Biostimulant Products Available for UK Cereals and Oilseeds*. (Stoneleigh Park: Agriculture and Horticulture Development Board (ADHB)).

Tripathy, B. C., and Oelmüller, R. (2012). Reactive oxygen species generation and signaling in plants. *Plant Signal. Behav.* 7, 1621–1633. doi: 10.4161/psb.22455

Tschiersch, H., Junker, A., Meyer, R. C., and Altmann, T. (2017). Establishment of integrated protocols for automated high throughput kinetic chlorophyll fluorescence analyses. *Plant Methods* 13:54. doi: 10.1186/s13007-017-0204-4

Voynikov, Y., Zheleva-Dimitrova, D., Gevrenova, R., Lozanov, V., Zaharieva, M. M., Tsvetkova, I., et al. (2016). Hydroxycinnamic acid amide profile of *Solanum schimperianum* hochst by UPLC-HRMS. *Int. J. Mass Spectr.* 408, 42–50. doi: 10.1016/j.ijms.2016.08.008

Conflict of Interest Statement: MT is the owner and CEO of PSI (Photon Systems Instruments), Drásov, Czechia, and KlP is an employee of his company. KeP is an ex-employee of PSI, and an MS and a Ph.D. student both conducted the experiments at PSI. RC is the director of Nixe Company. HR is an employee of Italpollina Company (Anderson, CA, United States). GC is a member of the spin-off company Arcadia approved by Tuscia University, Italy.

The remaining authors declare that the research was conducted in the absence of any commercial or financial relationships that could be construed as a potential conflict of interest.

frontiers in Plant Science

ORIGINAL RESEARCH
published: 12 June 2019
doi: 10.3389/fpls.2019.00758

Hormonal Effects of an Enzymatically Hydrolyzed Animal Protein-Based Biostimulant (Pepton) in Water-Stressed Tomato Plants

Andrea Casadesús[1], Javier Polo[2] and Sergi Munné-Bosch[1,3]*

[1] Department of Evolutionary Biology, Ecology and Environmental Sciences, University of Barcelona, Barcelona, Spain, [2] R&D Department, APC Europe S.L., Granollers, Spain, [3] Institut de Nutrició i Seguretat Alimentària (INSA), University of Barcelona, Barcelona, Spain

Biostimulants may promote growth or alleviate the negative effects of abiotic stress on plant growth eventually resulting in enhanced yields. We examined the mechanism of action of an enzymatically hydrolyzed animal protein-based biostimulant (Pepton), which has previously been shown to benefit growth and yield in several horticultural crops, particularly under stressful conditions. Tomato plants were exposed to well-watered and water-stressed conditions in a greenhouse and the hormonal profiling of leaves was measured during and after the application of Pepton. Results showed that the Pepton application benefited antioxidant protection and exerted a major hormonal effect in leaves of water-stressed tomatoes by increasing the endogenous content of indole-3-acetic acid (auxin), *trans*-zeatin (cytokinin), and jasmonic acid. The enhanced jasmonic acid content may have contributed to an increased production of tocochromanols because plastochromanol-8 concentration per unit of chlorophyll was higher in Pepton-treated plants compared to controls. In conclusion, the tested Pepton application may exert a positive effect on hormonal balance and the antioxidant system of plants under water stress in an economically important crop, such as tomato plants.

Keywords: auxin, biostimulants, cytokinins, defenses, jasmonic acid, tocopherols, tomato

OPEN ACCESS

Edited by:
Oliver Körner, Leibniz-Institut für Gemüse- und Zierpflanzenbau (IGZ), Germany

Reviewed by:
Arafat Abdel Hamed Abdel Latef, South Valley University, Egypt
Klára Kosová, Crop Research Institute (CRI), Czechia

****Correspondence:***
Javier Polo
javier.polo@apc-europe.com

Specialty section:
This article was submitted to Crop and Product Physiology, a section of the journal Frontiers in Plant Science

Received: *20 January 2019*
Accepted: *24 May 2019*
Published: *12 June 2019*

Citation:
Casadesús A, Polo J and Munné-Bosch S (2019) Hormonal Effects of an Enzymatically Hydrolyzed Animal Protein-Based Biostimulant (Pepton) in Water-Stressed Tomato Plants. Front. Plant Sci. 10:758. doi: 10.3389/fpls.2019.00758

INTRODUCTION

Biostimulants are re-emerging as important tools to improve yields and alleviate the negative effects of stress in horticultural crops (du Jardin, 2015). Among the different categories of biostimulants, enzymatically hydrolyzed animal protein-based biostimulants represent a cost-effective approach to alleviate the negative effects of different types of stress in horticultural crops (Polo et al., 2006;

Abbreviations: ABA, abscisic acid; CKs, cytokinins; DW, dry weight; F_v/F_m, photosystem II maximum quantum yield efficiency; GAs, gibberellins; IAA, indole-3-acetic acid; IPA, isopentenyladenosine; JA, jasmonic acid; RWC, relative water content; SA, salicylic acid; *t*Z, *trans*-zeatin; *t*ZR, *trans*-zeatin riboside.

Phelan et al., 2009; Colla et al., 2014, 2017; Polo and Mata, 2018). Pepton 85/16® (Pepton) is a natural biostimulant product obtained by proprietary enzymatic hydrolysis of animal protein available in micro-granular form and highly soluble in water (APC Europe S.L., Spain). Pepton has demonstrated beneficial effects on commercial crops, especially under abiotic stress conditions. Pepton reduced the negative effects caused by intense cold or heat episodes in lettuce and, at the highest inclusion level tested, Pepton completely reversed the negative impact of the cold or heat induced thermal stress (Polo et al., 2006). Similarly, in strawberry plants stressed by being transplanted and subjected to conditions of intense cold ambient temperatures, Pepton application accelerated newly formed roots, flowering, and production of fruit (Marfà et al., 2009). Recently, in a study using gold cherry tomatoes grown under mild stress ambient field conditions, Pepton application at different inclusion levels (from 2 to 4 kg/ha) resulted in a linear improvement of all vegetative growth parameters and yield was 27% higher compared to the control treatment (Polo and Mata, 2018).

Phytohormones are crucial to vegetative growth regulation. Their cross-talk is responsible for the coordination of several plant growth and developmental processes (Davies, 2010). They coordinate most plant developmental processes in response to internal and external factors, with auxins, CKs, and GAs generally promoting vegetative growth (Wolters and Jürgens, 2009; Davies, 2010). Auxin bioactive form (IAA) promotes vegetative growth of the whole plant through polar transport mediated by PIN proteins that result in a basipetal auxin gradient that regulates cell expansion, cell differentiation, morphogenesis and organogenesis (Pacifici et al., 2015). CKs are adenosine- and isoprenoid-derived compounds and the most common bioactive form, *t*Z, plays an essential role in modulation of cell division and the establishment of source-sink relations within the plant (Mok and Mok, 2001). Bioactive GAs (e.g., GA_1 and GA_3) are essential compounds in the regulation of plant size, play a key role in the regulation of cell expansion, and regulate key developmental processes in the plant life cycle, such as seed germination and flower development (Pacifici et al., 2015). Major stress-related phytohormones include ABA, ethylene, jasmonates, and salicylates (Wolters and Jürgens, 2009). ABA, which is derived from carotenoids (violaxanthin or neoxanthin), mediates the response to environmental stress stimuli, i.e., drought stress or salt stress, to typically close stomata, accumulate compatible osmolytes and modulate the expression of stress-related genes that will ultimately reduce vegetative growth and provide tolerance to desiccation (Wolters and Jürgens, 2009; Pacifici et al., 2015). JA is an oxylipin derived from chloroplasts-located fatty acids, such as linolenic acid, with a major role in the coordination of the defense against biotic stress, such as herbivore attack or necrotrophic pathogen infection (Wang and Wu, 2013). Also, JA has been associated with abiotic stress resistance to ultraviolet radiation or ozone, and with different aspects of development (Huang et al., 2017). SA is a phenolic acid derived from chorismate that is also involved in biotic stress response (mainly biotrophic pathogens) retarding plant growth and inducing pathogen-related genes, mainly in interaction with ABA and JA (Ku et al., 2018).

Phytohormones have been previously linked with biostimulants in horticultural crops. Several seaweed and plant extracts have been shown to possess CKs-like and auxin-like activities (Stirk et al., 2014), and also hormonal activity due to the presence of GAs, brassinosteroids, ABA, SA, and/or JA (Stirk et al., 2014; Elzaawely et al., 2016; Kulkarni et al., 2019). Higher endogenous auxin and GAs contents in plants have been reported after biostimulant application using humic substances (Aremu et al., 2015). Abbas (2013) observed higher endogenous ABA contents in *Vicia faba* treated with microbial biostimulants, seaweed extracts, and humic substances. Furthermore, Desoky et al. (2018) showed higher auxins, GAs and CKs contents in sorghum plants under salt stress conditions that were treated with humic substances or vegetable extracts as biostimulants. Despite several examples of hormone-like effects for biostimulants in horticultural crops (Cohen and Bandurski, 1982; Philipson, 1985; Parrado et al., 2008), the mechanism of action of hydrolyzed animal peptides as biostimulants remains largely unknown. Hydrolyzed animal peptides contain several amino acids, in particular aromatic amino acids such as tryptophan and phenylalanine which are precursors for the synthesis of auxin, which has been suggested as a key to explain their action (Dai et al., 2013; Zhao, 2014). Furthermore, glutamic acid, glycine and, to a lesser extent, alanine and arginine have been considered fundamental metabolites in the process of chlorophyll synthesis (Von Wettstein et al., 1995). However, research is needed to better understand possible mechanisms of action of these products in economically interesting crops under stress conditions. Therefore, the aim of this work was to establish a mechanism of action for an enzymatically hydrolyzed animal protein-based biostimulant (Pepton), which has previously been shown to promote growth and yield in several horticultural crops, particularly under stressful conditions.

MATERIALS AND METHODS

Growth Conditions, Treatments, and Samplings

Tomatoes were chosen as a model crop for studying actions of biostimulant products because they are a high value crop produced worldwide with a relatively short growing period and less expensive to use in research studies compared with other crops. In addition, there is considerable information on the variation of physiological components of tomatoes associated with stress conditions. Seeds of tomato (*Lycopersicon esculentum*, var. "Ailsa Craig"), which were obtained from the Experimental Field Facilities of the University of Barcelona, were sown in 1 dm^3 pots under long-day conditions in a growth chamber (12 h light/12 h dark) on March 23, 2018. After 1 month of growth, seedlings were transplanted to 3 dm^3 pots and placed in a greenhouse with a distance between pots of 20 cm. Four treatments were established, including well-watered plants without Pepton, well-watered plants with Pepton, water-stressed plants without Pepton and water-stressed plants with Pepton. The well-watered condition was developed with daily, constant

irrigation of plants with Hoagland nutritive solution maintaining whole pot field capacity, according to their evapotranspirative demand. Water deficit conditions were established by irrigating plants with 0.5 dm^3 of Hoagland nutritive solution every 2 days during the first 6 weeks, and later with 1 dm^3 every 2 days to the end of the treatment period, responding to the increase of evapotranspirative demand as the season progressed toward mid-summer (**Supplementary Figure 1**). Pepton was applied by ferti-irrigation once every 2 weeks at a dose equivalent of 4 Kg/ha, that is 200 mg of Pepton dissolved in 0.5 dm^3 of irrigation water, which corresponds to the highest level of supplier recommendation for this crop. Applications were performed 1 h before sunset. Leaf samples were collected May 10 (week 0, start of the experiment), May 25 (week 2), June 8 (week 4), July 5 (week 8), and August 2 (week 12) at predawn (1 h before sunrise). Two young, fully developed leaves were sampled. One leaf was immediately frozen in liquid nitrogen and stored at −80°C for hormonal profile analyses. The other leaf was used to determine the F_v/F_m *in situ* with the mini-PAM II (Photosynthesis Yield Analyser, Walz, Germany). Then the leaf blade was cut in two symmetric parts. One part was used to determine the RWC [calculated as 100 × (FW − DW)/TW − DW), where FW is the fresh weight, TW is the turgid weight after 24 h immersed in water, and DW is the dry weight after drying in the oven at 80°C]. The other part was immediately frozen in liquid nitrogen and stored at −80°C to perform analyses of tocochromanols and photosynthetic pigments.

Pepton Composition

Pepton is an enzymatically hydrolyzed animal protein product that contains L-α amino acids (84.8%), free amino acids (16.5%), organic-nitrogen (12.0%), iron (3000 ppm), and potassium (4.0%). The average molecular weight distribution of Pepton is around 2,000–3,000 Da, from which 66% of the peptides are considered short-chain (with less than 50 amino acids per chain) and 16% are considered long-chain peptides (>50 amino acids). A complete chemical composition of Pepton is reported by Polo and Mata (2018) and is available as **Supplementary Table 1**.

Hormonal Profiling

Hormonal profiling was performed by liquid chromatography coupled to electrospray ionization tandem mass spectrometry (UHPLC/ESI-MS/MS) as described by Müller and Munné-Bosch (2011). ABA, GA_1 and GA_3, IAA, JA, and SA were analyzed using negative ion mode and the CKs, IPA, *t*Z, and *t*ZR using positive ion mode. Extracts were performed using 100 mg of well powdered fresh leaf with a mixture of methanol and acetic acid (99:1, v/v) as a solvent. Deuterium-labeled plant hormones were added to the extract and 250 mm^3 of the final mixture was

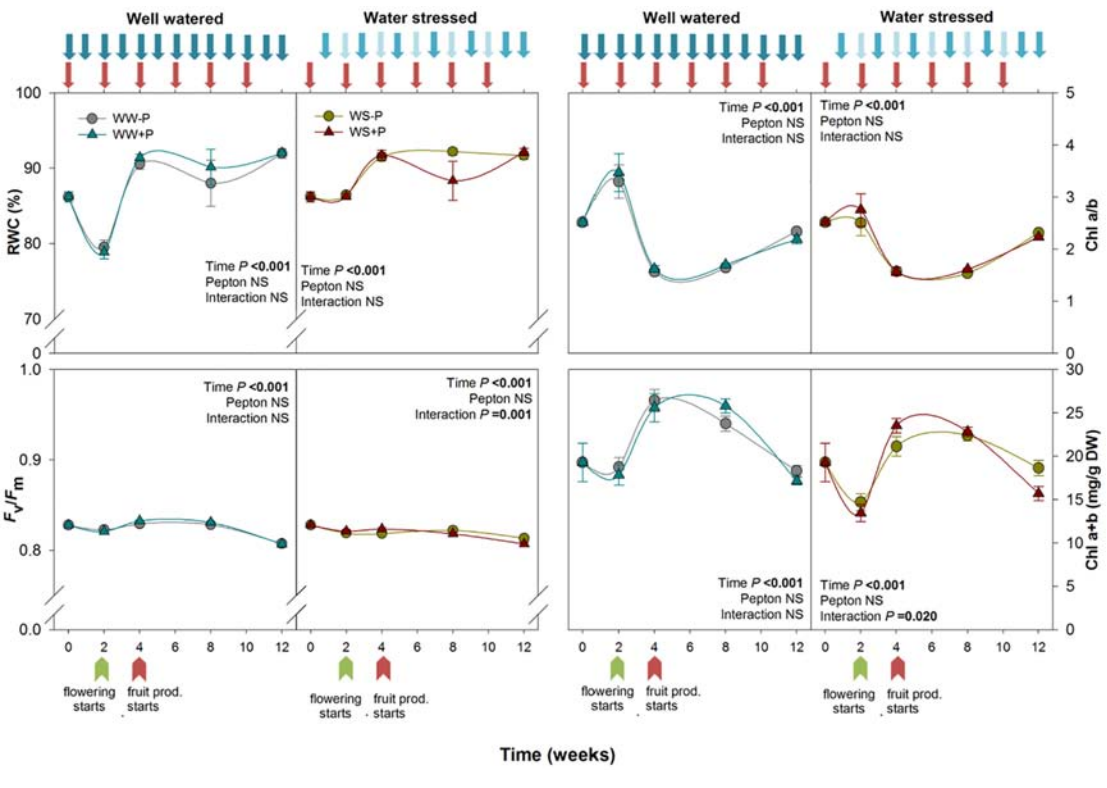


FIGURE 1 | Variations in stress indicators [including the relative water content (RWC), photosystem II maximum quantum yield efficiency (F_v/F_m), chlorophyll *a*/*b* ratio, and total chlorophyll content] in Pepton-treated tomato plants compared to controls under well-watered or water-stressed conditions. Data are the means ± SE of n = 10 individuals. Results of statistics (two-way ANOVA with Tukey HSD *post hoc* test) are shown in the inlets. Arrows represent days of watering and Pepton application.

vortexed and ultra-sonicated for 30 min (Branson 2510 ultrasonic cleaner, Bransonic, Danbury, CT, United States). Then the extract was vortexed again and centrifuged for 10 min at 4°C and 200 g. The supernatant was collected, filtered using a 0.22 µm PTFE filter (Waters, Milford, MA, United States) and injected into the HPLC/ESI-MS/MS system.

Tocochromanols Analyses

Tocochromanols analyses included the determination of α, β, γ, δ-tocopherol, α, β, γ, δ-tocotrienol and plastochromanol-8. Analyses were performed by high-performance liquid chromatography (HPLC) using methanolic extracts as described by Cela et al. (2011). 100 mg of well powdered fresh leaf were used for the extract, and 300 mm^3 was filtered using a 0.22 µm PTFE filter and injected into the HPLC system (consisting of a Waters 600 controller pump, Waters 714 plus auto-sampler and Jasco FP-1520 fluorescence detector). The mobile phase was a mixture of *n*-hexane and 1,4-dioxane (95.5:4.5, v/v) at a flow rate of 0.7 cm^3/min. Tocopherol homologues were separated on a normal-phase column (Inertsil 100A, 5 µm, 30 × 250 mm, GL Sciences Inc., Japan). Fluorescence detection was at an excitation wavelength of 295 nm and emission at 330 nm. Standards of α, β, γ, δ-tocopherol, α, β, γ, δ-tocotrienol and plastochromanol-8 (Sigma-Aldrich) were used for calibration.

Photosynthetic Pigments Analyses

Photosynthetic pigments, including chlorophyll *a*, *b* and total carotenoids, were analyzed using UV/Visible spectroscopy of double beam as described by Oliván and Munné-Bosch (2010). Methanolic extracts were prepared using 100 mg of well powdered fresh leaf and were diluted 1:5 (v/v). The absorbance was read at 470, 653, and 666 nm using a CE Aquarius UV/Visible Spectrophotometer (Cecyl Instruments Ltd., Cambridge, United Kingdom) and pigment concentrations were obtained using the equations developed by Lichtenthaler (1987).

Statistical Analyses

To determine the effect of "Pepton" and "time," multifactorial analyses of two fixed factors using two-way ANOVA and Tukey HSD *post hoc* test were performed. Differences were considered significant when $P \leq 0.05$. Normality and homoscedasticity of residues were checked as described by Zuur et al. (2009). Principal Component Analysis (PCA) was performed using all variables measured, previously standardized in a range value from 0 to 1 to avoid effects of the magnitude of values. All statistical tests were performed using R statistical software (R Foundation for Statistical Computing, Vienna, Austria).

RESULTS AND DISCUSSION

Protein hydrolysates have been demonstrated to exert beneficial effects in alleviating stress in horticultural crops (du Jardin, 2015). The signaling role of amino acids and peptides and their effect on hormone profiling is suggested to play a key role in improving plant performance (Yakhin et al., 2017). Positive effects of protein hydrolysates have been reported under a different type of stress, such as drought, salinity, heavy metals (Phelan et al., 2009; Colla et al., 2017) and thermal stress (Polo et al., 2006; Kauffman et al., 2007; Marfà et al., 2009) but products of animal origin remain poorly studied. Understanding mechanisms of action of animal-derived biostimulants will undoubtedly contribute to better management of horticultural crops under non-favorable conditions which

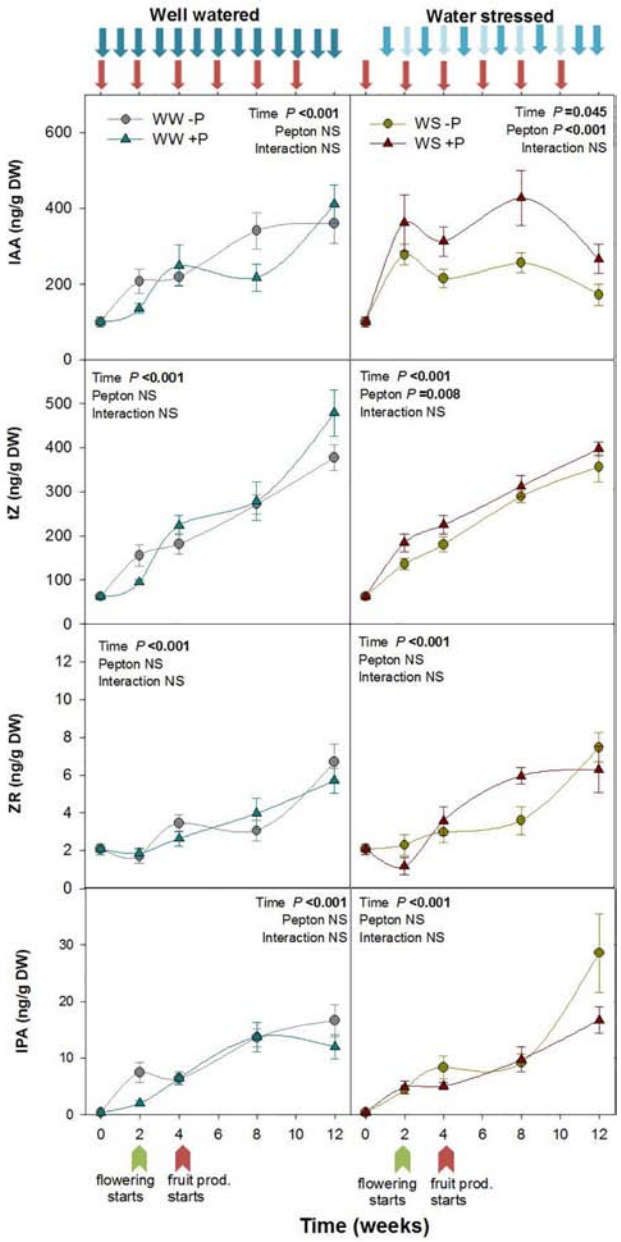


FIGURE 2 | Variations in the endogenous content of auxin and cytokinins in Pepton-treated tomato plants compared to controls under well-watered or water-stressed conditions. Data are the means ± SE of *n* = 10 individuals. Results of statistics (two-way ANOVA with Tukey HSD *post hoc* test) are shown in the inlets. Arrows represent days of watering and Pepton application. DW, dry weight.

are increasingly important in the current global climate change scenario (Le Quéré et al., 2018; Nogueira et al., 2019). In the present study, the average temperature in the greenhouse was 26.3°C. The minimum and maximum mean daily temperatures occurred at the start and end of the experiment (18.5 and 30.9°C during May 13 and August 2, respectively). The mean daily relative humidity during the study was 58.2%, with a maximum of 75.8% during week 4 and a minimum of 46.0% near the end of the experiment (**Supplementary Figure 1**). Both well-watered and water-stressed plants showed RWC above 80% and F_v/F_m values above 0.75 throughout the study. The water deficiency in water-stressed plants was mild compared to irrigated plants because it did not cause differential effects on RWC of fully developed young leaves and the youngest leaves recovered first, but it did cause strong reductions in plant growth. Plant biomass was reduced in water-stressed plants compared to well-watered ones and Pepton slightly alleviated the water-stressed phenotype (**Supplementary Figure 2**). Chlorophyll *a*/*b* ratio ranged between 1.5 and 4 for well-watered plants and between 1.5 and 3 for water-stressed plants, respectively. Total chlorophyll was around 20–25 mg/g DW in well-watered plants, and between 13 and 23 mg/g DW in water-stressed plants. Pepton had no effect on photosynthetic pigments (**Figure 1**).

Pepton Increased Growth-Related Phytohormones Under Stressful Conditions

Recent studies suggest that biostimulants based on protein hydrolysates improve crop performance by stimulating carbon, nitrogen and hormonal metabolism of plants (Colla et al., 2017). Several studies have reviewed hormone-like activity of protein hydrolysates of animal origin in crops (Phelan et al., 2009; Lachhab et al., 2014; Colla et al., 2017). However, in the present study we directly evaluated endogenous changes in hormone profile produced by biostimulant application to better understand how biostimulants can improve crop development

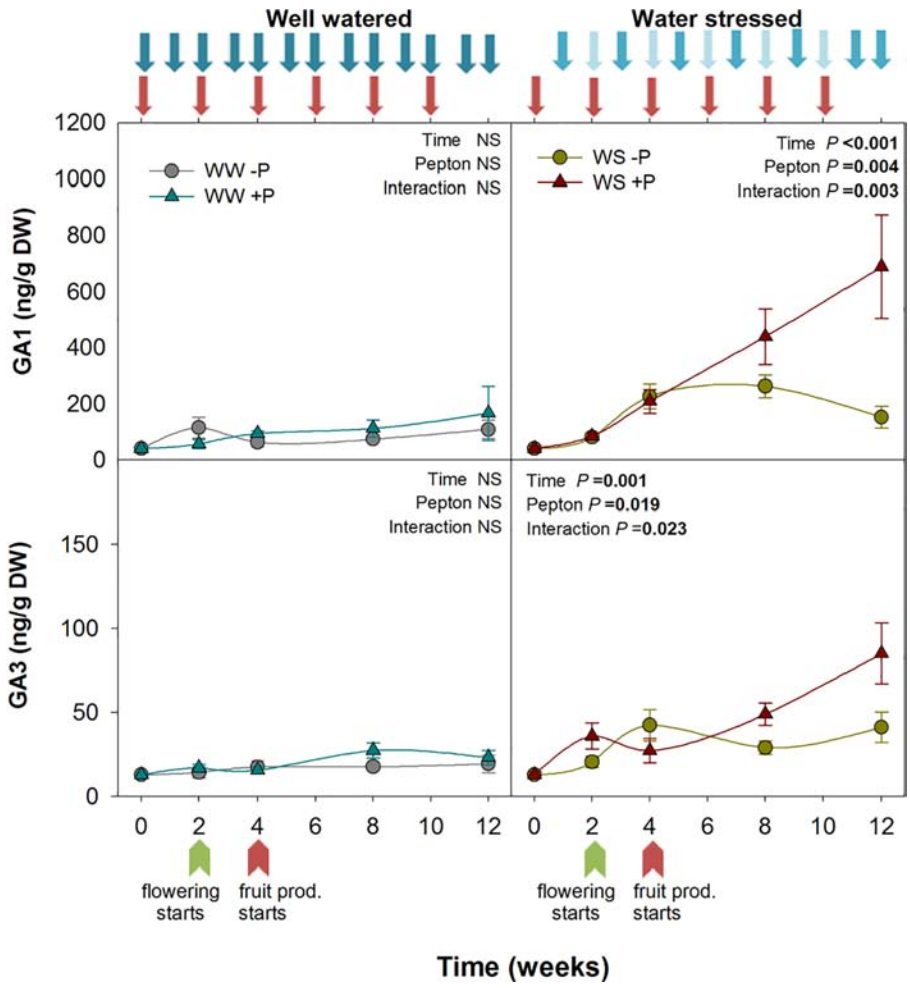


FIGURE 3 | Variations in the endogenous content of gibberellins in Pepton-treated tomato plants compared to controls under well-watered or water-stressed conditions. Data are the means ± SE of n = 10 individuals. Results of statistics (two-way ANOVA with Tukey HSD *post hoc* test) are shown in the inlets. Arrows represent days of watering and Pepton application. DW, dry weight.

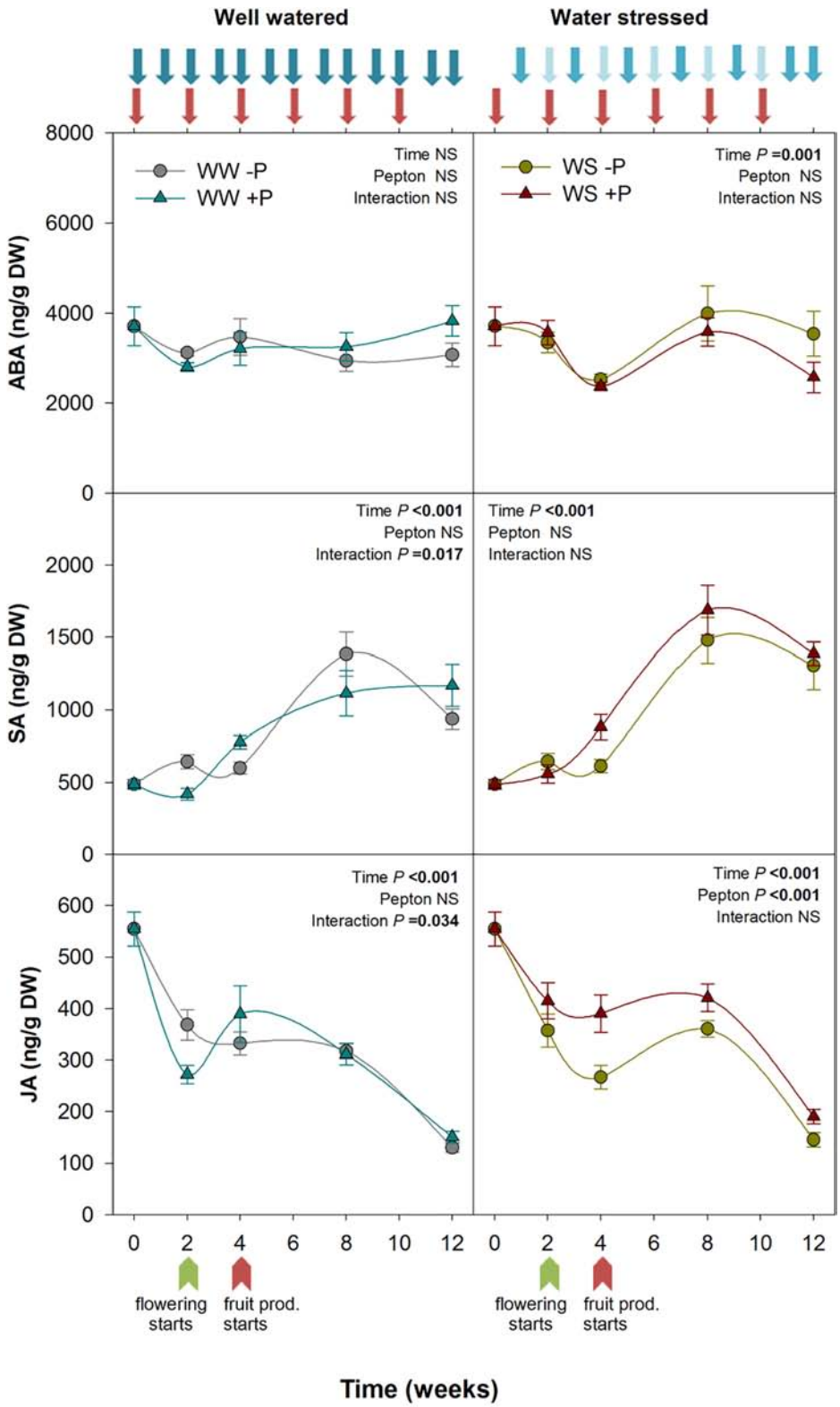


FIGURE 4 | Variations in the endogenous content of abscisic acid, salicylic acid, and jasmonic acid in Pepton-treated tomato plants compared to controls under well-watered or water-stressed conditions. Data are the means ± SE of n = 10 individuals. Results of statistics (two-way ANOVA with Tukey HSD *post hoc* test) are shown in the inlets. Arrows represent days of watering and Pepton application. DW, dry weight.

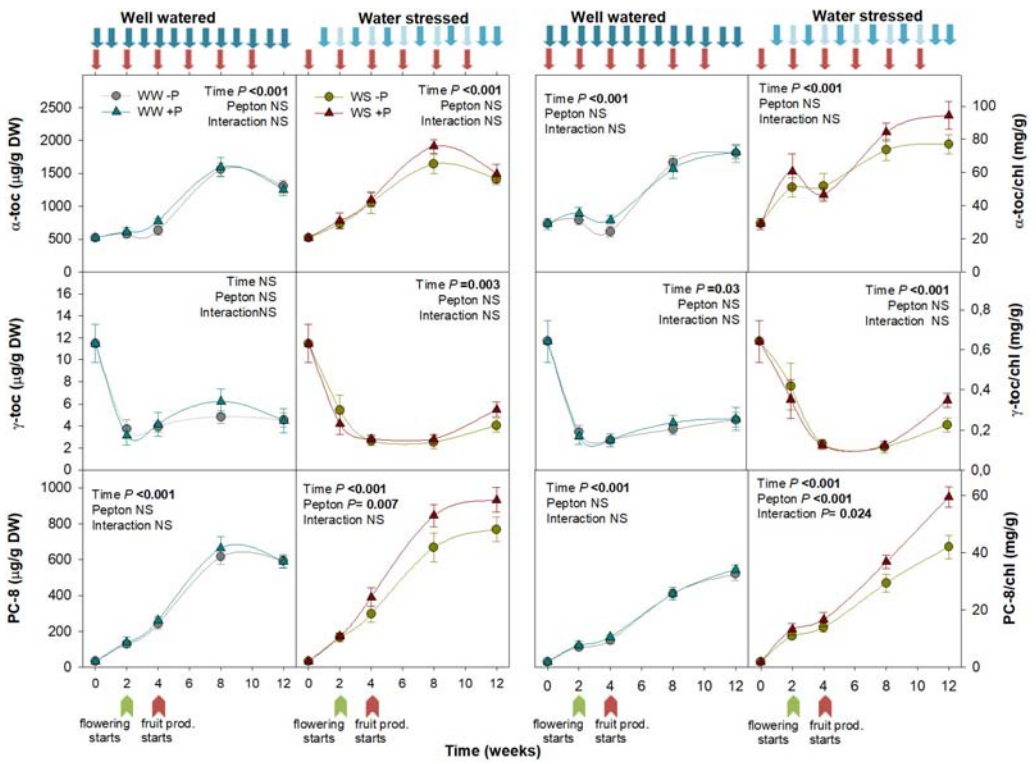


FIGURE 5 | Variations in the endogenous content of tocochromanols in Pepton-treated tomato plants compared to controls under well-watered or water-stressed conditions. Data are the means ± SE of n = 10 individuals. Results of statistics (two-way ANOVA with Tukey HSD *post hoc* test) are shown in the inlets. Arrows represent days of watering and Pepton application. α-toc, α-tocopherol; γ-toc, γ-tocopherol; DW, dry weight; PC-8, plastachromanol-8.

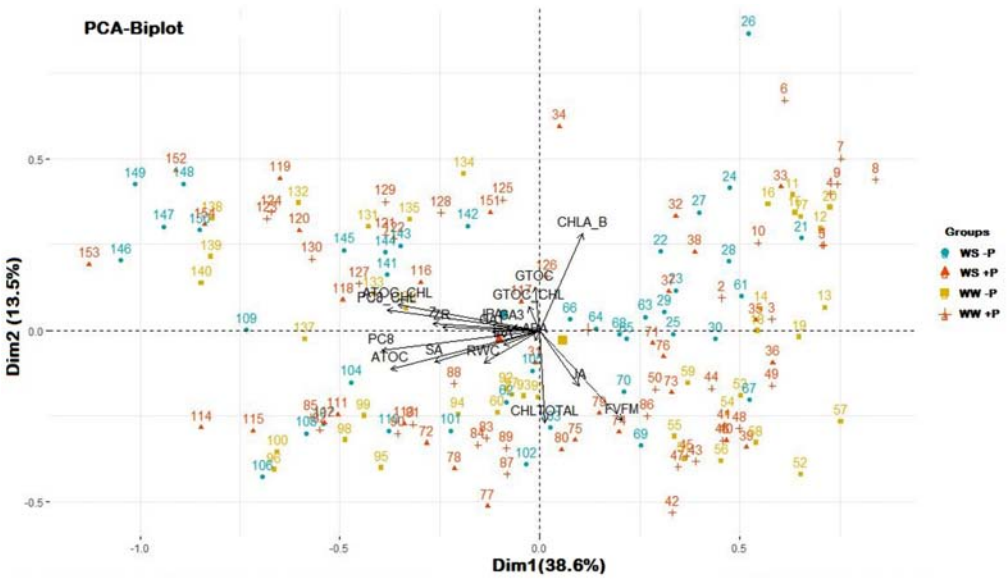


FIGURE 6 | Principal Component Analysis of all variables measured in the study. Numbers represent each observation used to perform the analysis (n = 157) and symbols with colors represent each treatment (WS, water stressed; WW, well-watered; –P, without Pepton application; +P, with Pepton application, as shown in the legend). Symbols without number are the coordinates for a given group, calculated as the mean coordinates of the individuals in the group. Arrows represent each variable measured in the study. Dim1, principal component 1; Dim2, principal component 2. Percentages indicate to what extent the component explains observed variability. ATOC, α-tocopherol; ATOC_CHL, α-tocopherol/chlorophyll; CHLA_B, chlorophyll *a*/chlorophyll *b* ratio; CHLTOTAL, chlorophyll *a* + chlorophyll *b;* GTOC, γ-tocopherol; GTOC_CHL, γ-tocopherol/chlorophyll; PC8, plastochromanol-8; PC8_CHL, plastocromanol-8/chlorophyll.

under stressful environments. Pepton treatment revealed a significant effect on the contents of major growth-related phytohormones, but only under water stress conditions. Under water-stressed conditions, auxin IAA was higher in Pepton-treated plants compared to controls at all sampling times of the study ($P < 0.001$; **Figure 2**). The highest magnitude of differences was observed during weeks 4 and 8 with Pepton-treated plants having 44 to 66% higher auxin IAA than the controls (**Figure 2**). An enhanced growth due to the auxin-like activity has also been previously observed (Colla et al., 2014; Ugolini et al., 2015; Elzaawely et al., 2016; Desoky et al., 2018), in part related to an activation of expansions and other auxin-responsive genes (Ertani et al., 2017), using protein hydrolysate products of plant origin. Additionally, the enhanced production of auxin may also in part be related to the high levels of phenylalanine (5.93%) and moderate levels of Trp (1.25%) in Pepton, two well-known precursors of auxin synthesis.

The active cytokinin (*tZ*) concentration was also higher in Pepton-treated plants compared to controls under water stress conditions ($P = 0.008$) with 25–30% higher tZ observed during weeks 2 and 4. The concentration of non-active CKs, including the ribosides ZR and IPA, did not differ between Pepton-treated plants and controls under water stress or irrigated conditions (**Figure 2**). Elzaawely et al. (2016) and Desoky et al. (2018) also observed higher CKs in leaves using plant extracts as a biostimulant, and the same results were observed by Aremu et al. (2016) and Kulkarni et al. (2019) using seaweed extracts as a biostimulant.

Bioactive GAs are involved in several developmental processes of plants, i.e., plant size or flowering. Pepton treatment also led to significantly higher GA_1 and GA_3 under water stress conditions ($P = 0.004$ and $P = 0.019$, respectively). GA_1 contents increased progressively from the start to the end of the study in Pepton-treated plants, while this increase did not occur in non-treated plants. At week 12, GA_1 and GA_3 contents of Pepton-treated plants were six and threefolds higher, respectively, compared to non-treated plants. GAs did not show the same pattern as other growth related phytohormones under well-watered conditions. Under non-stressful conditions, GA_1 and GA_3 concentrations remained between 40 and 100 ng/g DW and between 13 and 20 ng/g DW for GA_1 and GA_3, respectively, in both Pepton and non-treated plants (**Figure 3**). Other studies using plant extracts as biostimulants have confirmed GAs-like activity of these products with greater shoot elongation (Colla et al., 2014; Ertani et al., 2017; Desoky et al., 2018), and flowering (Elzaawely et al., 2016). Most of these studies attributed this effect to the presence of GAs in the plant extract.

Pepton Improved Defense Response Under Water-Stress Conditions

Stress related phytohormones are crucial to improve plant performance under non-favorable conditions. JAs are closely related to biotic stress (Wolters and Jürgens, 2009) but they are also involved in abiotic stress response such as drought (Jubany-Marí et al., 2010; Riemann et al., 2015; Ahmad et al., 2016). Using protein hydrolysates of plant origin, Ertani et al. (2017) demonstrated higher expression of several ethylene/JA/ABA responsive genes including wound-induced proteins and heat shock proteins which are crucial in both biotic and abiotic stress response. In the present study, we found a significant effect of Pepton application on stress-related phytohormones under stressful conditions, specifically for JA contents ($P < 0.001$; **Figure 4**). Pepton-treated plants under water stress conditions maintained higher JA values than non-treated plants throughout the study. We observed that JA concentrations markedly declined from the start to the end of the study. At week 0, JA was 555 ng/g DW, while by week 4 it had decreased 52% in non-treated plants but only 30% in Pepton-treated plants. By the end of the study, JA in Pepton-treated plants was 66% lower than at the start, but 73% lower in non-treated plants (**Figure 4**). Overall, the enhanced JA content observed in Pepton-treated plants may lead to greater expression of stress responsive genes. The JA biosynthetic pathway has been thoroughly studied in tomato plants. Because it is derived from fatty acids of the cell membrane (Ahmad et al., 2016), the enhanced JA synthesis observed in the present study appears to be indirect. Tejada et al. (2011) reported that animal protein hydrolysate biostimulants stimulate activity of soil microorganisms, which in turn can induce systemic resistance immunity mediated by JAs from below ground organs to above ground parts, triggering JAs signaling in the whole plant (Jung et al., 2012).

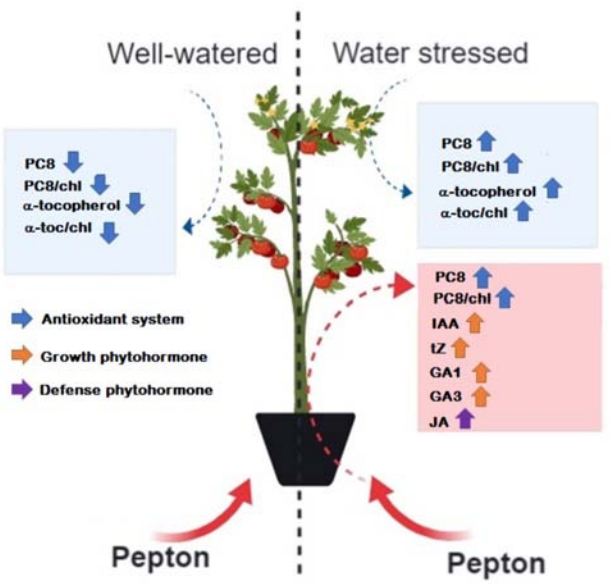


FIGURE 7 | Schematic view of Pepton effects on tomato plants under water stress conditions. Information provided by PCA and ANOVA tests were considered. Blue boxes include main water stress effects on the variables studied as indicated by PCA and red boxes indicate Pepton effects on the variables studied as indicated by ANOVA tests. α-toc, α-tocopherol; chl, chlorophyll; PC8, plastochromanol-8. This figure was made with the help of web-based program BioRender (BioRender, Toronto, Canada).

Pepton Improved Antioxidant Protection Under Water Stress

Pepton significantly affected tocochromanol dynamics under water stress conditions. In particular, we found this biostimulant impacted the plastochromanol-8 content and plastochromanol-8/chlorophyll ratio ($P = 0.007$ and $P < 0.001$, respectively; **Figure 5**). While tocopherols are ubiquitous in photosynthetic tissues of all plant species, plastochromanol-8 and tocotrienols distribution is more limited. Both tocopherols and plastochromanol-8, but not tocotrienols, were found in tomato leaves, which is in agreement with previous studies (Kruk et al., 2014). Higher contents of plastochromanol-8 by the end of our study were probably due to increasing ambient temperature as the season progressed. Plastochromanol-8 and tocopherols have been shown to provide stress tolerance (Loyola et al., 2012; Fleta-Soriano and Munné-Bosch, 2017). Limited evidence of improved tocochromanol contents using biostimulants have been reported. Zhang and Schmidt (2000) observed enhanced α-tocopherol contents using hormone containing products and humic substances. In our study, the significant effect of Pepton on higher plastochromanol-8 content indicates that Pepton improves the antioxidant capacity of tomato plants. Interestingly, the higher plastochromanol-8 content was observed in parallel with higher JA content in Pepton-treated plants exposed to water stress. Since jasmonates can increase the expression of the gene encoding tyrosine aminotransferase (Sandorf and Holländer-Czytko, 2002), which is involved in the biosynthesis of homogentisate, it is likely that Pepton-related effects on jasmonates may exert a positive effect on platochromanol-8 accumulation. Additionally, the content of tyrosine (1.92%) in Pepton can partially explain the increase observed in tococromanols.

A PCA was performed to unravel the main component explaining variability in this study (**Figure 6**). Principal component 1 (Dim1) captured 38.6% of the variance observed with plastochromanol-8, expressed as per unit of chlorophyll or per dry weight, as the greatest variable (17.52 and 16.39% of Dim1, respectively), followed by α-tocopherol and α-tocopherol/chlorophyll ratio. Observations were scattered through principal component 2 (Dim 2), which explained 13.5% of the whole variance, driven by the chlorophyll *a*/*b* ratio (25.19% of Dim2), chlorophylls (23.22% of Dim2) and F_v/F_m ratio (22.05% of Dim2), but no clustering produced by treatments was observed. Even though observations were not clustered among treatments, they separated coordinate means in two groups through Dim1, WW, and WS, suggesting that factor "irrigation" may drive main variables involved in Dim1, which is in accordance with their response in stressful conditions. Antioxidants and chlorophyll related variables show opposite directions (Loyola et al., 2012; Cao et al., 2015; Fleta-Soriano and Munné-Bosch, 2017).

CONCLUSION

It is concluded from the results obtained in this study that the enzymatically hydrolyzed animal protein-based biostimulant (Pepton) exerts a positive effect on the hormonal profile of tomato leaves and enhances abiotic defenses under water stress conditions, including defense-related phytohormones and antioxidants (a summary is depicted in **Figure 7**). Additional research is needed to more fully understand the mechanisms of action of hydrolyzed animal protein-based biostimulants to provide more accurate guidelines for its application in horticultural and agroecosystems.

AUTHOR CONTRIBUTIONS

JP and SM-B conceived and designed the experiments with the help of AC. AC wrote the manuscript with the help of JP and SM-B. AC prepared all the figures. All authors contributed to the discussion of ideas, revised, and approved the final manuscript.

FUNDING

This work was partially supported by APC Europe S.L., manufacturer of Pepton, the biostimulant used in this experiment.

ACKNOWLEDGMENTS

The authors are deeply indebted to Alba Arabia and Ricard Pujolriu for their help and assistance in the execution of the experiment and the sampling process.

SUPPLEMENTARY MATERIAL

The Supplementary Material for this article can be found online at: https://www.frontiersin.org/articles/10.3389/fpls.2019.00758/full#supplementary-material

REFERENCES

Abbas, S. (2013). The influence of biostimulants on the growth and on the biochemical composition of *Vicia faba* CV. *Giza* 3 beans. *Rom. Biotechnol. Lett.* 18, 8061–8068.

Ahmad, P., Rasool, S., Gul, A., Sheikh, S., Akram, N., Ashraf, M., et al. (2016). Jasmonates: multifunctional roles in stress tolerance. *Front. Plant Sci.* 7:813. doi: 10.3389/fpls.2016.00813

Aremu, A., Plačková, L., Gruz, J., Bíba, O., Novák, O., Stirk, W. A., et al. (2016). Seaweed-derived biostimulant (Kelpak®) influences endogenous cytokinins and bioactive compounds in hydroponically grown *Eucomis autumnalis*. *J. Plant Growth Regul.* 35, 151–162. doi: 10.1007/s00344-015-9515-8

Aremu, A., Stirk, A., Kulkarni, M., Tarkowská, D., Turečková, V., Gruz, J., et al. (2015). Evidence of phytohormones and phenolic acids variability in garden-waste-derived vermicompost leachate, a well-known plant growth stimulant. *Plant Growth Regul.* 75, 483–492. doi: 10.1007/s10725-014-0011-0

Cao, B., Ma, Q., Zhao, Q., Wang, L., and Xu, K. (2015). Effects of silicon on absorbed light allocation, antioxidant enzymes and ultrastructure of chloroplasts in tomato leaves under simulated drought stress. *Sci. Hortic.* 194, 53–62. doi: 10.1016/j.scienta.2015.07.037

Cela, J., Chang, C., and Munné-Bosch, S. (2011). Accumulation of γ-rather than α-tocopherol alters ethylene signaling gene expression in the vte4 mutant of *Arabidopsis thaliana*. *Plant Cell Physiol.* 52, 1389–1400. doi: 10.1093/pcp/pcr085

Cohen, J. D., and Bandurski, R. S. (1982). Chemistry and physiology of bound auxins. *Annu. Rev. Plant Physiol.* 33, 403–430. doi: 10.1146/annurev.pp.33.060182.002155

Colla, G., Hoagland, L., Ruzzi, M., Cardarelli, M., Bonini, P., Canaguier, R., et al. (2017). Biostimulant action of protein hydrolysates: Unraveling their effects on plant physiology and microbiome. *Front. Plant Sci.* 8:2202. doi: 10.3389/fpls.2017.02202

Colla, G., Rouphael, Y., Canaguier, R., Svecova, E., and Cardarelli, M. (2014). Biostimulant action of a plant-derived protein hydrolysate produced through enzymatic hydrolysis. *Front. Plant Sci.* 5:448. doi: 10.3389/fpls.2014.00448

Dai, X., Mashiguchi, K., Chen, Q., Kasahara, H., Kamiya, Y., Ojha, S., et al. (2013). The biochemical mechanism of auxin biosynthesis by an Arabidopsis YUCCA flavin-containing monooxygenase. *J. Biol. Chem.* 288, 1448–1457. doi: 10.1074/jbc.M112.424077

Davies, P. J. (ed.) (2010). *Plant Hormones: Biosynthesis, Signal Transduction, Action!*. Dordrecht: Springer.

Desoky, E. S., Merwad, A. R., and Rady, M. (2018). Natural biostimulants improve saline soil characteristics and salt stressed-sorghum performance. *Commun. Soil Sci. Plant Anal.* 49, 967–983. doi: 10.1080/00103624.2018.1448861

du Jardin, P. (2015). Plant biostimulants: definition, concept, main categories and regulation. *Sci. Hortic.* 16, 3–14. doi: 10.1016/j.scienta.2015.09.021

Elzaawely, A., Ahmed, M., Maswada, H., and Xuan, T. (2016). Enhancing growth, yield, biochemical and hormonal contents of snap bean (*Phaseolus vulgaris* L.) sprayed with moringa leaf extract. *Arch. Agron. Soil Sci.* 63, 687–699. doi: 10.1080/03650340.2016.1234042

Ertani, A., Schiavon, M., and Nardi, S. (2017). Transcriptome-wide identification of differentially expressed genes in *Solanum lycopersicon* L. in response to an alfalfa-protein hydrolysate using microarrays. *Front. Plan Sci.* 8:1159. doi: 10.3389/fpls.2017.01159

Fleta-Soriano, E., and Munné-Bosch, S. (2017). Enhanced plastochromanol-8 accumulation during reiterated drought in maize (*Zea mays* L.). *Plant Physiol. Biochem.* 112, 283–289. doi: 10.1016/j.plaphy.2017.01.016

Huang, H., Liu, B., Liu, L., and Song, S. (2017). Jasmonate action in plant growth and development. *J. Exp. Bot.* 68, 1349–1359. doi: 10.1093/jxb/erw495

Jubany-Marí, T., Prinsen, E., Munné-Bosch, S., and Alegre, L. (2010). The timing of methyl jasmonate, hydrogen peroxide and ascorbate accumulation during water deficit and subsequent recovery in the Mediterranean shrub *Cistus albidus L. Environ. Exp. Bot.* 69, 47–55. doi: 10.1016/j.envexpbot.2010.02.003

Jung, S., Martinez-Medina, A., Lopez-Raez, J., and Pozo, M. (2012). Mycorrhiza-induced resistance and priming of plant defenses. *J. Chem. Ecol.* 38, 651–664. doi: 10.1007/s10886-012-0134-6

Kauffman, G. L. III, Kneivel, D. P., and Watschke, T. L. (2007). Effects of a biostimulant on the heat tolerance associated with photosynthetic capacity, membrane thermos stability, and polyphenol production of perennial ryegrass. *Crop Sci.* 47, 261–267. doi: 10.2135/cropsci2006.03.0171

Kruk, J., Szymańska, R., Cela, J., and Munné-Bosch, S. (2014). Plastochromanol-8: fifty years of research. *Phytochemistry* 108, 9–16. doi: 10.1016/j.phytochem.2014.09.011

Ku, Y. S., Sintaha, M., Cheung, M. Y., and Lam, H. M. (2018). Plant hormone signaling corsstalks between biotic and abiotic stress responses. *Int. J. Mol. Sci.* 19:3206. doi: 10.3390/ijms19103206

Kulkarni, M., Rengasamy, K., Pendota, S., Gruz, J., Plačková, L., Novák, O., et al. (2019). Bioactive molecules derived from smoke and seaweed *Ecklonia maxima* showing phytohormone-like activity in *Spinaca oleracea* L. *New Biotechnol.* 48, 83–89. doi: 10.1016/j.nbt.2018.08.004

Lachhab, N., Sanzani, S. M., Adrian, M., Chiltz, A., Balacey, S., Boselli, M., et al. (2014). Soybean and casein hydrolysates induce grapevine immune responses and resistance against *Plasmopara viticola*. *Front. Plant Sci.* 5:716. doi: 10.3389/fpls.2014.00716

Le Quéré, C., Andrew, R., Friedlingstein, P., Sitch, S., Hauck, J., Pongratz, J., et al. (2018). Global carbon budget 2018. *Earth Syst. Sci. Data* 10, 2141–2194.

Lichtenthaler, H. K. (1987). Chlorophylls and: pigments of photosynthetic biomembranes. *Meth. Enzymol.* 148, 350–382. doi: 10.1016/0076-6879(87)48036-1

Loyola, J., Verdugo, I., González, E., Casaretto, J., and Ruiz-Lara, S. (2012). Plastidic isoprenoid biosynthesis in tomato: physiological and molecular analysis in genotypes resistant and sensitive to drought stress. *Plant Biol.* 14, 149–156. doi: 10.1111/j.1438-8677.2011.00465.x

Marfà, O., Cáceres, R., Polo, J., and Ródenas, J. (2009). Animal Protein Hydrolysate as a biostimulant for transplanted strawberry plants subjected to cold stress. *Acta Hortic.* 842, 315–318. doi: 10.17660/actahortic.2009.842.57

Mok, D., and Mok, M. (2001). Cytokinin metabolism and action. *Annu. Rev. Plant Physiol. Plant Mol. Biol.* 52, 89–118.

Müller, M., and Munné-Bosch, S. (2011). Rapid and sensitive hormonal profiling of complex plant samples by liquid chromatography coupled to electrospray ionization tandem mass spectrometry. *Plant Methods* 7:37. doi: 10.1186/1746-4811-7-37

Nogueira, C., Werner, C., Rodrigues, A., and Caldeira, M. (2019). A prolonged dry season and nitrogen deposition interactively affect CO2 fluxes in an annual Mediterranean grassland. *Sci. Total Environ.* 654, 978–986. doi: 10.1016/j.scitotenv.2018.11.091

Oliván, A., and Munné-Bosch, S. (2010). Diurnal patterns of α-tocopherol accumulation in Mediterranean plants. *J. Arid Environ.* 74, 1572–1576. doi: 10.1016/j.jaridenv.2010.05.003

Pacifici, E., Polverari, L., and Sabatini, S. (2015). Plant hormone cross-talk: the pivot of root growth. *J. Exp. Bot.* 66, 1113–1121. doi: 10.1093/jxb/eru534

Parrado, J., Bautista, J., Romero, E. J., Garcia-Martinez, A. M., Friaza, V., and Tejada, M. (2008). Production of a carob enzymatic extract: potential use as a biofertilizer. *Bioresour. Technol.* 99, 2312–2318. doi: 10.1016/j.biortech.2007.05.029

Phelan, M., Aherne, A., FitzGerald, J., and O'Brien, N. M. (2009). Casein-derived bioactive peptides: biological effects, industrial uses, safety aspects and regulatory status. *Int. Dairy J.* 19, 643–654. doi: 10.1016/j.idairyj.2009.06.001

Philipson, J. J. (1985). The promotion of flowering in large field-grown Sitka spruce by girdling and stern injections of gibberellin A4/7. *Can. J. Forest Res.* 15, 166–170. doi: 10.1139/x85-027

Polo, J., Barroso, R., Ródenas, J., Azcón-Bieto, J., Cácerres, R., and Marfá, O. (2006). Porcine hemoglobin hydrolysate as a biostimulant for lettuce plants subjected to conditions of thermal stress. *HortTechnology* 16, 483–487. doi: 10.21273/horttech.16.3.0483

Polo, J., and Mata, P. (2018). Evaluation of a biostimulant (Pepton) based in enzymatic hydrolyzed animal protein in comparison to seaweed extracts on root development, vegetative growth, flowering, and yield of gold cherry tomatoes grown under low stress ambient field conditions. *Front. Plant Sci.* 8:2261. doi: 10.3389/fpls.2017.02261

Riemann, M., Dhakarey, R., Hazman, M., Miro, B., Kohli, A., and Nick, P. (2015). Exploring jasmonates in the hormonal network of drought and salinity responses. *Front. Plant Sci.* 6:1077. doi: 10.3389/fpls.2015.01077

Sandorf, I., and Holländer-Czytko, H. (2002). Jasmonate is involved in the induction of tyrosine aminotransferase and tocopherol biosynthesis in *Arabidopsis thaliana*. *Planta* 216, 173–179. doi: 10.1007/s00425-002-0888-0

Stirk, W., Tarkowská, D., Turečová, V., Strnad, M., and Staden, J. (2014). Abscisic acid, gibberellins and brassinosteroids in Kelpak®, a commercial seaweed extract made from *Ecklonia maxima*. *J. Appl. Phycol.* 26, 561–567. doi: 10.1007/s10811-013-0062-z

Tejada, M., Benítez, C., Gómez, I., and Parrado, J. (2011). Use of biostimulants on soil restoration: effects on soil biochemical properties and microbial community. *Appl. Soil Ecol.* 49, 11–17. doi: 10.1016/j.apsoil.2011.07.009

Ugolini, L., Cinti, S., Righetti, L., Stefan, A., Matteo, R., D'Avino, L., et al. (2015). Production of an enzymatic protein hydrolysate from defatted sunflower seed meal for potential application as a plant biostimulant. *Ind. Crops Prod.* 75, 15–23. doi: 10.1016/j.indcrop.2014.11.026

Von Wettstein, D., Gough, S., and Kannangara, C. G. (1995). Chlorophyll biosynthesis. *Plant Cell.* 7, 1039–1057.

Wang, L., and Wu, J. (2013). The essential role of jasmonic acid in plant-herbivore interactions- using the wild tobacco *Nicotiana attenuata* as a model. *J. Genet. Genomics* 40, 597–606. doi: 10.1016/j.jgg.2013.10.001

Wolters, H., and Jürgens, G. (2009). Survival of the flexible: hormonal growth control and adaptation in plant development. *Nat. Rev.* 10, 305–317. doi: 10.1038/nrg2558

Yakhin, O., Lubyanov, A., Yakhin, I., and Brown, P. (2017). Biostimulants in plant science: a global perspective. *Front. Plant Sci.* 7:2049. doi: 10.3389/fpls.2016.02049

Zhang, X., and Schmidt, R. E. (2000). Hormone containing products impact on antioxidant status of tall fescue and creeping bent grass subjected to drought. *Crop Sci.* 40, 1344–1349.

Zhao, Y. (2014). Auxin biosynthesis. *Arabidopsis Book Am. Soc. Plant Biol.* 12:e0173. doi: 10.1199/tab.0173

Zuur, A., Ieno, E., and Elphick, C. (2009). A protocol for data exploration to avoid common statistical problems. *Methods Ecol. Evol.* 1, 3–14. doi: 10.1111/j.2041-210x.2009.00001.x

Conflict of Interest Statement: JP is employed by APC Europe S.L., Granollers, Spain.

The remaining authors declare that the research was conducted in the absence of any commercial or financial relationships that could be construed as a potential conflict of interest.

frontiers in Microbiology

ORIGINAL RESEARCH
published: 23 July 2019
doi: 10.3389/fmicb.2019.01639

Isolation and Characterization of Halotolerant Plant Growth Promoting Rhizobacteria From Durum Wheat (*Triticum turgidum* subsp. *durum*) Cultivated in Saline Areas of the Dead Sea Region

Randa N. Albdaiwi[1], Hala Khyami-Horani[1], Jamal Y. Ayad[2], Kholoud M. Alananbeh[3] and Rabea Al-Sayaydeh[4]*

[1] Department of Biological Sciences, Faculty of Science, The University of Jordan, Amman, Jordan, [2] Department of Horticulture and Crop Science, Faculty of Agriculture, The University of Jordan, Amman, Jordan, [3] Department of Plant Protection, Faculty of Agriculture, The University of Jordan, Amman, Jordan, [4] Shoubak College, Al-Balqa' Applied University, Shoubak, Jordan

OPEN ACCESS

Edited by:
Youssef Rouphael, University of Naples Federico II, Italy

Reviewed by:
Zisis Vryzas, Democritus University of Thrace, Greece
Mariateresa Cardarelli, Council for Agricultural and Economics Research, Italy

***Correspondence:**
Hala Khyami-Horani
horani-h@ju.edu.jo

Specialty section:
This article was submitted to Plant Microbe Interactions, a section of the journal Frontiers in Microbiology

Received: *25 August 2018*
Accepted: *02 July 2019*
Published: *23 July 2019*

Citation:
Albdaiwi RN, Khyami-Horani H, Ayad JY, Alananbeh KM and Al-Sayaydeh R (2019) Isolation and Characterization of Halotolerant Plant Growth Promoting Rhizobacteria From Durum Wheat (Triticum turgidum subsp. durum) Cultivated in Saline Areas of the Dead Sea Region. Front. Microbiol. 10:1639. doi: 10.3389/fmicb.2019.01639

Plant growth promoting rhizobacteria (PGPR) are beneficial microorganisms that can be utilized to improve plant responses against biotic and abiotic stresses. In this study, 74 halotolerant bacterial isolates were isolated from rhizosphere and endorhizosphere of durum wheat (*Triticum turgidum* subsp. *durum*) plants cultivated in saline environments in the Ghor region near the east of the Dead Sea. *16S rDNA* partial sequences and phylogenetic analysis of 62 isolates showed clear clustering of the isolates into three phyla: Firmicutes (61.3%), Proteobacteria (29.0%), and Actinobacteria (9.7%). At the genus level, the majority of them were grouped within the *Bacillus, Oceanobacillus,* and *Halomonas* genera. The isolates, which possessed plant growth promoting traits including nitrogen fixation, ACC deaminase activity, auxin production, inorganic phosphate solubilization and siderophore production, were selected. The effect of the inoculation of selected PGPR strains on growth of salt sensitive and salt tolerant durum wheat genotypes under high salt stress conditions was evaluated. Six halotolerant PGPR strains were able to improve survival in inoculated plants under high salinity stress conditions as reflected in higher germination percentages and seedling root growth when compared with non-inoculated plants. Furthermore, three halotolerant PGPR strains were able to improve durum wheat tolerance to water deficit stress. In addition, antagonistic effect in four halotolerant PGPR strains against an aggressive pathogenic isolate of *Fusarium culmorum* that causes crown rot disease was observed in a dual culture assay. In conclusion, the halotolerant PGPR strains described in this study might have great potential to improve durum wheat productivity under different stress conditions.

Keywords: ACC deaminase, durum wheat, endophyte, halotolerant PGPR, salinity

INTRODUCTION

Agricultural productivity is severely affected by major biotic and abiotic factors including drought, salinity, extreme temperatures and pathogens, which can limit the growth and development of any given crop. Salinity is an adverse condition affecting crop productivity in arid and semi-arid areas around the world where it causes an annual loss of 1–2% of arable land (Shrivastava and Kumar, 2015). Salinity alters cellular metabolism causing many physiological, morphological, biochemical, and molecular changes in plants (Gupta and Huang, 2014). Salinity impact on plant growth and development is primarily due to imposing osmotic stress, which has an immediate effect on water availability, accumulation of toxic ions such as Na^+ and Cl^- in the cells, nutrient imbalances, and oxidative stress damage (Munns and Tester, 2008).

Wheat (*Triticum* spp.) is considered one of the most important crops in the world and it is a staple food for over 35% of the world's population where it provides more calories and proteins than any other cultivated crop (FAOSTAT, 2017). Durum wheat (*Triticum turgidum* subsp. *durum*) is grown on 10% of all wheat cultivated areas in the world, and it is a major cereal crop in the Mediterranean region. Several studies indicated that the wild progenitor of modern durum wheat is widely distributed in the Jordan Valley region nearby the Dead Sea (Nevo et al., 2013) with archeological evidences of durum wheat utilization near the Dead Sea region before 9500 years ago (Weide, 2015).

High salt stress has more pronounced effects on durum wheat growth and development when compared with other cereals (Munns et al., 2006). This is mainly due to its inability to exclude Na^+ from its tissues (Roy et al., 2014). Several approaches were used to reduce salinity effects on durum wheat, which included proper soil practices and irrigation managements (Katerji et al., 2009) as well as traditional breeding and genetic engineering (Munns et al., 2006).

Microorganisms associated with the rhizosphere play a significant role in alleviating salt stress in plants resulting in better crop productivity (Etesami and Beattie, 2018). Among these, bacteria known as plant growth promoting rhizobacteria (PGPR), may possess multiple plant growth promoting traits able to increase plant growth and yield of crops and useful in directly and indirectly alleviating the effect of abiotic stresses. Directly, PGPR facilitate plant nutrients uptake from surrounding environments by producing siderophores to sequester iron and/or by phosphorus solubilization and/or by nitrogen fixation (Etesami and Beattie, 2018). Furthermore, PGPR can modulate plant growth by providing phytohormones such as indole acetic acid (IAA) or reducing the ethylene production by the activity of the 1-Aminocyclopropane-1-carboxylate (ACC) deaminase enzyme (Glick, 2014). On the other hand, indirect plant growth promotion by PGPR occurs when they limit or prevent plant damage caused by pathogenic agents such as bacteria, fungi and nematodes (Compant et al., 2005). Several studies have been conducted in order to understand the role of halotolerant PGPR on alleviating salinity damages on wheat plant (reviewed in Numan et al., 2018), with few reports on durum wheat. For instance, the inoculation of *Waha* durum wheat cultivar with *Azospirillum brasilense* NH strain, isolated from a saline soil in northern Algeria, improved the growth under salt stress conditions (Nabti et al., 2010).

The main objective of this study is to isolate halotolerant PGP rhizospheric and endophytic bacteria associated with durum wheat grown in saline manifested soils near the eastern side of the Dead Sea. The isolates were partially identified by *16S* rDNA sequencing and tested *in vitro* for their abilities to promote plant growth. Selected halotolerant PGPR isolates were evaluated for growth promoting ability in durum wheat plants grown under different levels of salinity.

MATERIALS AND METHODS

Collection Sites

During February and March 2017, soil and plant samples were collected from fields cultivated with durum wheat cv. *Haurani*, from three sites near the northern and eastern sides of the Dead Sea (−430 m, latitude: 31.3333° N, longitude: 35.5000° E): Ghor Haditha, Ghor Safi, and Ghor Sweimeh (**Supplementary Table S1**). These regions are characterized by an arid and very warm Mediterranean climate with a mean annual precipitation less than 100 mm and maximum temperatures above 40°C during summer (AL-Zu'bi and Al-Kharabsheh, 2003). From each field, three soil and three plant samples were collected in sterile plastic bags and placed in ice packs before their transfer to the laboratory of Plant Biotechnology, Faculty of Agriculture, The University of Jordan. Collected soil samples were analyzed for chemical and physical properties (**Supplementary Table S1**): the soil texture was analyzed by the Hydrometer method, organic matter by the Walkley-Black method and Electrical Conductivity (EC) and pH by saturated paste extract method (Estefan et al., 2014).

Isolation of Rhizospheric and Endophytic Bacteria

To isolate rhizospheric bacteria, plant roots were gently shaken to remove the clumps of loosely adhering soil to the roots, leaving behind the root-firmly adhering soil particles (rhizospheric soil), which was then suspended and vortexed in 100 ml of sterile 1% NaCl solution. Thereafter, 10 folded serial dilutions were prepared and 100 μl from each diluent were plated on nutrient agar (NA) medium (Thermo Fisher Scientific Oxoid, Ltd., Basingstoke, United Kingdom) supplemented with 10% NaCl. The plates were incubated at 28°C and monitored for colony formation up to 1 week (Fischer et al., 2007).

For the isolation of endophytic bacteria, the roots of the collected samples were washed carefully under running tap water for 10 min to remove adhering soil particles. The roots were disinfected with 70% ethanol for 1 min, then rinsed three times with sterile distilled water. The roots were then surface sterilized with 3% sodium hypochlorite solution containing few drops of Tween 20® (Sigma-Aldrich, Steinheim, Germany) for 10 min followed by six rinses with sterile distilled water. To confirm root surface sterilization efficiency, an aliquot (100 μl) from the sixth wash solution was spread on NA supplemented with 10% NaCl

and incubated at 28°C for 5 days. Thereafter, 1 g of the surface sterilized root tissue was macerated with a sterilized mortar and pestle in 10 ml of 1% NaCl solution, and serial dilutions were prepared and 1 ml from the tissue extract and the diluents were spread on NA medium supplemented with 10% NaCl. The plates were incubated at 28°C and monitored up to 1 week for bacterial colony formation (Ramadoss et al., 2013).

The colonies of rhizospheric and endophytic bacterial isolates were examined morphologically for their shape, size, margin, elevation, appearance, texture, pigmentation, and optical properties. In addition, cellular morphology, shape, gram staining and endospore formation were also examined (Robinson et al., 2016) (**Supplementary Table S2**). Colonies with distinct morphological characteristics were selected and purified by subculturing three times on NA media supplemented with 10% NaCl, before their storage in a 40% glycerol solution at −80°C till further use. Each sample was given a code representing the collection site (GHD: Ghor Haditha, GSF: Ghor Safi, and GSW: Ghor Sweimeh), followed by either R for Rhizospheric or E for Endophytic and by the isolate number (**Supplementary Table S2**).

Halotolerance Assay

Bacterial isolates were screened for halotolerance using NA media supplemented with various levels of NaCl (1, 5, 10, 15, 20, and 25%). The plates were inoculated with fixed volumes of starter inoculum (OD_{600} = 0.05) and the cultures were incubated for 7 days at 28°C (Ramadoss et al., 2013).

16S rDNA Sequencing and Phylogenetic Analysis

The selected bacterial isolates were partially identified by *16S rDNA* sequencing and phylogenetic analysis. For this purpose, 5 ml cultures grown in liquid NA media were used for total genomic DNA isolation using the Wizard® Genomic DNA Purification Kit (Promega, Madison, WI, United States) following the manufacturer's instruction. The isolated gDNA from each isolate was assessed by gel electrophoresis in a 1% agarose gel stained with Red Safe (Intron, BioTek, South Korea) and visualized by using Gel Doc™ XR Gel Documentation System (Bio-Rad, Hercules, CA, United States). The DNA concentration and quality were determined by spectrophotometry (Smart-Spec™ plus spectrophotometer, Bio-Rad, Hercules, CA, United States) and a stock solution (30 ng/μl) for each isolate was prepared and stored at −20°C for further use.

For the amplification of the *16S rDNA* region, PCR assays were performed by using the *16S rDNA* bacterial universal primers: Forward (5′-AGAGTTTGATCCTGGCTCAG-3′) and Reverse (5′-AAGGAGGTGATCCAGCCGCA-3′) (Edwards et al., 1989). The PCR reaction was performed in a 25 μl reaction mixture containing 30 ng of gDNA as a template, 2.5 μl of primers mix (10 μM of each primer), 5 μl of (5X) PCR buffer, 1.5 μl of (25 mM) $MgCl_2$, 0.5 μl of (10 mM) dNTPs, 0.5 μl of (50 unit/ μl) *Taq* polymerase (Promega, Madison, WI, United States) and the final volume was brought to 25 μl by using nuclease free water. PCR was carried out by using thermal cycler (BIO-RAD C1000™ Thermal Cycler, United States) with the following amplification conditions: 94°C for five min for initial DNA denaturation, 30 cycles at 94°C for 1 min (denaturation), 57°C for one min (annealing) and 72°C for 1.5 min (extension), and a final elongation step at 72°C for five min. The amplified products were analyzed by gel electrophoresis as described earlier and were purified by Wizard® SV Gel and PCR Clean-Up System (Promega, Madison, WI, United States), following the manufacturer's instructions. The final purified PCR products were sequenced from both directions using the *16S rDNA* Forward and Reverse primers at Macrogene, Inc., (Seoul, South Korea) using an ABI 3730XL capillary electrophoresis sequencing station (Applied Biosystem, United States).

The *16S rDNA* sequences of the bacterial isolates were compared against sequences available in the GenBank by the BLASTn tool[1] using non-redundant (nr) and microbes databases. The phylogenetic analysis of the *16S rDNA* sequences of bacterial isolates with reference bacterial sequences identified in the BLAST search were carried out using the MEGA6.0 software package[2] (Tamura et al., 2013). The sequences were aligned using the embedded Muscle algorithm and the output was used to build a phylogenetic tree by calculating distance matrices for neighbor joining (NJ) analysis with the Kimura two-parameter model and a bootstrapping analysis with 10000 replicates to test the robustness of internal branches. Several *16S rDNA* sequences of previously identified PGPR were included as references in the phylogenetic tree.

In vitro Assessment of Plant Growth Promoting Traits

Plant growth promoting traits of bacteria isolates were assessed for nitrogen fixation, inorganic phosphate solubilization, siderophore production, IAA production, ACC deaminase activity, and antifungal activity against *Fusarium culmorum*. A reference PGPR strain, *Azospirillum lipoferum* (ATCC® 29707™), was used as positive control in all assays except for ACC deaminase assay as it served as a negative control. All assays were carried out in triplicates and were replicated at least three times for each assay.

Nitrogen Fixation Assay and PCR Amplification of the *nifH* Gene

The qualitative estimation of nitrogen fixation was conducted as described by Baldani et al. (1986). The nitrogen free semi-solid (NFb) medium was prepared and inoculated with fixed volumes of a starter inoculum (OD_{600} = 0.05) and the cultures were then incubated at 30°C for 5 days and monitored for the formation of a pellicle at the subsurface level. Blue color development was considered a positive sign for nitrogen fixation. To confirm the positive results, the bacteria were inoculated on NFb solid medium [supplemented with 10 g Agar (Thermo Fisher Scientific Oxoid, Ltd., Basingstoke, United Kingdom)] at 28°C for 7 days

[1]http://blast.ncbi.nlm.nih.gov

[2]http://www.megasoftware.net/

and bacterial growth and the formation of blue color were used as qualitative evidences for nitrogen fixation.

Further confirmation of nitrogen fixation ability was carried out by PCR amplification of a targeted fragment within the *nitrogenase iron protein* (*nifH*) gene. For this purpose, all isolates that gave positive results in the qualitative assay and selected negative isolates were tested for *nifH* genes (Ji et al., 2014). For this purpose, a *nifH* gene fragment (∼390 bp) was amplified by using two universal primers: 19F (5′-GCIWTYTAYGGIAAR GGIGG-3′) and 407R (5′-AAICCRCCRCAIACIACRTC-3′). The amplified products were analyzed by gel electrophoresis were carried out as previously described.

Phosphate Solubilization Assay

The ability of inorganic phosphate solubilization was conducted by spot inoculation of bacterial isolates on modified Pikovskaya agar plates using tricalcium phosphate as a substrate (Goswami et al., 2014). The formation of transparent halo zones around the bacterial colonies after 7 days of incubation at 28°C were considered an indication of phosphate solubilizing activity.

Siderophores Production Assay

The siderophore production was assayed by spot inoculation of selected bacterial isolates on chrome azurol S (CAS) blue agar plates as described by Schwyn and Neilands (1987). The cultures were incubated for 7 days at 28°C on CAS blue agar plates. The formation of halo zones around the growing colonies was monitored and bacterial isolates with clear zones were scored as siderophore producers.

Indole Acetic Acid Production Assay

Indole acetic acid production by selected bacterial isolates was determined using a modified quantification method developed by Bric et al. (1991). Briefly, the bacterial isolates were cultured for 24 h in 10 ml of NB and then, 100 μl of bacteria inoculum (OD_{600} = 0.5) for each selected isolate was transferred into 5 ml of NB medium supplemented with 1% of L-tryptophan (Sigma-Aldrich, Germany). After the incubation in the dark conditions at 30°C with continuous shaking (150 rpm) for 4 days, 2 ml of the bacterial cultures were centrifuged (8,000 rpm and 4°C) and 70 μL of the supernatant were transferred into a well in a microtitre plate. Thereafter, 140 μl of Salkowski reagent (50 ml of 35% $HClO_4$ +1 ml of 0.5M $FeCl_3$) was added to the supernatant and the mixture was kept at room temperature for 25 min. The absorbance at 530 nm was measured using an Epoch plate reader (BioTek, United States) (Tsavkelova et al., 2007). The un-inoculated tryptophan containing medium mixed with the Salkowski reagent was used as a negative control. The development of pink color in the well-indicated the production of IAA, and the amount of IAA produced was estimated against a standard curve prepared with different concentrations of IAA (Sigma-Aldrich, Germany).

ACC Deaminase Assay and PCR Amplification of the *acdS* Gene

The ACC deaminase activity of bacterial isolates was assessed based on the ability of the respective isolate to use ACC as a sole nitrogen source in a nitrogen free broth (Glick et al., 1995). Selected bacterial isolates were grown in 10 ml of NB medium at 28°C for 24 h. The cultures were then centrifuged for five min at 8,000 rpm and the pellets were washed twice in one ml of normal saline before spot inoculation on Burks' media supplemented with three mM ACC (Sigma-Aldrich, United States) as the sole nitrogen source. ACC-free Burks' media with and without 0.2% Ammonium Sulfate ($(NH_4)_2SO_4$) were used as positive and negative control, respectively. The cultures were incubated for 7 days at 28°C and the growth of bacterial isolates on ACC-supplemented plates were compared with the negative and positive controls (Ali et al., 2014).

To confirm the ACC deaminase assay results, PCR amplification of an *ACC deaminase* (*acdS*) gene fragment (∼800 bp) was conducted in positive and negative isolates using gene specific primers as described by Jha et al. (2012). For this purpose, *acdS* gene was amplified by using two universal primers: Forward (5′-GCCAARCGBGAVGACTGCAA-3′) and Reverse (5′-TGCATSGAYTTGCCYTC-3′). The amplified products were analyzed by gel electrophoresis and visualized by using Gel Documentation System as previously described.

Antifungal Activity Against *F. culmorum*

Antifungal activity of nine selected bacterial isolates against a *F. culmorum* aggressive isolate (accession number MH001550.1), which causes crown rot disease on wheat in Jordan (Alananbeh, unpublished), was tested using a dual culture method (Ji et al., 2014). The inoculated plates were incubated at 28°C for 10 days and the antagonistic effects of the isolates against an *F. culmorum* isolate were monitored for the formation of inhibition zones starting from day 3. The dual culture experiments were repeated three times.

Evaluation of Growth Promoting Ability in Durum Wheat

Salinity Experiments

Based on *in vitro* plant growth promoting assays, nine isolates possessing most of plant growth-promoting traits were selected and used to evaluate their effect on two durum wheat genotypes at the germination stage in the presence of different salinity levels. Two durum genotypes were selected: Tamaroi, a salt sensitive Australian cultivar, and Line 5004, a salt tolerant BC4F2 homozygous line carrying *NAX2* (*TmHKT1;5-A)* gene derived from backcrossing line 149 with Tamaroi (recurrent parent) (kindly provided by Dr. S. Udupa, ICARDA, Rabat, Morocco; James et al., 2006).

The seeds of the two lines were surface sterilized by washing with 70% ethanol for 1 min followed by three washings with sterile distilled water., Thereafter, the seeds were treated with 1.5% sodium hypochlorite (NaOCl) solution for 5 min followed by six times of successive washings in sterile water to remove all traces of the disinfectant (Rudolph et al., 2015). To check the efficacy of sterilization process, few seeds were placed on plates containing NA medium for 4 days and the plates were monitored for any microbial growth.

To prepare the bacteria inoculum, log phase cultures (OD_{600} of ∼0.6) of the nine selected bacterial isolates and *A. lipoferum*

(ATCC® 29707™) were used. The cultures were centrifuged at 5000 rpm for 10 min and the pellets were washed three times with sterile distilled water and then resuspended in a final volume of 25 ml sterile distilled water. Sterilized seeds were imbibed in the bacterial suspension for 1 h (for control treatment, seeds were imbibed in sterile water). Imbibed seeds were allowed to dry under laminar air flow cabinet for 2 h before transferring them into sterile plastic boxes lined with sterile filter papers soaked with 30 ml of different concentrations of NaCl (0, 80, 120, 160, and 200 mM). Three replicates for each treatment was performed with 20 seeds per plate (10 seeds of Tamaroi and 10 seeds of Line 5004) and the experiments were repeated twice. All boxes were incubated at 22°C under complete darkness for the first 3 days, then placed under a photoperiod cycle of 14 h light–10 h dark for other 7 days. Germination percentage was measured after 10 days and root length and seedling dry weight were recorded.

The same germination experiment was repeated twice with three selected bacterial isolates (out of nine) showing clear growth promoting activity and a negative control (non-inoculated seeds) using different concentrations of NaCl (80 and 160 mM of NaCl) and non-saline conditions to evaluate their effect on root projected area, root length, and root diameter using the WinRhizo root scanning software system (version 2009c; Regent Instruments, Inc., Quebec City, QC, Canada).

The effect of plant growth promoting activity of a selected isolate from wheat (GSW-E-7) on seedling growth under saline conditions was carried out using a hydroponic culture. For this purpose, sterilized seeds of the two durum genotypes (Tamaroi and Line 5004) were inoculated with GSW-E-7 bacterial strain, in addition to non-inoculated as a control. The seeds of both treatments germinated on filter paper wetted with distilled water as previously described above. Thereafter, 1-week old seedlings were transferred into a sterilized aerated hydroponic system that consisted of a dark plastic box (capacity 10 L) filled with a nutrient solution (Hoagland and Arnon, 1950) supplemented with different NaCl concentrations (0, 80, and 160 mM). Each box was aerated with an adjustable air pump that was operating continuously to ensure adequate air supply. In each box, six seedlings from each genotype inoculated with the bacterial isolate were grown for a period of 3 weeks in a controlled growth chamber under a daily temperature regime of 24 ± 1°C/ day and 18 ± 1°C/ night with a photoperiod of 12 h light and 12 h darkness. Each treatment was replicated three times and the experiments were repeated twice. At the end of each experiment, data were collected for shoot and root fresh weights, projected areas and lengths for shoots and roots.

Drought Experiments

The nine selected bacterial isolates described above were used to assess their effect on drought tolerance in 2-week-old of durum wheat cv. *Tamaroi*. For this purpose, 5 sterilized seeds inoculated with a selected bacterial isolate and non-inoculated seeds (negative control) were sown in a small pot (10 cm depth and 10 cm diameter) filled with sterile sand. Three replicates for each treatment were used. After seedling emergence, pots were kept moist for 2 weeks by irrigating them with nutrient solution (Hoagland and Arnon, 1950). Thereafter, drought conditions were imposed by continuous water withholding for 10 days followed by re-watering as described in Al-Abdallat et al. (2014). The wilting behavior of treated seedlings and the survival percentages of inoculated and the non-inoculated control were monitored and each bacteria strain was compared with its negative control.

Statistical Analysis

The data for salinity experiments were analyzed as a split-plot arrangement of treatments combination in a randomized complete block design. Combined analyses of variance (ANOVA) were performed for bacterial isolates, salinity level and genotypes and their possible combinations for each experiment using Statistical Analysis System (SAS) software (SAS Institute 2002, SAS/Stat software, Release 9.0., Cary, NC, United States) using mixed procedure and means for bacteria × genotype at the same salinity level were separated using Fisher's protected least significant difference (LSD) at probability level ($p \leq 0.05$).

RESULTS

Collection Sites

In this study, saline areas near the eastern side of the Dead Sea cultivated with durum wheat were surveyed for the isolation of rhizospheric and endophytic bacteria (**Supplementary Table S1**). The surveyed wheat plants varied in their growth and developmental stages. Ghor Haditha and Ghor Sweimeh plants were at the heading stage, while at Ghor Safi they were at the tillering stage. The plant material collected in Ghor Safi were suffering from environmental stresses and particularly salinity, thus only plants surviving and showing good growth were collected.

The soil texture in Ghor Haditha and Ghor Safi was sandy loam while in Ghor Sweimeh it was sandy-clay-loam. Soil pH ranged from 7.83 to 8.10 and the highest soil organic matter content (6.22%) was recorded in Ghor Safi location (**Supplementary Table S1**). For soil salinity level, the EC values varied and was 3.32 dS/m in Ghor Haditha, 6.64 dS/m in Ghor Sweimeh and in Ghor Safi it reached up to 17.7 dS/m, which is considered very high indicating a severe saline condition that had negative effect on plant growth and development (**Supplementary Table S1**).

Isolation of Rhizospheric and Endophytic Bacteria

Out of 74 isolates that grew on NA media supplemented with 10% NaCl (38 endophytic and 36 rhizospheric), 20 were isolated from Ghor Haditha (13 endophytic and 7 rhizospheric), 32 from Ghor Safi (17 endophytic and 15 rhizospheric), and 22 from Ghor Sweimeh (8 endophytic and 14 rhizospheric) (**Supplementary Table S2**). The majority of isolated colonies had creamy colored (44.5%), and 62.2% were Gram positive, and rod shaped and most of them were sporeformers. On the other hand, 37.8% of isolates were Gram negative with the majority of

them have rod shape. 71.0% of endophytic bacteria and 52.7% of rhizospheric bacteria were Gram positive (**Supplementary Table S2**). For halotolerance assay, thirty isolates were able to tolerate salinity levels up to 20% NaCl and only seven isolates tolerated up to 25%, six of which were isolated from Ghor Swiemeh (**Supplementary Table S3**).

16S rDNA Sequencing and Phylogenetic Analysis

Out of the 74 isolates, 62 were successful in producing the crossponding ∼1500 bp amplification products and their DNA sequencing data were of high quality that enabled their analysis using the blastn tool using the non-redundant (nr) and microbes' databases (**Supplementary Table S4**). The majority of the isolates (38 out of 62) belonged to the phylum Firmicutes, class Bacilli and were distributed as follows: 10 isolates belonged to the genus *Bacillus*, nine isolates belonged to genus *Oceanobacilllus*, five belonged to the genus *Salinococcus* and four belonged to genus *Halobacillus*. On the other hand, 18 isolates belonged to the phylum Proteobacteria, 17 of which belonged to class Gammaproteobacteria; with 14 isolates identified as genus *Halomonas*, two as genus *Pseudomonas* and one as genus *Psychrobacter*. A single isolate was identified as genus *Agrobacterium* belonging to class Alphaproteobacteria. Six isolates belonged to phylum Actinobacteria, class Actionobacteria. Interestingly, some endophytic isolates shared a high sequence similarity with rhizospheric isolates from the same collected sample in the same site, e.g., GSF-E-11 with GSF-R-11 from Ghor Safi location (**Supplementary Table S4**). Based on the collection site, all endophytic and the majority of rhizospheric isolates from Ghor Sweimeh belonged to family Bacillaceae, class Bacilli. Whereas clear diversity was found among isolates obtained from Ghor Safi location (**Supplementary Table S4**). Phylogenetic analysis with *16S rDNA* sequences of all isolates with reference sequences from related bacteria species confirmed the identification of the tested isolates and grouping at the genus level was observed (**Figure 1**). Furthermore, four clear clusters representing each identified taxonomical group were identified where Firmicutes was the largest, and Actinobacteria was the smallest. The accession numbers of all identified isolates and reference bacteria sequences are given in **Supplementary Table S5**.

In vitro Plant Growth Promoting Assays

Based on the phylogenetic analysis and the halotolerance assay results, 35 strains were selected for the *in vitro* growth promoting assays. The selection preference was based on removing redundant samples identified in blastn and phylogenetic analysis and the ability to tolerate high salinity levels in the halotolerance assay. These selected strains were tested for their ability to produce IAA, siderophores, nitrogen fixation, phosphate solubilization and ACC deaminase activity. A reference strain, *A. lipoferum* (ATCC® 29707™), was used as positive control in all assays except in ACC deaminase assay where it was used as negative control. The results of *in vitro* growth promoting assays in all strains are given in **Supplementary Table S6**. The data of nine selected strains that showed multiple PGP traits (three isolates from each location) plus *A. lipoferum* are presented in **Table 1**.

ACC Deaminase Activity

The activity of ACC deaminase in the selected strains was assessed based on their ability to utilize ACC as a sole nitrogen source and were confirmed by PCR amplification of a DNA fragment in the *acdS* gene. Only two endophytic strains (GSF-E-11 and GSW-E-6) were able to degrade ACC and used it as a sole nitrogen source in the media (**Table 1** and **Supplementary Table S6**). Both strains belonged to the Bacilli class with GSF-E-11 closely related to *O. iheyensis,* and GSW-E-6 closely related to *B. subtilis* (**Figure 1** and **Supplementary Table S4**). The PCR amplification of ∼800 bp fragment in the *acdS* gene in both strains confirmed the presence of n *acdS* gene and their ability to degraded the ACC in the media (**Figure 2A**).

Nitrogen Fixation

The ability of the selected strains to fix nitrogen was assessed by the formation of a pellicle in semi-solid NFb media and confirmed by PCR amplification of a 390 bp DNA fragment in the *nifH* gene. Five out of 35 strains were able to form growth pellicles in semi-solid NFb with clear blue coloration in solid NFb plates (**Table 1** and **Supplementary Table S6**). Among the five positive isolates, two (GHD-R-3 and GHD-E-12) were found to be closely related to *H. campaniensis* and *Gracilibacillus timonensis,* respectively; while GSW-E-5, GSW-E-6, and GSW-E-7 were closely related to *B. subtilus* and *B. licheniformis,* respectively (**Figure 1** and **Supplementary Table S4**). PCR amplification of ∼390 bp of *nifH* gene fragment confirmed its presence in the five positive strains as compared to *A. lipoferum* as positive control (**Figure 2B**).

Phosphate Solubilization and Siderophore Production

The ability of selected strains to solubilize inorganic phosphate from the media was tested. Among the 35 strains, five were able to solubilize phosphate and formed clear zones on modified Pikovskaya agar plates (**Supplementary Table S6**). The best strain in solubilizing phosphate was GSF-R-3, which was closely related to *H. titanicae* (**Figure 3A**).

The production of siderophore was also examined using CAS-blue agar assay; 17 out of 35 strains were able to form halo zones indicting their ability to produce siderophore (**Figure 3B**, **Table 1**, and **Supplementary Table S6**). Strains from Ghor Sweimeh, which belonged to the Bacilli class, were considered good siderophoregenic bacteria (**Supplementary Table S6**).

IAA Production

Compared to *A. lipoferum* strain, GSW-E-5 and GSW-E-6 strains produced the highest levels of IAA (**Table 1** and **Supplementary Table S6**). These two strains were shown to be closely related to *B. subtilis* (**Figure 1** and **Supplementary Table S4**). Only 10 strains were able to produce levels of IAA above 10 μg/ml, while 15 produced levels less than 5 μg/ml. Strains from Ghor Sweimeh, which belonged to the Bacilli class, were considered good IAA producers (six out of seven strains produced IAA levels higher

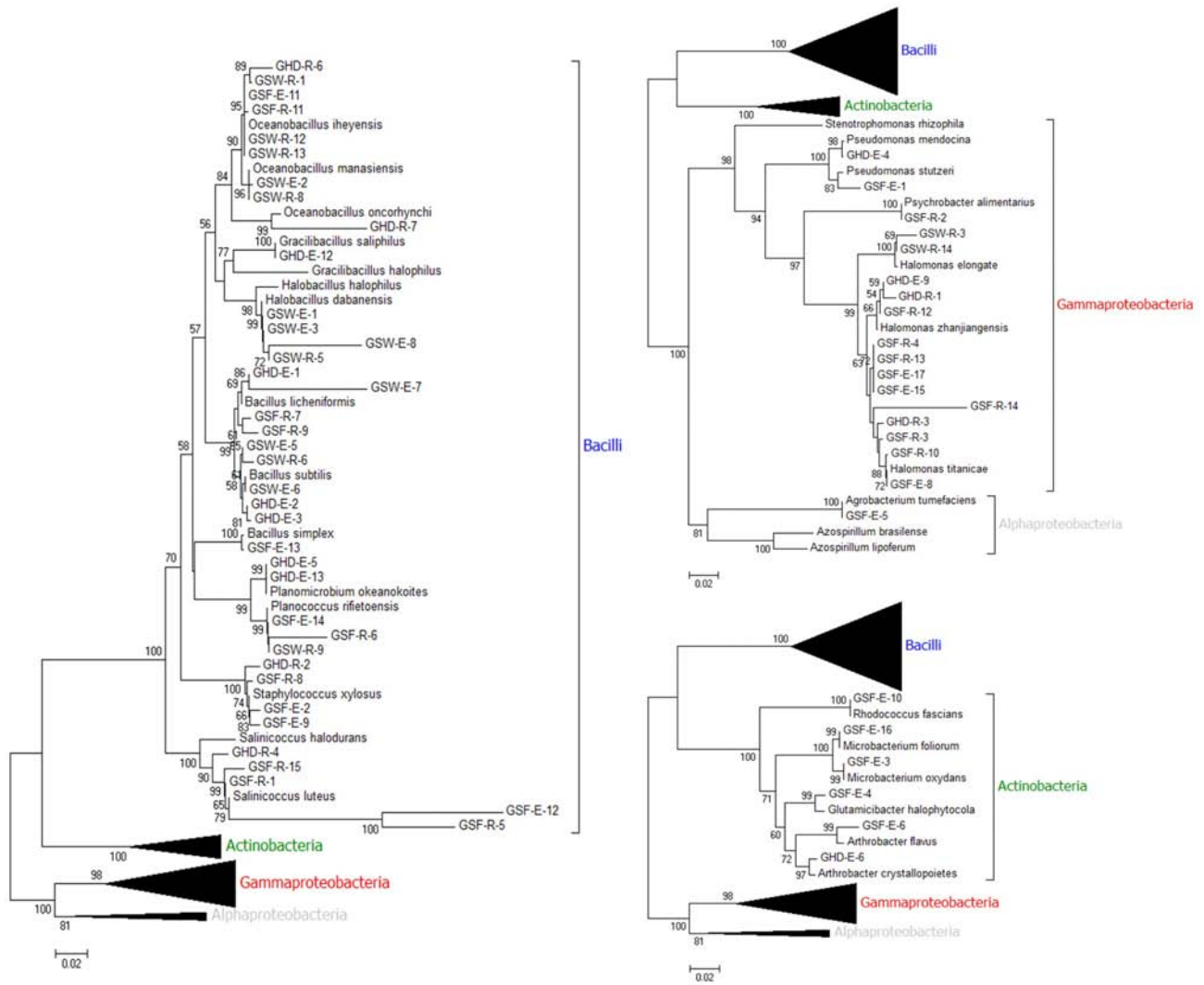


FIGURE 1 | Phylogenetic tree constructed by using neighbor-joining analysis between 62 bacterial isolates [endophytic (E) and rhizospheric (R) and grouped based on class] collected from three locations [Ghor Haditha (GHD), Ghor Safi (GSF), and Ghor Sweimeh (GSW)] nearby the Dead Sea region based on *16S rDNA* sequences and sequences from selected bacteria reference isolates (GenBank accession number for the collected isolates and reference strains are given in **Supplementary Table S5**). Bootstrap values (>50) are represented by numbers at the nodes based on 10000 replications.

TABLE 1 | Plant growth promoting traits of nine selected strains associated with durum wheat in this study (+ indicates positive; − indicates negative).

	***In vitro* growth promoting assay**				
Isolate	**Nitrogen fixation**	**Phosphate mobilization**	**Siderophore production**	**IAA production (μg/ml)**	**ACC deaminase activity**
GHD-E-6	−	−	+	14.76	−
GHD-E-12	+	−	+	9.09	−
GHD-R-3	+	−	+	14.9	−
GSF-E-8	−	−	+	10.69	−
GSF-E-10	−	+	−	13.53	−
GSF-E-11	−	−	−	6.28	+
GSW-E-5	+	+	+	22.41	−
GSW-E-6	+	−	+	18.28	+
GSW-E-7	+	+	+	13.63	−
A. lipoferum	+	+	+	27.55	−

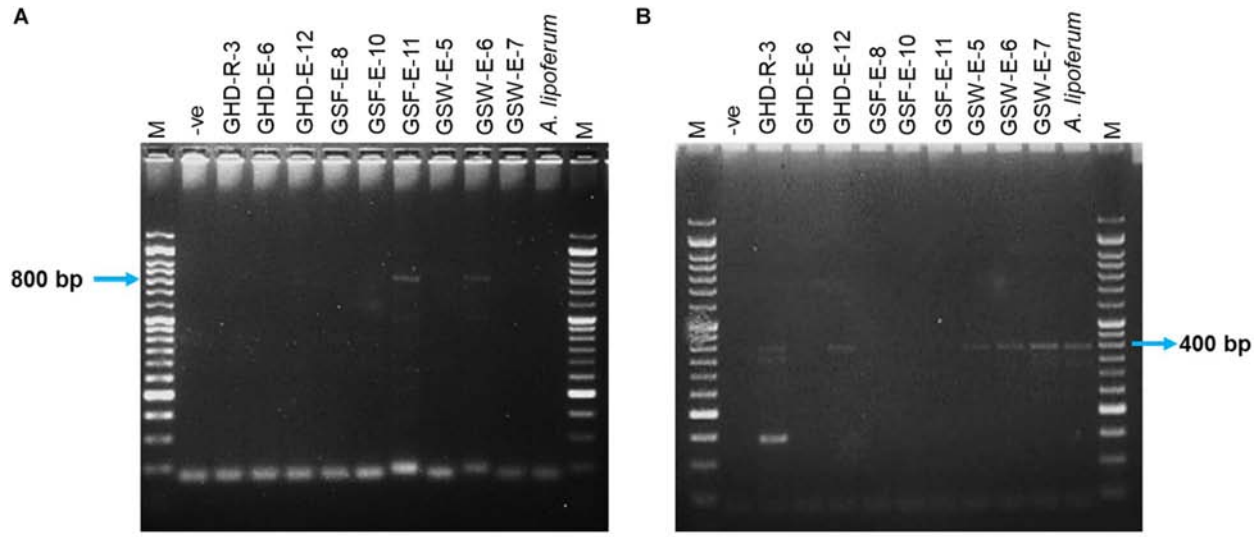


FIGURE 2 | PCR amplification of DNA fragments in *nifH* and *acdS* genes in 10 selected isolates and *Azospirillum lipoferum*. **(A)** The expected DNA fragment of *acdS* is ∼800 bp. **(B)** The expected DNA fragment of *nifH* is ∼390 bp. M: 50 bp DNA ladder (GeneDireX, South Koera); –ve: no bacteria gDNA.

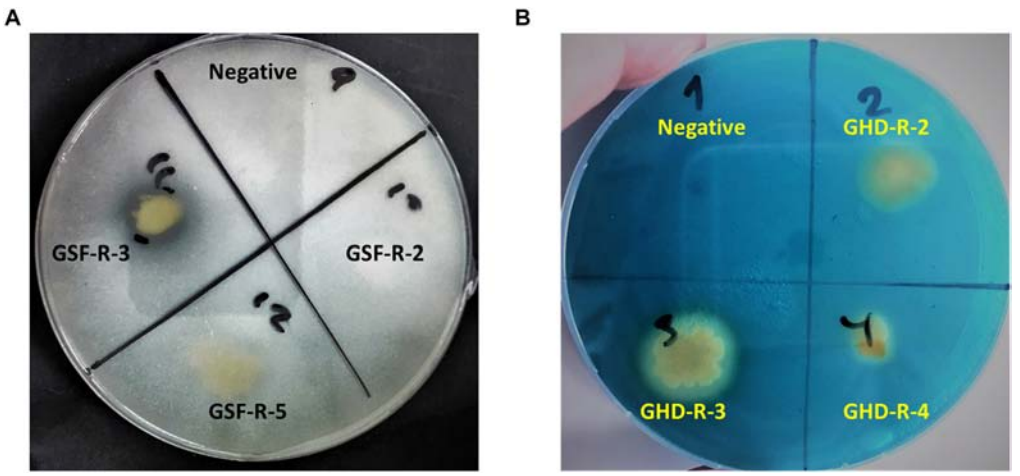


FIGURE 3 | (A) Phosphate-solubilizing activity **(B)** and siderophore production in selected halotolerant bacterial strains associated with durum wheat in this study. Negative indicates no bacteria inoculation.

than 12 μg/ml) when compared with strains from other locations (**Supplementary Table S6**).

Antifungal Activity of PGPR Against *F. culmorum*

Four endophytic bacterial strains, GHD-E-12, GSF-E-11, GSW-E-6 and GSW-E-7, showed antifungal activity against *F. culmorum* restricting mycelial growth in a dual culture plate assay (**Figure 4**). No antifungal effect against *F. culmorum* was observed in the rest of tested isolates beside *A. lipoferum*.

Plant Growth Promoting Ability in Durum Wheat Under Stress Conditions

Based on the *in vitro* plant growth promoting assays, nine strains (three per location) with distinguished plant growth promoting features were selected to study their effects on the growth of durum wheat under different stress conditions (**Table 1**).

Salinity Tolerance

The nine selected strains were used to study their effects on the growth of two durum wheat genotypes under different levels of salinity. The combined ANOVA for this experiment showed a high significant ($p < 0.001$) differences for all tested traits among bacteria, salt level, genotype and their interactions (**Supplementary Table S7**). Salinity levels above 80 mM resulted in significant reduction of germination percentage in inoculated seeds of both tested genotypes (**Supplementary Table S8**). On the other hand, Line 5004 (*NAX2*) seeds showed significantly higher germination percentages at high

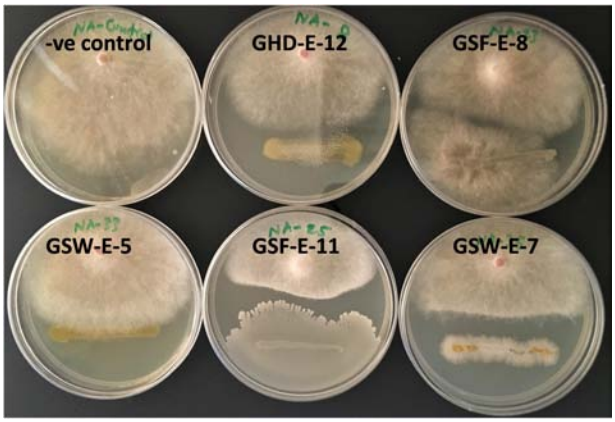


FIGURE 4 | Antifungal effect of halotolerant bacterial strains against *F. culumorum* in a dual culture assay.

NaCl levels (>80 mM) when compared with Tamaroi, the salt sensitive genotype. At salinity level of 200 mM, inoculated seeds with six bacterial strains produced significantly higher germination percentages for each tested genotype, while three strains (GSF-E-8, GSF-E-10, and GSF-E-11) showed no effect or had lower mean values (**Supplementary Table S8**). For instance, Tamaroi seeds inoculated with GSW-E-6 and GSW-E-7 strains produced mean values of 83.3 and 81.7% for germination percentages, respectively, which were significantly higher when compared with 53.3% in non-inoculated seeds. Whereas, Line 5004 (*NAX2*) inoculated with the same strains produced 100 and 96.7% germination percentages, respectively, which were significantly higher when compared with 66.7% in non-inoculated seeds. Increasing salinity levels up to 200 mM NaCl resulted in the reduction of roots number in all treatments with clear variation among tested strains and wheat genotypes (**Supplementary Table S8**).

Under non-saline conditions, a significant improvement in growth was observed in both genotypes inoculated with some bacterial strains as reflected in higher root length and seedling dry weight mean values (**Supplementary Table S8**). Only two bacterial strains, GSF-E-8 and GSF-E-10, did not show clear significant effect on growth promotion of both tested genotypes. In Tamaroi, strain GSW-E-6, which is closely related to *B. subtilis* produced the highest mean value for root length (20.67 cm) and was significantly different from non-inoculated seedlings mean value (17.42 cm) (**Figure 5A** and **Supplementary Table S8**). No significant differences in Tamaroi root length mean values were observed between GSW-E-6 and GHD-E-12, GSW-E-5 and GSW-E-7 strains under non-saline conditions (**Supplementary Table S8**). For Line 5004, GSW-E-7 produced the highest mean value under non-saline conditions and was significantly different from the non-inoculated seedlings mean value. For dry weight under non-saline conditions, the lowest mean value was observed with Tamaroi inoculated with GSF-E-8 with no significant difference with non-inoculated seedlings (**Supplementary Table S8**). In general, seedlings inoculated with seven selected bacterial strains and *A. lipoferum* improved root lengths and root dry weights in both tested genotypes under saline conditions (≥80 mM) when compared with non-inoculated seeds (**Figure 5B** and **Supplementary Table S8**). Salinity levels ≥ 80 mM resulted in a significant reduction in root length mean values in non-inoculated seedlings of both genotypes when compared with GHD-R-3, GHD-E-9, GHD-E-12, GSF-E-11, GSW-E-5, GSW-E-6, and GSW-E-7 with higher mean values observed in Line 5004 when compared with Tamaroi (**Supplementary Table S8**). A significant growth improvement was observed in both tested genotypes inoculated with *A. lipoferum* under saline conditions (**Supplementary Table S8**).

Root characteristics in terms of root projected area, total root length and root diameter were also analyzed under different NaCl levels (0, 80, and 160 mM) in both wheat genotypes either non-inoculated or inoculated with three selected halotolerant strains (GSF-E-11, GSW-E-6, and GSW-E-7). The combined ANOVA for this experiment showed a high significant ($p < 0.001$) differences for all tested traits among bacteria, salt level, genotype

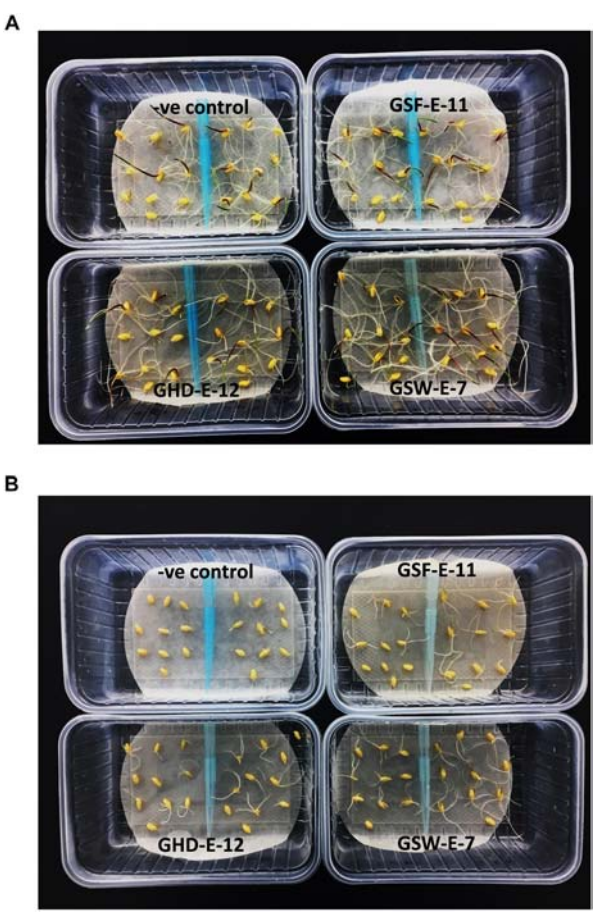


FIGURE 5 | Effect of inoculation with three PGPR bacterial strains, GHD-E-12, GSF-E-11 and GSW-E-7, on the germination of two durum wheat [right: Line 5004 (*NAX2*); left: Tamaroi] after 5 days of culture. **(A)** Non-saline conditions. **(B)** 160 mM NaCl.

and their interactions except for bacteria × salt level × genotype for root projected area trait (**Supplementary Table S7**). Under non-saline conditions, the inoculated seedlings with the three strains showed significantly higher mean values for root projected area and total root length in both tested genotypes when compared with non-inoculated seedlings (**Table 2**).

At salinity level of 80 mM, inoculated seedlings produced significantly higher mean values than non-inoculated with a clear difference in salinity tolerance behavior between the two tested genotypes. No significant differences were observed for root projected area between the three strains in Tamaroi genotype, while significant differences in the total root length mean values were observed between GSF-E-11 and GSW-E-6 and GSW-E-7 (**Table 2**). At salinity level of 160 mM, inoculated seedlings produced significantly higher mean values for both traits when compared with non-inoculated genotypes with clear significant differences between the two tested genotypes (**Table 2**). For root diameter, no significant differences were observed between treatments under non-saline conditions (**Table 2**). However, under saline conditions, non-inoculated seedlings produced significantly higher mean values when compared with inoculated seedlings.

To determine the effect of bacterial inoculation on plant growth under salinity stress in hydroponic culture, 1-week old seedlings of Tamaroi and Line 5004 genotypes either non-inoculated or inoculated with GSW-E-7 strain were transferred into a nutrient solution supplemented with different NaCl levels (0, 80, and 160 mM). The combined ANOVA for this experiment showed a high significant ($p < 0.001$) differences for all tested traits among bacteria, salt level, genotype and their interactions except for bacteria × salt level × genotype for root length and shoot fresh weight traits (**Supplementary Table S7**).

Under non-saline conditions, a significant growth increase was observed in inoculated seedlings of both tested genotypes when compared with non-inoculated seedlings as reflected in higher fresh weight, projected area and total length of both roots and shoots (**Figure 6** and **Table 3**). On the other hand, GSW-E-7 strain inoculated seedlings showed significant root growth at 80 mM salinity level for all measured traits (fresh weight, projected area, and total root length) in both tested genotypes with clear significant differences between the two tested genotypes (**Table 3**). At salinity level of 160 mM, clear significant differences between inoculated and non-inoculated seedlings were observed for all root growth parameters. However, a clear significant difference was observed between the two tested genotypes only in non-inoculated seedlings, while mean values of inoculated seedlings were non-significant between both genotypes (**Table 3**).

Interestingly, no significant effect of GSW-E-7 strain inoculation on shoot projected area and shoot length in Line 5004 genotype was observed, although a clear effect was observed on inoculated Tamaroi seedling when compared with non-inoculated control (**Table 3**).

Drought Tolerance

Exposing non-inoculated Tamaroi wheat seedling to water withholding for 10 continuous days resulted in pronounced wilting and subsequently the death of all treated seedlings and no recovery was observed after rewatering treatment (**Figure 7**). Similar responses were obtained for seedling inoculated with other six bacterial strains. On the other hand, the inoculation with GHD-E-6, GSF-E-11, and GSW-E-6 bacterial strains improved water deficit tolerance and survival percentages in Tamaroi seedlings when compared with non-inoculated seedlings (**Figure 7**).

DISCUSSION

The Dead Sea is the lowest point on earth (−430 m) and it is located at the tip of the western horn of the fertile crescent where agricultural revolution is believed to have started before

TABLE 2 | Effect of inoculation with three halotolerant bacterial strains, GHD-E-12, GSF-E-11 and GSW-E-7, on root characteristics [projected area (mm^2), length (mm), surface area (mm^2), and root diameter (mm)] in two durum wheat genotypes under different salinity levels.

		Projected area		Root length		Root diameter	
Salt level	**Bacteria**	**Tamaroi**	**Line 5004 (*NAX2*)**	**Tamaroi**	**Line 5004 (*NAX2*)**	**Tamaroi**	**Line 5004 (*NAX2*)**
0 mM	Control	80.94 d*	80.12 d	41.72 e	46.22 d	0.4905 a	0.5101 a
	GSF-E-11	109.37 a	98.55 bc	54.96 c	60.42 b	0.4392 a	0.4363 a
	GSW-E-6	103.45 ab	102.14 bc	62.09 b	65.80 a	0.4289 a	0.4203 a
	GSW-E-7	100.86 bc	95.34 c	53.32 c	61.36 b	0.4138 a	0.4177 a
80 mM	Control	26.70 e	45.56 d	10.09 f	18.28 e	0.6702 a	0.5864 a
	GSF-E-11	67.53 c	85.06 ab	33.86 d	53.95 a	0.4459 b	0.4377 b
	GSW-E-6	62.97 c	81.70 b	40.77 c	44.37 b	0.4461 b	0.4428 b
	GSW-E-7	68.67 c	90.98 a	39.04 c	41.12 bc	0.4130 b	0.4306 b
160 mM	Control	2.44 d	2.30 d	1.66 c	0.75 c	1.2627 b	1.9221 a
	GSF-E-11	15.85 bc	19.28 bc	10.29 b	13.68 b	0.5241 c	0.4853 c
	GSW-E-6	15.81 bc	33.66 a	10.96 b	18.02 a	0.4915 c	0.4872 c
	GSW-E-7	13.82 c	20.99 b	12.34 b	18.00 a	0.4810 c	0.4694 c

**Means carrying different letters at the same salinity level are significantly different using LSD values at $p \leq 0.05$.*

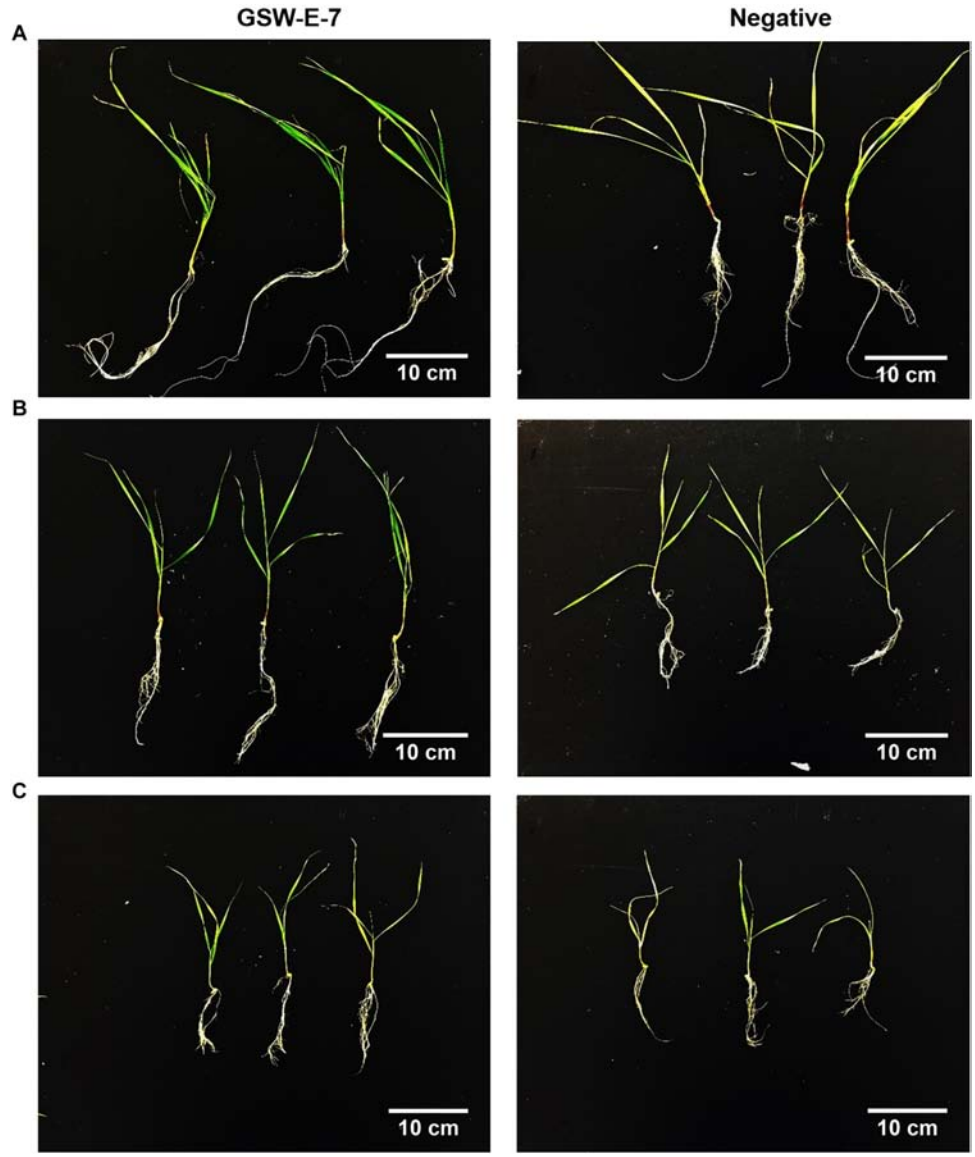


FIGURE 6 | Effect of inoculation with GSW-E-7 strain on growth of durum wheat cv. Tamaroi seedlings after 30 days of culture in hydroponic system. **(A)** 0 mM NaCl; **(B)** 80 mM NaCl; **(C)** 160 mM NaCl. Negative: non-inoculated seeds.

10000 years ago. To the north of the Dead Sea basin, the Jordan valley is considered a core diversity area of wild emmer wheat (*T. turgidum* subsp. *dicoccoides*), the direct progenitor of durum wheat (Weide, 2015). The Dead Sea region climate is considered hot and dry with an annual rainfall level less than 100 mm (Loss and Siddique, 1994) and saline soil conditions is prevalent in many nearby cultivated areas reaching a mean value of EC 14.0 dS/m hindering irrigated agriculture and crop productivity (Ammari et al., 2013).

Targeting such harsh environment where durum wheat had evolved might help in identifying halotolerant PGPR with positive effects on durum wheat growth and development under saline conditions. The isolation of halotolerant PGPR with promoting effect on growth of wheat under saline conditions were reported previously from saline environments (Nabti et al., 2007; Tiwari et al., 2011; Ramadoss et al., 2013) and roots of bread wheat plants (Fischer et al., 2007; Egamberdieva and Kucharova, 2009; Tiwari et al., 2011). Targeting saline soils from northern Algeria resulted in the isolation of the halotolerant *A. brasilense* strain NH that had improved durum wheat growth and development under high salt stress conditions (Nabti et al., 2007). In another study, the *Azotobacter chroococcum* AZ6 strain was isolated from rhizospheric soil surrounding durum wheat plants cultivated in an arid location in Algeria (Silini et al., 2016). Inoculation of durum wheat plants with the AZ6 strain either alone or in combination with osmolytes improved growth under high salt stress. In this study, 74 halotolerant rhizospheric and endophytic bacteria associated with durum wheat were isolated

TABLE 3 | Effect of inoculation with GSW-E-7 strain on root and shoot characteristics [fresh weight (gm), projected area (mm^2), and length (mm)] in two durum wheat genotypes under different salinity levels after 3 weeks in a hydroponic culture.

		Root projected area		Root length		Root fresh weight	
Salt level	**Bacteria**	**Tamaroi**	**Line 5004**	**Tamaroi**	**Line 5004**	**Tamaroi**	**Line 5004**
0	Control	94.98 b*	89.29 c	152.47 c	158.67 c	0.0894 b	0.0776 c
	GSW-E-7	115.32 a	117.04 a	208.28 a	194.87 b	0.1067 a	0.1144 a
80	Control	28.27 d	40.21 c	49.12 d	68.15 c	0.0597 d	0.0709 c
	GSW-E-7	48.05 b	56.87 a	120.03 b	136.63 a	0.0926 b	0.1045 a
160	Control	23.40 c	31.83 b	30.78 c	45.83 b	0.0261 b	0.0434 a
	GSW-E-7	38.94 a	42.34 a	73.68 a	72.89 a	0.0419 a	0.0442 a
		Shoot projected area		**Shoot length**		**Shoot fresh weight**	
Salt level	**Bacteria**	**Tamaroi**	**Line 5004**	**Tamaroi**	**Line 5004**	**Tamaroi**	**Line 5004**
0	Control	92.68 b	99.81 b	67.15 d	72.75 c	0.498 b	0.518 b
	GSW-E-7	110.48 a	112.96 a	80.25 b	88.52 a	0.593 a	0.609 a
80	Control	74.58 b	95.68 a	41.15 c	57.33 a	0.165 d	0.2563 c
	GSW-E-7	98.41 a	95.89 a	50.32 b	54.28 a	0.377 b	0.4293 a
160	Control	46.73 c	59.45 ab	27.29 c	37.51 a	0.109 a	0.130 a
	GSW-E-7	56.24 b	64.89 a	32.79 b	37.32 a	0.141 a	0.131 a

**Means carrying different letters at the same salinity level are significantly different using LSD values at $p \leq 0.05$.*

from saline areas in the Ghor region nearby the Dead Sea. Among them, 46 isolates were found to be Gram-positive and 28 isolates were able to tolerate salinity levels up to 20% NaCl. However, to the best of our knowledge, no previous report described the isolation of plant growth promoting endophytic bacteria from durum wheat plants cultivated in hypersaline soils as described in this study. The association between halotolerant PGP rhizospheric and endophytic isolates with a salt sensitive plant such as durum wheat indicates the possibility to mitigate salinity stress and the ability to promote growth under such conditions by indigenous PGPR. This is highly probable knowing that plant samples collected in this study were from saline fields cultivated with the durum wheat cv. *Haurani*, a salt sensitive genotype with salinity threshold for grain yield about 20% at an EC of 7 dS/m (Katerji et al., 2005).

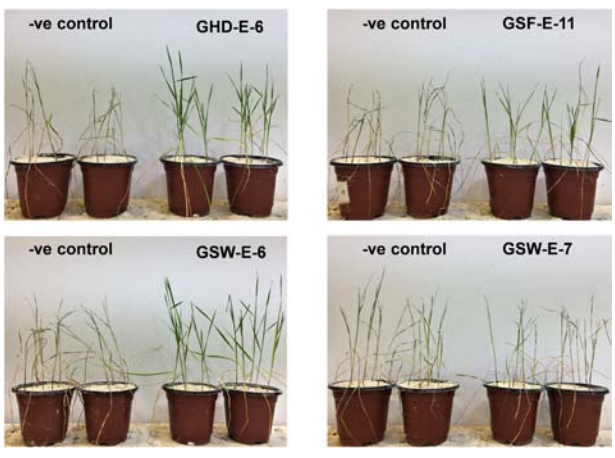


FIGURE 7 | Effect of inoculation with four PGPR bacterial strains, GHD-E-6, GSF-E-11, GSW-E-6 and GSW-E-7, on durum wheat cv. Tamaroi seedling after 10 days of water with-holding. –ve control: non-inoculated seedlings.

Phylogenetic analysis of 62 halotolerant isolates using *16S rDNA* partial sequences revealed that the obtained isolates were highly diverse at the genus level. The majorities of the isolates were found to belong to the class Bacilli and included eight different genera. Ten isolates were closely related to *Bacillus* spp., five isolates clearly grouped with *B. subtilis* and four isolates were closely related to *B. licheniformis*, all of which are well-known to be beneficial PGPR with growth promotion abilities under different stress conditions that can also induced resistance against different pathogens (Vurukonda et al., 2016; Etesami and Beattie, 2018). Furthermore, nine isolates from the Bacilli class were closely related to *Oceanobacillus* spp., which were previously reported as PGP endophytic bacteria in different plant species (Siddikee et al., 2010; Mapelli et al., 2013) including wheat (Orhan, 2016). On the other hand, 14 isolates were closely related to the genus *Halomonas* (**Figure 1**), which was reported previously as halotolerant PGPR that can induce tolerance against salinity in wheat plant (Tiwari et al., 2011). Similarly, Upadhyay et al. (2009) reported that the majority of halotolerant bacterial genera identified from rhizospheric soil surrounding bread wheat under saline conditions belonged to the class Bacilli.

The plant growth promoting abilities including nitrogen fixation, inorganic phosphate solubilization, siderophore production, auxin production, ACC deaminase activity, and antifungal effect against *F. culmorum* were assessed in 35 selected isolates. The utilization of halotolerant PGPR with nitrogen fixation ability is considered a good strategy to improve the growth of salt sensitive plants (Etesami and Beattie, 2018). Only five isolates were found to fix nitrogen, three of them (GSW-E-5, GSW-E-6, and GSW-E-7) were closely related to

Bacillus spp., while GHD-E-12 was related to *Gracilibacillus* spp. and GHD-R-3 was related to *Halomonas* spp. The low incidence of halotolerant isolates with nitrogen fixation in this study is in consistence with Upadhyay et al. (2009), who reported that two out of 24 halotolerant isolates were capable of fixing nitrogen. *Bacillus* spp. with nitrogen fixation ability was identified previously in wheat (Beneduzi et al., 2008) and rice plants (Ji et al., 2014). The halotolerant *B. licheniformis* HSW-16 strain associated with salinity tolerance in wheat was able to fix nitrogen (Singh and Jha, 2016a).

Five halotolerant strains were able to solubilize insoluble phosphate indicating their potential to promote growth of wheat under phosphate-limited conditions as described previously by Upadhyay and Singh (2015). Two PGPR strains closely related to *Halomonas* spp. and *Halobacillus* spp. were able to solubilize phosphate in the presence of salinity and had positive effects on plant growth under such conditions (Desale et al., 2014). Siderophores producing halotolerant PGPR can enhance the uptake of iron by plants (Kloepper et al., 1980) and can also improve plant health by producing antimicrobial compound and depleting metals from rhizosphere affecting pathogen growth and infection (Haas and Défago, 2005). In this study, 17 bacterial strains were found to produce siderophores, which is similar to previous reports where several halotolerant PGPR isolated from wheat were siderophore producers and had growth promoting abilities under saline conditions (Beneduzi et al., 2008; Upadhyay et al., 2009).

The ability of halotolerant PGPR to produce IAA was associated with improved growth of wheat under saline conditions (Nabti et al., 2007; Egamberdieva, 2009; Tiwari et al., 2011). Two strains closely related to *B. subtilis*, GSW-E-5 and GSW-E-6, were able to produce high levels of IAA, which was close to the levels produced by *A. lipoferum* reference strain. Ramadoss et al. (2013) reported the isolation of halotolerant isolates that are related to *Bacillus* spp. with IAA production and growth promoting abilities in wheat. Upadhyay et al. (2012) reported that *B. subtilis* SU47 strain was able to produce IAA and promoted growth of wheat under saline conditions.

Two strains (GSW-E-6 and GSF-E-11) out of 35 were able to utilize ACC as a sole nitrogen source in the media. GSW-E-6 was closely related to *B. subtilis,* while GSF-E-11 was closely related to *O. picturae*. Halotolerant bacteria with ACC deaminase activities closely related to *Bacillus* spp. were reported previously in wheat (Upadhyay et al., 2009; Orhan, 2016; Singh and Jha, 2016a). The ability of PGPR to produce the enzyme ACC deaminase can result in reducing the levels of ethylene hormone by degrading its precursor ACC and subsequently results in promoting plant growth under stress conditions (Glick, 2014).

Recently, several PGPR strains with multiple growth promoting traits improved wheat growth and tolerance against high salinity (Singh and Jha, 2016a,b,c). In this study, three strains closely related to *Bacillus* spp. (GSW-E-5, GSW-E-6, and GSW-E-7) were isolated from the same field site and were found to possess several growth promoting traits such as IAA production, nitrogen fixation and siderophore production that resulted in improving growth of wheat plants under saline conditions. The three strains had pronounced effects on durum wheat seed germination and seedling growth under severe saline conditions. *Bacillus* spp. strains with multiple plant growth promoting traits were found more effective in enhancing wheat growth when compared with single trait strains (Baig et al., 2012). Similarly, strain GSW-E-6, which is closely related to *B. subtilis*, was found to possess multiple plant growth promoting traits including ACC deaminase activity, nitrogen fixation, siderophore and IAA production and had a positive effect on durum wheat germination and seedling growth under severe saline conditions. Furthermore, the same strain improved seedling survival in response to drought by maintaining plant water status and delaying wilting even after 10 days of water withholding. The ability of *Bacillus* strains with ACC deaminase and IAA production activities to improve drought tolerance in different species was reported previously (Vurukonda et al., 2016; Saleem et al., 2018). Strain GSW-E-7, which is closely related to *B. licheniformis* improved substantially wheat growth and development under saline conditions when compared with non-inoculated plants. Similarly, the ACC deaminase and nitrogen fixation bacterium *B. licheniformis* HSW-16 improved wheat growth under saline condition and it possessed other growth promoting traits such as auxin production and phosphate solubilization activity (Singh and Jha, 2016a). Beside salinity tolerance, strain GSW-E-7 has antagonistic effect against an aggressive *F. culmorum* as observed in a dual culture assay. *B. licheniformis* HSW-16 had antagonistic effect against different pathogens including *F. oxysporum* and *F. graminearum* (Singh and Jha, 2016a). Other *Bacillus* spp. were reported to have antifungal effect against *F. culmorum* and induced resistance in durum wheat (Mnasri et al., 2017). The GHD-E-12 strain was found to be closely related to *Gracilibacillus* spp., which was previously reported as an endophytic PGPR in *Arthrocnemum macrostachyum*, a halophyte plant growing in saline agricultural soils (Navarro-Torre et al., 2017).

Few reports described the promoting effect of PGPR on durum wheat growth and development under saline conditions (Nabti et al., 2007, 2010; Silini et al., 2016). In this study, several PGPR strains that belong to different genera were identified as endophytic PGPR in durum wheat, a salt sensitive plant (Gorham, 1990), which was cultivated in saline soils. Introducing the high-affinity K^+ transporters, *TmHKT1;5-A* (*NAX2*) and *TmHKT7-A2* (*NAX1*) from *T. monococcum* into durum wheat, improved high salt tolerance through sodium unloading from xylem tissues reducing its accumulation into toxic levels in the leaf blade (Huang et al., 2006; James et al., 2006). In this study, the inoculation of durum salt-sensitive and salt-tolerant (carries *NAX2*) genotypes with halotolerant PGPR improved germination and seedling growth under high salinity conditions. In the salt sensitive genotype, the inoculation with halotolerant PGPR isolates improved growth under saline conditions, although growth performance was still less than *NAX2* inoculated plants at the same salinity level. This might be related to its weak ability to exclude Na^+ ions from its root tissue causing reduced growth when compared with the *NAX2* carrying line. Previously, the inoculation of two durum wheat varieties with different levels of salinity tolerance with *A. chroococcum* AZ6 didn't affect the differential accumulation

of the Na^+ in plant tissues where the salt sensitive Bousselam variety accumulated more Na^+ in leaves tissues when compared with Waha, a more salt tolerant variety (Silini et al., 2016). In another study, *B. subtilis* strain GB03 induced salt tolerance in bread wheat that was associated with reduced accumulation of Na^+ in plant tissues accompanied with improved K^+/Na^+ ratio (Zhang et al., 2014); the same strain was found to modulate tissue-specific expression of *HKT1* gene in Arabidopsis plant under salt stress conditions and failed to induce salinity tolerance in *athkt1* mutant lines (Zhang et al., 2008). In this study, PGPR strains were successful in improving the growth in a salt sensitive durum wheat genotype lacking *TmHKT1;5-A*, an ortholog to *HKT1;* but whether they have affected spatial accumulation of Na^+ in plant tissue or modulated the expression of other *HKT* genes in durum wheat, need to be investigated. The substantial increase in roots growth only but not in the shoots under high salt stress conditions in inoculated seedling of *NAX2* carrying line might indicate that ability of the halotolerant PGPR strains to improve tolerance against salinity by other mechanisms beside sodium exclusion that is activated by *TmHKT1;5-A*. Such mechanisms might include exopolysaccharides production, which can bind sodium and decreases its uptake by plants, osmolytes accumulation, ions hemostasis, biofilms formation, and production of phytohormones (Etesami and Beattie, 2018).

CONCLUSION

Characterization of several halotolerant PGPR isolated from the rhizosphere of durum wheat plants cultivated in hypersaline environments revealed several growth promoting traits. This was reflected on their ability to alleviate the negative effects of high salinity on durum wheat seed germination and seedling growth. Furthermore, halotolerant PGPR strains with antifungal effect against *F. culmorum* were identified that might have a potential to induce resistance against an aggressive isolate causing crown root rot in durum wheat plants. The identification of halotolerant-multifarious PGPR associated with durum wheat might be used commercially in the future to improve of durum wheat and other crops productivity under saline conditions. However, field testing and studying their efficiency in promoting growth under natural conditions should be considered. Future studies are needed to investigate the mechanisms of induced salinity tolerance by the identified PGPR strains from durum wheat at molecular and physiological levels. Whole genome sequencing of the most promising halotolerant PGPR from this study might also shed the light on and PGP mechanisms associated with induced salinity tolerance in durum wheat.

AUTHOR CONTRIBUTIONS

RA conceived most of the research and experimental work, and helped in drafting the manuscript. HK-H designed and conceived the research, helped in data analysis, and drafted the manuscript. JA significantly contributed to the management of the salinity experiments, data analysis, and the interpretation of the results. KA contributed to the antifungal experiments and the interpretation of the results. RA-S contributed to the management of the drought experiments and data analysis.

FUNDING

The authors would like to thank the Deanship of Scientific Research, The University of Jordan (Amman, Jordan) for their financial support.

ACKNOWLEDGMENTS

We sincerely acknowledge the Jordanian farmers from Ghor Haditha, Ghor Safi, and Ghor Sweimeh for their assistance and permission to use their fields for sampling and material collection. We would like to thank Prof. Dr. Muhanad Akash for his assistance in statistical analysis. We would also like to thank Miss. Shireen Hasan for her technical assistance. We thank Dr. Sripada Udupa from the ICARDA for providing seeds of the wheat genotypes used in this study.

SUPPLEMENTARY MATERIAL

The Supplementary Material for this article can be found online at: https://www.frontiersin.org/articles/10.3389/fmicb.2019.01639/full#supplementary-material

REFERENCES

Al-Abdallat, A. M., Ayad, J. Y., Abu Elenein, J. M., Al-Ajlouni, Z., and Harwood, W. A. (2014). Overexpression of the transcription factor HvSNAC1 improves drought tolerance in barley (*Hordeum vulgare* L.). *Mol. Breed.* 33, 401–414. doi: 10.1007/s11032-013-9958-1.

Ali, S. Z., Sandhya, V., and Rao, L. V. (2014). Isolation and characterization of drought-tolerant ACC deaminase and exopolysaccharide-producing fluorescent *Pseudomonas* sp. *Ann. Microbiol.* 64, 493–502. doi: 10.1007/s13213-013-0680-3

AL-Zu'bi, Y. A., and Al-Kharabsheh, A. (2003). Multicriteria analysis for water productivity in the jordan valley. *Water Int.* 28, 501–511. doi: 10.1080/02508060308691727

Ammari, T. G., Tahhan, R., Abubaker, S., Al-Zu'bi, Y., Tahboub, A., Ta'Any, R., et al. (2013). Soil salinity changes in the jordan valley potentially threaten sustainable irrigated agriculture. *Pedosphere* 23, 376–384. doi: 10.1016/S1002-0160(13)60029-6

Baig, K. S., Arshad, M., Shaharoona, B., Khalid, A., and Ahmed, I. (2012). Comparative effectiveness of *Bacillus* spp. possessing either dual or single growth-promoting traits for improving phosphorus uptake, growth and yield of wheat *(Triticum aestivum L.)*. *Ann. Microbiol.* 62, 1109–1119. doi: 10.1007/s13213-011-0352-0

Baldani, J. I., Baldani, V. L. D., Seldin, L., and Döbereiner, J. (1986). Characterization of Herbaspirillum seropedicae gen nov, sp nov, a root-associated nitrogen-fixing bacterium. *Int. J. Syst. Bacteriol.* 4:86–93. doi: 10.1099/00207713-36-1-86

Beneduzi, A., Peres, D., Vargas, L. K., Bodanese-Zanettini, M. H., and Passaglia, L. M. P. (2008). Evaluation of genetic diversity and plant growth promoting activities of nitrogen-fixing bacilli isolated from rice fields in South Brazil. *Appl. Soil Ecol.* 39, 311–320. doi: 10.1016/j.apsoil.2008.01.006

Bric, J. M., Bostock, R. M., and Silverstone, S. E. (1991). Rapid in situ assay for indoleacetic acid production by bacteria immobilized on a nitrocellulose membrane. *Appl. Environ. Microbiol.* 57, 535–538.

Compant, S., Duffy, B., Nowak, J., Clément, C., and Barka, E. A. (2005). Use of plant growth-promoting bacteria for biocontrol of plant diseases: principles, mechanisms of action, and future prospects. *Appl. Environ. Microbiol.* 71, 4951–4959. doi: 10.1128/AEM.71.9.4951-4959.2005

Desale, P., Patel, B., Singh, S., Malhotra, A., and Nawani, N. (2014). Plant growth promoting properties of *Halobacillus* sp. and *Halomonas sp*. in presence of salinity and heavy metals. *J. Basic Microbiol.* 54, 781–791. doi: 10.1002/jobm.20120077

Edwards, U., Rogall, T., Blöcker, H., Emde, M., and Böttger, E. C. (1989). Isolation and direct complete nucleotide determination of entire genes. Characterization of a gene coding for 16S ribosomal RNA. *Nucleic Acids Res.* 17, 7843–7853. doi: 10.1093/nar/17.19.7843

Egamberdieva, D. (2009). Alleviation of salt stress by plant growth regulators and IAA producing bacteria in wheat. *Acta Physiol. Plant.* 31, 861–864. doi: 10.1007/s11738-009-0297-0

Egamberdieva, D., and Kucharova, Z. (2009). Selection for root colonising bacteria stimulating wheat growth in saline soils. *Biol. Fertil. Soil.* 45, 563–571. doi: 10.1007/s00374-009-0366-y

Estefan, G., Sommer, R., and Ryan, J. (2014). *Analytical Methods for Soil-Plant and Water in the Dry Areas. A Manual of Relevance to the WEST Asia and North Africa Region*. 3rd Edn. Aleppo: International Center for Agricultural Research in the Dry Areas

Etesami, H., and Beattie, G. A. (2018). Mining halophytes for plant growth-promoting halotolerant bacteria to enhance the salinity tolerance of non-halophytic crops. *Front. Microbiol.* 9:148. doi: 10.3389/fmicb.2018.00148

FAOSTAT (2017). *Food and Agriculture Organization of the United Nations*. Rome: FAOSTAT.

Fischer, S. E., Fischer, S. I., Magris, S., and Mori, G. B. (2007). Isolation and characterization of bacteria from the rhizosphere of wheat. *World J. Microbiol. Biotechnol.* 23, 895–903. doi: 10.1007/s11274-006-9312-4

Glick, B. R. (2014). Bacteria with ACC deaminase can promote plant growth and help to feed the world. *Microbiol. Res.* 169, 30–39. doi: 10.1016/j.micres.2013.09.009

Glick, B. R., Karaturovíc, D. M., and Newell, P. C. (1995). A novel procedure for rapid isolation of plant growth promoting pseudomonads. *Can. J. Microbiol.* 41, 533–536. doi: 10.1139/m95-070

Gorham, J. (1990). Salt tolerance in the Triticeae: K/Na discrimination in synthetic hexaploid wheats. *J. Exp. Bot.* 41, 623–627. doi: 10.1093/jxb/41.5.623

Goswami, D., Dhandhukia, P., Patel, P., and Thakker, J. N. (2014). Screening of PGPR from saline desert of Kutch: growth promotion in Arachis hypogea by Bacillus licheniformis A2. *Microbiol. Res.* 169, 66–75. doi: 10.1016/j.micres.2013.07.004

Gupta, B., and Huang, B. (2014). Mechanism of salinity tolerance in plants: physiological, biochemical, and molecular characterization. *Int. J. Genom.* 2014, 1–18. doi: 10.1155/2014/701596

Haas, D., and Défago, G. (2005). Biological control of soil-borne pathogens by fluorescent pseudomonads. *Nat. Rev. Microbiol.* 3, 307–319. doi: 10.1038/nrmicro1129

Hoagland, D. R., and Arnon, D. I. (1950). The water-culture method for growing plants without soil. *Cir. California Agric. Exp. Station* 347:32.

Huang, S., Spielmeyer, W., Lagudah, E. S., James, R. A., Platten, J. D., Dennis, E. S., et al. (2006). A sodium transporter (HKT7) is a candidate for Nax1, a gene for salt tolerance in durum wheat. *Plant Physiol.* 142, 1718–1727. doi: 10.1104/pp.106.088864

James, R. A., Davenport, R. J., and Munns, R. (2006). Physiological characterization of two genes for Na+ exclusion in durum wheat, Nax1 and Nax2. *Plant Physiol.* 142, 1537–1547. doi: 10.1104/pp.106.086538

Jha, B., Gontia, I., and Hartmann, A. (2012). The roots of the halophyte Salicornia brachiata are a source of new halotolerant diazotrophic bacteria with plant growth-promoting potential. *Plant Soil* 356, 265–277. doi: 10.1007/s11104-011-0877-9

Ji, S. H., Gururani, M. A., and Chun, S. C. (2014). Isolation and characterization of plant growth promoting endophytic diazotrophic bacteria from Korean rice cultivars. *Microbiol. Res.* 169, 83–98. doi: 10.1016/j.micres.2013.06.003

Katerji, N., Mastrorilli, M., Van Hoorn, J. W., Lahmer, F. Z., Hamdy, A., and Oweis, T. (2009). Durum wheat and barley productivity in saline–drought environments. *Eur. J. Agron.* 31, 1–9. doi: 10.1016/j.eja.2009.01.003

Katerji, N., Van Hoorn, J. W., Fares, C., Hamdy, A., Mastrorilli, M., and Oweis, T. (2005). Salinity effect on grain quality of two durum wheat varieties differing in salt tolerance. *Agric. Water Manag.* 75, 85–89. doi: 10.1016/j.agwat.2004.12.005

Kloepper, J. W., Leong, J., Teintze, M., and Schroth, M. N. (1980). Enhanced plant growth by siderophores produced by plant growth-promoting rhizobacteria. *Nature* 286:885. doi: 10.1038/286885a0

Loss, S. P., and Siddique, H. M. (1994). Morphological and physiological traits associated with wheat yield increases in mediterranean environments. *Adv. Agron.* 52, 229–276. doi: 10.1016/S0065-2113(08)60625-2

Mapelli, F., Marasco, R., Rolli, E., Barbato, M., Cherif, H., Guesmi, A., et al. (2013). Potential for plant growth promotion of rhizobacteria associated with Salicornia growing in Tunisian hypersaline soils. *Biomed. Res. Int.* 2013, 1–13. doi: 10.1155/2013/248078

Mnasri, N., Chennaoui, C., Gargouri, S., Mhamdi, R., Hessini, K., Elkahoui, S., et al. (2017). Efficacy of some rhizospheric and endophytic bacteria in vitro and as seed coating for the control of *Fusarium culmorum* infecting durum wheat in Tunisia. *Eur. J. Plant Pathol.* 147, 501–515. doi: 10.1007/s10658-016-1018-3

Munns, R., James, R. A., and Läuchli, A. (2006). Approaches to increasing the salt tolerance of wheat and other cereals. *J. Exp. Bot.* 57, 1025–1043. doi: 10.1093/jxb/erj100

Munns, R., and Tester, M. (2008). Mechanisms of salinity tolerance. *Annu. Rev. Plant Biol.* 59, 651–681. doi: 10.1146/annurev.arplant.59.032607.092911

Nabti, E., Sahnoune, M., Adjrad, S., Van Dommelen, A., Ghoul, M., Schmid, M., et al. (2007). A halophilic and osmotolerant *Azospirillum brasilense* strain from Algerian soil restores wheat growth under saline conditions. *Eng. Life Sci.* 7, 354–360. doi: 10.1002/elsc.200720201

Nabti, E., Sahnoune, M., Ghoul, M., Fischer, D., Hofmann, A., Rothballer, M., et al. (2010). Restoration of growth of durum wheat (Triticum durum var. *waha*) under saline conditions due to inoculation with the rhizosphere bacterium *Azospirillum brasilense* NH and extracts of the marine alga Ulva lactuca. *J. Plant Growth Regul.* 29, 6–22. doi: 10.1007/s00344-009-9107-6

Navarro-Torre, S., Barcia-Piedras, J. M., Mateos-Naranjo, E., Redondo-Gómez, S., Camacho, M., Caviedes, M. A., et al.,, (2017). Assessing the role of endophytic bacteria in the halophyte *Arthrocnemum macrostachyum* salt tolerance. *Plant Biol.* 19, 249–256. doi: 10.1111/plb.12521

Nevo, E., Korol, A. B., Beiles, A., and Fahima, T. (2013). *Evolution of Wild Emmer and Wheat Improvement: Population Genetics, Genetic Resources, and Genome Organization of Wheat's Progenitor, Triticum Dicoccoides*. Berlin: pringer Science & Business Media

Numan, M., Bashira, S., Khana, Y., Mumtaza, R., Shinwaric, Z., Khanb, A., et al. (2018). Plant growth promoting bacteria as an alternative strategy for salt tolerance in plants: a review, *Microbiol. Res.* 209, 21–32. doi: 10.1016/j.micres.2018.02.003

Orhan, F. (2016). Alleviation of salt stress by halotolerant and halophilic plant growth-promoting bacteria in wheat (*Triticum aestivum*). *Braz. J. Microbiol.* 47, 621–627. doi: 10.1016/j.bjm.2016.04.001

Ramadoss, D., Lakkineni, V. K., Bose, P., Ali, S., and Annapurna, K. (2013). Mitigation of salt stress in wheat seedlings by halotolerant bacteria isolated from saline habitats. *SpringerPlus* 2:6. doi: 10.1186/2193-1801-2-6

Robinson, R. J., Fraaije, B. A., Clark, I. M., Jackson, R. W., Hirsch, P. R., and Mauchline, T. H. (2016). Endophytic bacterial community composition in wheat (*Triticum aestivum*) is determined by plant tissue type, developmental stage and soil nutrient availability. *Plant Soil* 405, 381–396. doi: 10.1007/s11104-015-2495-4

Roy, S. J., Negrão, S., and Tester, M. (2014). Salt resistant crop plants. *Curr. Opin. Biotechnol.* 26, 115–124. doi: 10.1016/j.copbio.2013.12.004

Rudolph, N., Labuschagne, N., and Aveling, T. A. S. (2015). The effect of plant growth promoting rhizobacteria on seed germination and seedling growth of maize. *Seed Sci. Technol.* 43, 507–518. doi: 10.15258/sst.2015.43.3.04

Saleem, A. R., Brunetti, C., Khalid, A., Della Rocca, G., Raio, A., Emiliani, G., et al. (2018). Drought response of Mucuna pruriens (L.) DC. inoculated with ACC deaminase and IAA producing *rhizobacteria*. *PloS One* 13:e0191218. doi: 10.1371/journal.pone.0191218

Schwyn, B., and Neilands, J. B. (1987). Universal chemical assay for the detection and determination of siderophores. *Anal. Biochem.* 160, 47–56. doi: 10.1016/0003-2697(87)90612-9

Shrivastava, P., and Kumar, R. (2015). Soil salinity: a serious environmental issue and plant growth promoting bacteria as one of the tools for its alleviation. *Saudi J. Biol. Sci.* 22, 123–131. doi: 10.1016/j.sjbs.2014.12.001

Siddikee, M. A., Chauhan, P. S., Anandham, R., Han, G. H., and Sa, T. (2010). Isolation, characterization, and use for plant growth promotion under salt stress, of ACC deaminase-producing halotolerant bacteria derived from coastal soil. *J. Microbiol. Biotechnol.* 20, 1577–1584. doi: 10.4014/jmb.1007.07011

Silini, A., Cherif-Silini, H., and Yahiaoui, B. (2016). Growing varieties durum wheat (*Triticum durum*) in response to the effect of osmolytes and inoculation by *Azotobacter chroococcum* under salt stress. *Afr. J. Microbiol. Res.* 10, 387–399.

Singh, R. P., and Jha, P. N. (2016a). A halotolerant bacterium Bacillus licheniformis HSW-16 augments induced systemic tolerance to salt stress in wheat plant (*Triticum aestivum*). *Front. Plant Sci.* 7:18090 doi: 10.3389/fpls.2016.01890

Singh, R. P., and Jha, P. N. (2016b). Mitigation of salt stress in wheat plant (*Triticum aestivum*) by ACC deaminase bacterium *Enterobacter* sp. SBP-6 isolated from Sorghum bicolor. *Acta Physiol. Plant* 38:110. doi: 10.1007/s11738-016-2123-9

Singh, R. P., and Jha, P. N. (2016c). The multifarious PGPR *Serratia marcescens* CDP-13 augments induced systemic resistance and enhanced salinity tolerance of wheat (*Triticum aestivum* L.). *PLoS One* 11:0155026. doi: 10.1371/journal.pone.0155026

Tamura, K., Stecher, G., Peterson, D., Filipski, A., and Kumar, S. (2013). MEGA6: molecular evolutionary genetics analysis version 6.0. *Mol. Biol. Evol.* 30, 2725–2729. doi: 10.1093/molbev/mst197

Tiwari, S., Singh, P., Tiwari, R., Meena, K. K., Yandigeri, M., Singh, D. P., et al. (2011). Salt-tolerant rhizobacteria-mediated induced tolerance in wheat (*Triticum aestivum*) and chemical diversity in rhizosphere enhance plant growth. *Biol. Fert. Soil.* 47:907. doi: 10.1007/s00374-011-0598-5

Tsavkelova, E. A., Cherdyntseva, T. A., Botina, S. G., and Netrusov, A. I. (2007). Bacteria associated with orchid roots and microbial production of auxin. *Microbiol. Res.* 162, 69–76. doi: 10.1016/j.micres.2006.07.014

Upadhyay, S. K., Maurya, S. K., and Singh, D. P. (2012). Salinity tolerance in free living plant growth promoting rhizobacteria. *Int. J. Sci. Res.* 3, 73–78.

Upadhyay, S. K., and Singh, D. P. (2015). Effect of salt tolerant plant growth promoting rhizobacteria on wheat plants and soil health in a saline environment. *Plant Biol.* 17, 288–293. doi: 10.1111/plb.12173

Upadhyay, S. K., Singh, D. P., and Saikia, R. (2009). Genetic diversity of plant growth promoting rhizobacteria isolated from rhizospheric soil of wheat under saline condition. *Curr. Microbiol.* 59, 489–496. doi: 10.1007/s00284-009-9464-1

Vurukonda, S. S. K. P., Vardharajula, S., Shrivastava, M., and SkZ, A. (2016). Enhancement of drought stress tolerance in crops by plant growth promoting rhizobacteria. *Microbiol. Res.* 184, 13–24. doi: 10.1016/j.micres.2015.12.003

Weide, A. (2015). On the identification of domesticated emmer wheat, *Triticum turgidum* subsp. *dicoccum* (*Poaceae*), in the aceramic neolithic of the fertile crescent. *Arch. Inf.* 38, 381–424.

Zhang, H., Kim, M. S., Sun, Y., Dowd, S. E., Shi, H., and Paré, P. W. (2008). Soil bacteria confer plant salt tolerance by tissue-specific regulation of the sodium transporter HKT1. *Mol. Plant Microbe Interact.* 21, 737–744. doi: 10.1094/MPMI-21-6-0737

Zhang, J. L., Aziz, M., Qiao, Y., Han, Q. Q., Li, J., Wang, Y. Q., et al. (2014). Soil microbe Bacillus subtilis (GB03) induces biomass accumulation and salt tolerance with lower sodium accumulation in wheat. *Crop Pasture Sci.* 65, 423–427. doi: 10.1071/CP13456

Conflict of Interest Statement: The authors declare that the research was conducted in the absence of any commercial or financial relationships that could be construed as a potential conflict of interest.

frontiers in Plant Science

ORIGINAL RESEARCH
published: 30 July 2019
doi: 10.3389/fpls.2019.00911

Trichoderma erinaceum Bio-Priming Modulates the WRKYs Defense Programming in Tomato Against the *Fusarium oxysporum* f. sp. *lycopersici* (*Fol*) Challenged Condition

Mohd Aamir[1], Sarvesh Pratap Kashyap[2,3], Andleeb Zehra[1], Manish Kumar Dubey[1], Vinay Kumar Singh[4], Waquar Akhtar Ansari[2,3], Ram S. Upadhyay[1] and Surendra Singh[1]*

[1] Laboratory of Mycopathology and Microbial Technology, Centre of Advanced Study in Botany, Institute of Science, Banaras Hindu University, Varanasi, India, [2] Division of Crop Improvement and Biotechnology, Indian Institute of Vegetable Research, Indian Council of Agricultural Research, Varanasi, India, [3] Department of Botany, Institute of Science, Banaras Hindu University, Varanasi, India, [4] Centre for Bioinformatics, School of Biotechnology, Institute of Science, Banaras Hindu University, Varanasi, India

OPEN ACCESS

Edited by:
Youssef Rouphael, University of Naples Federico II, Italy

Reviewed by:
Dierk Wanke, University of Tübingen, Germany
Dhananjaya Pratap Singh, National Bureau of Agriculturally Important Microorganisms (ICAR), India

***Correspondence:**
Mohd Aamir
khan.aamir18@gmail.com

Specialty section:
This article was submitted to Plant Microbe Interactions, a section of the journal Frontiers in Plant Science

Received: *02 August 2018*
Accepted: *27 June 2019*
Published: *30 July 2019*

Citation:
Aamir M, Kashyap SP, Zehra A, Dubey MK, Singh VK, Ansari WA, Upadhyay RS and Singh S (2019) Trichoderma erinaceum Bio-Priming Modulates the WRKYs Defense Programming in Tomato Against the Fusarium oxysporum f. sp. lycopersici (Fol) Challenged Condition. Front. Plant Sci. 10:911. doi: 10.3389/fpls.2019.00911

The beneficial association and interaction of rhizocompetent microorganisms are widely used for plant biofertilization and amelioration of stress-induced damage in plants. To explore the regulatory mechanism involved in plant defense while associating with beneficial microbial species, and their interplay when co-inoculated with pathogens, we evaluated the response of tomato defense-related *WRKY* gene transcripts. The present study was carried out to examine the qRT–PCR-based relative quantification of differentially expressed defense-related genes in tomato (*Solanum lycopersicum* L.; variety S-22) primed with *Trichoderma erinaceum* against the vascular wilt pathogen (*Fusarium oxysporum* f. sp. *lycopersici*). The tissue-specific and time-bound expression profile changes under the four different treatments "(unprimed, *Fol* challenged, *T. erinaceum* primed and *Fol* + *T. erinaceum*)" revealed that the highest upregulation was observed in the transcript profile of *SlWRKY31* (root) and *SlWRKY37* (leaf) in *T. erinaceum* bioprimed treated plants at 24 h with 16.51- and 14.07-fold increase, respectively. In contrast, *SlWRKY4* showed downregulation with the highest repression in *T. erinaceum* bioprimed root (24 h) and leaf (48 h) tissue samples with 0.03 and 0.08 fold decrease, respectively. Qualitative expression of PR proteins (chitinases and glucanases) was found elicited in *T. erinaceum* primed plants. However, the antioxidative activity of tomato superoxide dismutase and catalase increased with the highest upregulation of *SOD* and *SlGPX1* in *Fol* + *T. erinaceum* treatments. We observed that these expression changes were accompanied by 32.06% lesser H_2O_2 production in *T. erinaceum* bioprimed samples. The aggravated defense response in all the treated conditions was also reflected by an increased lignified stem tissues. Overall, we conclude that *T. erinaceum* bio-priming modulated the defense transcriptome of tomato after the *Fol* challenged conditions, and were accompanied by enhanced accumulation of defense-related *WRKY* transcripts, increased antioxidative enzyme activities, and the reinforcements through a higher number of lignified cell layers.

Keywords: bio-priming, defense transcriptome, *WRKY* genes, lignification, gene expression

INTRODUCTION

Microbial bio-priming represents an adaptive strategy to improve the defensive capacity of plants that result in increased resistance/stress tolerance, and/or a more aggravated defense response against the stress challenged conditions. Plant growth-promoting fungi (PGPF) include many strains of *Trichoderma* spp., which have been used as a potential biocontrol agent. The rhizocompetent nature of *Trichoderma* spp. allows it to colonize roots, stimulates the plant immune system (induced systemic resistance; ISR), and pre-activation (priming) of the molecular mechanisms of defense against several potent phytopathogens (Hermosa et al., 2012; Pieterse et al., 2014; Martínez-Medina et al., 2017). Furthermore, colonization of this beneficial fungi promotes plant growth and also ameliorates the host plants against various abiotic and biotic stresses (Brotman et al., 2013; Zhang et al., 2016; Fu et al., 2017).

The WRKY family of transcription factors (Tfs) play a central role in plant development and the defense response against various abiotic and biotic stresses (Kiranmai et al., 2018; Singh et al., 2018; Vives-Peris et al., 2018) are plant-specific zinc finger type regulatory proteins. The WRKY proteins regulate the gene expression directly or indirectly by modulating the downstream target genes, by activating or repressing the other genes (encoding Tfs) or by self-regulating their own expression (Shankar et al., 2013). In tomato (*Solanum lycopersicum*), a total of 83 *WRKY* genes (previously documented 81 genes; Huang et al., 2012) has been identified (Bai et al., 2018; Karkute et al., 2018). WRKY TFs play an indispensable role in the regulation of diverse biological processes, but most notably are the key players in plant responses to biotic and abiotic stresses (Bai et al., 2018). The plant defense regulation involving WRKY proteins can be determined through dynamic changes in the levels of accumulated *WRKY* transcripts inside the cell (Chi et al., 2013). One of the most crucial aspects of *WRKY* gene regulation is that despite of having the functional diversity among different WRKY members, almost all analyzed WRKY proteins recognize and bind conserved TTGACC/T W-box sequences (de Pater et al., 1996; Rushton et al., 1996; Eulgem et al., 2000; Ciolkowski et al., 2008). However, DNA binding assays revealed the importance of the invariant "GAC" core consensus sequences of the core promoter element in a feasible DNA-protein interaction (Sun et al., 2003; Ciolkowski et al., 2008; Brand et al., 2010, 2013). Further, some WRKY members exhibit differences in their DNA binding preferences, which are partly dependent on additional adjacent DNA sequences lying outside of the TTGACY-core motif (Ciolkowski et al., 2008). By homology modeling, *in vitro* DNA-protein interaction-enzyme-linked immunosorbent assay and molecular simulation studies performed with different AtWRKY proteins revealed differences in DNA binding specificities (Brand et al., 2013). The two most important questions arise if all the WRKY factors bind with the W-box DNA; how the specificity for certain promoters is accomplished, and how the stimulus-specific responses are mediated by diverse members of the *WRKY* gene family. Later, studies revealed that besides the W-box-specific DNA binding, *WRKY* specific stimulus-response is facilitated by other essential components that regulate the biological function of different WRKY members (Brand et al., 2013). Furthermore, activation or repression through W-box and W-box like sequences is regulated at transcriptional, translational, and domain level (Phukan et al., 2016) and the interaction of WRKY Tfs with the W-box (with core motif TTGACC/T) and clustered W-boxes present in the promoters of downstream genes, regulate a dynamic web of signaling through the kinase or other phosphorylation cascades. Epigenetic, retrograde and proteasome-mediated regulation pathways enable *WRKYs* to accomplish a dynamic cellular homeostatic reprograming (Bakshi and Oelmüller, 2014; Phukan et al., 2016). Microbial priming is also associated with chromatin modification in promoters of WRKY Tfs genes that regulates SA dependent defenses, thereby promoting the enhanced expression of WRKYs upon pathogen challenged condition (Jaskiewicz et al., 2011). Furthermore, *WRKYs* role in transcriptional reprogramming becomes clearer because of *in silico* analysis of *cis*-acting DNA regulatory elements from the promoter region of stress-responsive *WRKYs* revealed the presence of various abiotic stress-responsive elements (Karkute et al., 2018). In this way, characterization of the promoter elements could help in understanding the functional dimension and regulation of WRKY members at the molecular level which is particularly important for those WRKYs that have been reported in the crosstalk between abiotic and biotic stress tolerance (Bai et al., 2018). Because of the tight regulation involved in the specific recognition and binding of WRKYs to the downstream promoters, they have been considered as a promising candidate for crop improvement (Karkute et al., 2018).

The rhizospheric microbiome (beneficial) are known to play an indispensable role in the transcriptional reprogramming required for plant defense against pathogens (Spence et al., 2014) and requires complex signaling cascades, involving multiple Tfs that primarily function as transcriptional regulators. At the molecular level, the biocontrol mechanism induced by *Trichoderma* is mediated through the adaptive recruitment and reprogramming of defense-related transcripts (Shaw et al., 2016). The transcriptional regulation of stress-inducible genes and the activation of an adaptive response (microbial symbiosis) are mediated and modulated through an immediate early expression of the *WRKY* genes (Chen et al., 2012). Therefore, the level of the WRKY proteins inside the cell accumulate sharply, which further directs the transcriptional regulation of the target genes through regulation with other *cis*-acting response elements (Chen et al., 2012). In addition, bio-priming with *Trichoderma* spp. has been found to be associated with the expression of several genes involved in regulating the general oxidative stress as well as osmoprotection. Further, the signaling cascade that regulates the plant pathogen interaction may involve classical phytohormones such as salicylic acid (SA), jasmonic acid (JA), and ethylene (ET) or may incorporate auxin, abscisic acid (ABA), cytokinins (CKs), and brassinosteroids (Robert-Seilaniantz et al., 2011). The pathogen-triggered immunity (PTI) and effector-triggered immunity (ETI) has been reported to function at differently regulatory levels and their signaling network is regulated by WRKY proteins (Shen et al., 2007; Mangelsen et al., 2008; Bakshi and Oelmüller, 2014). In one prominent example, it was found that in barley (*Hordeum vulgare*) *HvWRKY1* and *HvWRKY2* were found to activate by the FLG22 (a MAMP) and were reported to

play a critical role by acting as a negative regulator (repressor) of the PTI against the powdery mildew fungus, *Blumeria graminis* f. sp. *hordei* (Bai et al., 2018).

Trichoderma induced bioprimed signaling involves a systemic resistance type immune response (Tucci et al., 2011; Yoshioka et al., 2012; Martínez-Medina et al., 2013), interconnected in a complex network of cross-communicating hormone pathways involving the JA/ET-induced systemic resistance (ISR) or SA dependent signaling mechanism. It has been documented that the early signaling events following the interaction of *Trichoderma* spp. with the host plant, is mediated through the pattern recognition receptors (PRRs) that activate the MAMPs/DAMPs-triggered immunity (MTI/DTI) (Hermosa et al., 2013; Ruocco et al., 2015). The WRKY mediated defense response regulates the signaling crosstalk through the activation of JA/ET-mediated signaling pathways, encoding repressors that suppress the SA regulated gene expression (Lai et al., 2008). Therefore, WRKY Tfs also acts as an important node of convergence between the SA and JA signaling (Pieterse et al., 2012). During pathogen challenged conditions, plants protect themselves through a plethora of mechanisms including activation of the ROS system, accumulation of H_2O_2, cellular reinforcement at the infection sites (through deposition of the suberin and lignin), expression of the plant pathogenesis-related (PR) proteins (Pusztahelyi et al., 2015) which further reflects the role of the SA mediated systemic acquired resistance (SAR) pathway (Gharbi et al., 2017). Among the differentially expressed PR proteins, chitinases and β-1, 3-glucanases are two major hydrolytic enzymes abundant in many plant species following the infection of different fungal pathogens (Ebrahim et al., 2011; Balasubramanian et al., 2012). Both chitinases and glucanases play a crucial role in plant defense against fungal pathogens since the cell wall of pathogenic fungi is composed of the two most crucial elements chitin and β-1, 3-glucan, and constitute the structural barrier of pathogenic cell wall fungi. Further, β-1, 3- glucanases appear to be coordinately expressed along with chitinases after fungal infection (Balasubramanian et al., 2012). The lignification event is an important mechanism which is accompanied by the accumulation of lignin or lignin-like phenolic compounds following the pathogenic attack and has been reported to occur in the plethora of plant-microbe interactions during the plant defense responses (Ebrahim et al., 2011). As a part of the host defense mechanism, lignification plays an indispensable role in preventing pathogen growth and dissemination. The lignified tissues represent the structural barrier and provide resistance in plants against biotic damages.

Trichoderma spp. induced bio-priming is characterized by the secretion of various antimicrobial compounds through the participation of the phenylpropanoid pathway that not only delimits the infection and dissemination of pathogens but also confers tolerance against various abiotic stresses (Mastouri et al., 2012; Ahmad et al., 2015). The inoculation of *Trichoderma* spp. leads to the activation of an efficient reactive oxygen species (ROS) detoxification system (De Palma et al., 2019). Further, mycoparasitic colonization by *Trichoderma* spp. leads into induction and accumulation of the PR proteins at early stages of root colonization (Yedidia et al., 2000). In many studies, it has been demonstrated that treatment of plants with beneficial microbes, particularly *Trichoderma*, enhances plant resistance against pathogens through ROS generation and lignification (Patel et al., 2017; Meshram et al., 2019).

In recent years, experimental studies done with *Arabidopsis thaliana* co-inoculated with *T. asperelloides* T203 provides sufficient data showing an increased expression of the specific *WRKY* genes and activation of the JA pathway that stimulates JA signaling through repression of the jasmonate ZIM domain (JAZ) repressors (Brotman et al., 2013). However, the signaling cascades and molecular events involved in *Trichoderma*-root association in the presence of elicitors or effectors from the pathogen *Fusarium oxysporum* f. sp. *lycopersici* (*Fol*) have not been investigated in light of *WRKYs* gene-mediated defense regulation of the host. Therefore, the objective of the present study is to provide a comparative approach for unraveling the *WRKY* gene-mediated defense signaling in the presence and absence of the beneficial microbe (*Trichoderma* spp.). The study was carried out to examine the real-time based relative quantification of differently expressed defense-related *WRKY* genes in tomato plants primed with *T. erinaceum* against the fungal pathogen *Fusarium oxysporum* f. sp. *lycopersici.* Additionally, we have also analyzed the time-dependent and tissue-specific expression profile changes in genes that constitute the ROS detoxification system, including superoxide dismutase (*SOD*), glutathione peroxidase (*GPX1*) and PR proteins. The biochemical response of the host has been investigated in terms of antioxidative enzyme activities, H_2O_2 content, and lignin deposition.

MATERIALS AND METHODS

Fungal Inoculum Preparation

The pathogenic cultures *Fusarium oxysporum* f. sp. *lycopersici* (*Fol*) was brought from the Department of Mycology and Plant pathology, Institute of Agricultural Sciences, Banaras Hindu University (BHU). The fungal inoculum was prepared from the 7 day old culture of *Fol* pathogen as per the method suggested by Taylor et al. (2013). In brief, the Petri dish containing the culture was suspended with sterile distilled water. The spores were gently removed using a glass spreader, and then the heterogeneous suspension was filtered using muslin cloth for removing the mycelial mat. The filtered suspension was diluted with sterile distilled water to maintain a minimum density of 2×10^5 to 2×10^6 spores mL^{-1} as quantified through the hemocytometer.

Pathogenicity Test

The pathogenicity test was performed with the *Fol* isolate to validate the Koch postulates for confirmation of the role of the pathogen in developing vascular wilt disease symptoms. The spore suspension of the *Fol* pathogen prepared above was used for pathogenicity testing. Twenty days old healthy tomato seedlings from both control and bioprimed plants were inoculated by the standard root dip method (Nirmaladevi et al., 2016). The healthy seedlings were uprooted from pots with gentle care without disturbing and disrupting the root integrity, shaken for removal of adhered soil particles and washed gently under the running tap water. The sterilized scissor was used for trimming the root apex portion (about 1 cm) and then the trimmed root portion

was dipped in the prepared conidial suspension (2×10^5 to 2×10^6 spores mL^{-1}) of the *Fol* pathogen for 30–40 min, for soaking in the conidial suspension. The inoculated seedlings were then used for transplantation to mini pots (15 cm diameter, surface sterilized with 0.1% mercuric chloride) containing the sterilized soil and sand mixed in a 2:1 ratio. Three seedlings per pot were used for transplantation. Plants were maintained in a greenhouse under 16 h light/8 h dark conditions with temperature ranging 28–29°C. Seedlings were watered on a daily basis. The symptoms of vascular wilt disease were initially observed 15–20 days after post inoculation of the *Fusarium oxysporum* f. sp. *lycopersici* (*Fol*) pathogen.

In vitro Antagonistic Assay

Ten different isolates of *Trichoderma* spp. (T_1–T_{10}) were brought about from NBAIMCC, Kushmaur, Mau, Uttar Pradesh, India. The isolates were as follows: *Trichoderma fasiculatum* (T_1) (NAIMCC-F-01714), *T. hamatum* (T_2) (NAIMCC-F-01717), *T. koningii* (T_3) (NAIMCC-F-01757), *T. longibrachiatum* (T_4) (NAIMCC-F-01770), *T. pseudokoningii* (T_5) (NAIMCC-F-01775), *T. asperellum* (T_6) (NAIMCC-F-02170), *T. erinaceum* (T_7) (NAIMCC-F-02171), *T. virense* (T_8) (NAIMCC-F-02231), *T. piluliferum* (T_9) (NAIMCC-F-02227), *T. viride* (T_{10}) (NAIMCC-F-02500). The detailed information regarding the *Trichoderma* spp. used in this study including accession identities, source of isolation and isolation code has been provided in **Supplementary Table S1**. The *in vitro* antagonistic activity was checked for all the selected 10 isolates of *Trichoderma* spp. against the *Fol* pathogen, to find out the best isolate that could be used for seed bio-priming and further experiments. A 5 mm mycelial plug was cut with the help of cork borer from both the *Fol* Petri plate and each of the *Trichoderma* isolates (T_1–T_{10}) culture plates. The disks from *Fol* and each of the *Trichoderma* isolates were placed in a fresh culture plate with a separation of 3 cm apart from each other. The plates were then incubated at 27 ± 2°C for 5 days. The mycelial growth was recorded, and the percent inhibition was calculated. The experiment was done in replicates of three and the percentage inhibition of radial growth was measured from formula [100 × (C–T)/C] where C = radial growth of the pathogen in control and T = radial growth of the pathogen in dual culture with antagonist (Garrett, 1956).

Preparation of *T. erinaceum* Spore Suspension and Seed Bio-Priming

The *Trichoderma* isolates showing the maximum growth inhibition (among the 10 isolates under study) of the *Fol* pathogen (*T. erinaceum*; NAIMCC-F-02171) was further grown on PDB medium for 7 days at 27 ± 2°C. The spores were harvested in sterile saline (NaCl 0.85%) and filtered with a sterile muslin cloth. The optical density OD was measured at 600 nm with OD of 1.026 that contained 2.26×10^7 spores mL^{-1}. The spore suspension was centrifuged at 10,000 rpm for 10 min. The pellet was re-suspended in the same volume of autoclaved 1.5% CMC (carboxy methyl cellulose) (Jain et al., 2012). The tomato seeds (S-22 variety) susceptible (83.67%) to the *Fol* pathogen (Chopada et al., 2014) were used in this experiment. For seed bio-priming with spore suspension of *T. erinaceum* the fresh and healthy seeds of tomato were surface sterilized with 0.01% aqueous solution of mercuric chloride followed by repeated washing with double-distilled water and further dried under laminar air flow on autoclaved blotting paper (Jain et al., 2012). The surface sterilized and dried seeds were treated by soaking in the spore suspensions of *T. erinaceum*. The control seeds were treated with only CMC without suspension. Further, all the seeds were placed in the moist chamber at 98 % relative humidity and 28–30°C and maintained for 24 h (Jensen et al., 2004).

Pot Trials

The *T. erinaceum* bioprimed and control seeds were further sowed in the fresh plastic pots (having 08 cm diameter) containing the sterilized soil mixed with vermiculite (2:1). A total of 4 seeds per pot were sown for each treatment and a control set was maintained. The pots were irrigated manually on every alternate day. A total of five replicates for each treatment were prepared and maintained at 16 h light/8 h dark in greenhouse conditions with temperature 28–29°C and relative humidity in the range of 50–70% following the protocol (Zehra et al., 2017). All the seeds were allowed to grow in greenhouse conditions for 5–6 weeks. The 5–6 weeks old plants following the seed germination (height 15 cm) were treated with the *Fol* suspension following the protocol (Zehra et al., 2017). The root and leaf tissues from all the four treatments including control, *Fol* challenged, *T. erinaceum* bioprimed and the *T. erinaceum* bioprimed + *Fol* challenged were collected at the different time interval 0 h (control), 24 and 48 h for qRT-PCR analysis. Further, the biochemical assessment was done using the leaf tissues from all the samples collected at different time intervals.

Morphological Growth Characteristic

The monitoring of the bioprimed plant and control (unprimed) plants was done regularly. The plants were first observed at 15 days and later on after 45 days interval for recording the characteristic changes observed in morphological growth parameters and other attributes such as increased plant height, root and shoot length, number of leaves, and thickness of the stem. The data were compared with the same day untreated control samples.

RNA Extraction and cDNA Synthesis

The relative expression of distinctly upregulated *WRKY* transcripts under all the treated conditions were measured both quantitatively and semi-quantitatively in root and leaf tissues at different time intervals (0, 24, and 48 h), respectively. The total RNA was extracted using TRIZOL reagent (Invitrogen) following the manufacturer's protocol. The 1.0% agarose gel (prepared in DEPC treated water) was used to check the quality of the extracted RNA. Further, the quantification, purity, and integrity of the extracted RNA were evaluated using Nanophotometer (Implen, CA, United States) at absorption ratio of 230/260/280 nm. The first strand of cDNA was synthesized through the iscript^{TM} cDNA synthesis kit (Bio-Rad Laboratories,

United States) using 1.0 μg of the extracted RNA as per the given recommendation.

Real-Time Quantitative PCR Analysis

The quantitative and semi quantitative studies were done for evaluating the spatial and temporal expression of accumulated *WRKY* transcripts as well as other defense related genes in all the four treatment conditions. Real-time quantitative PCR (qRT-PCR) reactions were performed using SsoFast™ EvaGreen® Supermix detection chemistry (Bio-Rad) with an iQ5 thermocycler (BioRad Laboratories, United States). The qPCR was performed in three independent biological replicates with each biological replicate performed in triplicates using the SYBR Green fluorescence dye (Qiagen, United States) and analyzed using iQ-SYBR Green Supermix (Bio-Rad, CA, United States) on iQ5 thermocycler (Bio-Rad, CA, United States) with iQ5 Optical System Software version 2.0 (Bio-Rad, CA, United States) following the protocols as mentioned. The PCR reactions were performed in a 20 μl final volume reaction mixture that contains 2 μl of the template cDNA (20 ng), 1 μl of each gene-specific primer (0.2 μM) and 10 μl of 2 × SsoFast™ EvaGreen® Supermix. The *WRKY* gene-specific primer was designed through the Primer 3 http://primer3.ut.ee/ (Untergasser et al., 2012) (**Table 1**). The protein sequences of glutathione peroxidase (SlGPX1; NP_001234567) and PR proteins including both PR2 (NP_001234158) and PR3 (XP_004237833.1) were used for designing gene specific primers. Further, sequences of all the primers were validated using Primer-Blast at https://www.ncbi.nlm.nih.gov/tools/primer-blast/ (Ye et al., 2012). The qRT PCR reaction program was set as an initial denaturation at 95°C for 10 min followed by 45 cycles of denaturation at 95°C for 15 s, annealing at 60°C for the 30 s and extension at 72°C for 30 s. The heat map was generated using the replicated count data Bio-conductor R[1] obtained from expression values for both root and leaf tissues. The tomato *ACTIN* gene was used as a reference gene due to its constitutive and stable expression (Vergne et al., 2007). The ΔC_t value was calculated from the difference observed between the C_t values given for our target *WRKY* gene and the housekeeping *ACTIN* gene (that act as the constitutive control). The relative quantification was analyzed by using the $2\text{-}^{\Delta\Delta CT}$ method given by Livak and Schmittgen (2001) and then normalized to the C_t data about the transcript level of the *ACTIN* gene as an internal control because of its constitutive expression. The heat map was generated using Bioconductor R (see footnote 1) software tool.

Gene Prediction, Chromosomal Map, and *Cis*-Acting *DNA* Regulatory Element Analysis

We have predicted the location of three characterized *WRKY* genes (including *SlWRKY4*, *SlWRKY31*, and *SlWRKY37*) having clear-cut upregulation in all the *Fol* challenged tissues through the gene prediction tool. Further, the chromosomal location of each *WRKY* gene in the tomato genome was searched and a promoter scan was done to identify the putative *cis*-acting DNA regulatory elements including the position of the W-box DNA. For gene prediction, the topmost hit identifiers for each respective SlWRKY member having maximum query cover and percent identity values were selected (based on Blast-p annotation). The protein sequences of SlWRKY4 (XP_004235494.1), SlWRKY31 (NP_001306910.1; previously renamed as SlWRKY33A) and SlWRKY37 (NP_001308885.1) were collected from the NCBI. The CDS sequences were analyzed using BLASTx tool to check the full-length protein sequence. Further, the full-length protein sequences were searched using tBLASTn program of NCBI and was searched across the whole genome shotgun contigs (wgs) database with selecting organism name (*Solanum lycopersicum* taxid: 4081). The promoter region prior to translational start site was searched for each *SlWRKY* gene and was confirmed through Blastx tool [where TSS; represent the position of transcription start site (TATA-box position)] and polyadenylation (Poly A; Poly A represents the 3′ polyadenylation site) tail. The orientation of each *SlWRKY* gene in positive frame was determined through the Fgenesh[2] server. The Gene display server (GSDS 2.0)[3] (Hu et al., 2015) was used to map the position of promoter, coding sequences, intronic region along the length of each *SlWRKY* gene. Further, to locate the position of each characterized *SlWRKY* gene on tomato chromosome, the respective protein sequences were annotated using tBLASTn tool across the wgs contigs database. Further, the chromosomal location of each *WRKY* gene was retrieved from Ensembl-Blast tool. The Plant CARE promoter database http://bioinformatics.psb.ugent.be/webtools/plantcare/html/ (Lescot et al., 2002) was used for finding the *cis*-acting DNA regulatory elements that bind to the promoter region of the characterized *WRKY* genes.

Biochemical Assessment of Plant Defense Response

H_2O_2 Quantification

The amounts of H_2O_2 produced and accumulated in leaf tissues collected from all the treated samples (control, the *Fol* challenged, *T. erinaceum* bioprimed and the *Fol* + *T. erinaceum*) were quantified following the method as suggested by Jana and Choudhuri (1981). The leaves at different time intervals (0, 24, 48, and 72 h) were collected from all the treated samples. 200 mg of leaf tissue from each sample was crushed in 50 mM sodium phosphate (NaH_2PO_4) buffer (pH 6.5). The sample was then centrifuged at 8000 rpm for 20 min. The supernatant thus obtained was amended with 0.1% titanium sulfate (TiS_2O_8). The sample was again centrifuged and the intensity of the yellow colored solution was measured spectrophotometrically at 410 nm. The amount of H_2O_2 produced was calculated with an extinction coefficient of 0.28 $\mu M^{-1}cm^{-1}$ and was expressed as $\mu mol\ g^{-1}$ fresh weight (FW).

[1]http://www.bioconductor.org

[2]http://www.softberry.com/

[3]http://gsds.cbi.pku.edu.cn

TABLE 1 | List of gene specific primers used for quantitative and semi-quantitative real time PCR studies.

S.No	Gene name	Primer sequence (5-3′)	T_m	GC%
1	*SlWRKY4* (Forward Primer)	CGTTGCACATACCCTGGATG	58.98	55.00
2	*SlWRKY4* (Reverse Primer)	GGCCTCCAAGTTGCAATCTC	59.19	55.00
3	*SlWRKY31* (Forward Primer)	CCACCTCCTTCACTTCCATT	57.11	50.00
4	*SlWRKY31* (Reverse Primer)	GATGGAAAACTCCCAGTCGT	57.53	50.00
5	*SlWRKY37* (Forward Primer)	CAGATGCAGCAGTTCAAAGG	57.37	50.00
6	*SlWRKY37* (Reverse Primer)	CTTCGAGGGACACATGTTGA	57.54	50.00
7	Chloroplast Cu/Zn-superoxide dismutase (SOD) (Forward Primer)	CTGGACTTCACGGGTTTCAT	57.81	50.00
8	Chloroplast Cu/Zn-superoxide dismutase (Reverse Primer)	TTTGGACCGGTCAATGGTAT	56.81	45.00
9	Chitinase (*CHI1*) (Forward)	GTCAAGGGGGACCTTGTTTT	60.20	50.00
10	Chitinase (*CHI1*) (Reverse	CATGTGTGACATGAGCGAAG	58.81	50.00
11	β-1,3-Glucanase (GNSL) (Forward)	AGACAACGTCCGAGGGTATG	59.18	55.00
12	β-1,3-Glucanase (GNSL) (Reverse)	TTTTTCAAGGGCCGAGTATG	56.03	45.00
13	Phospholipid hydroperoxide glutathione peroxidase (GPX1) (Forward)	ACCAGTTTGGTGGACAGGAG	60.00	55.00
14	Phospholipid hydroperoxide glutathione peroxidase (GPX1) (Reverse)	GCTGGAGAAGTGGTTGGAGA	60.39	55.00
15	Actin (Constitutive control) Forward primer	GAAATAGCATAAGATGGCAGACG	58.90	45.00
16	Actin (Reverse)	ATACCCACCATCACACCAGTAT	58.40	45.00

Assessment of Antioxidative Enzyme Activities

SOD Activity

The superoxide dismutase (SOD; EC 1.15.1.1) activity was measured following the method as suggested by Beauchamp and Fridovich (1971). 200 mg leaf tissues from all the treated leaf samples were crushed in a 5 mL extraction buffer that contained 0.1 M phosphate buffer (pH 7.5) and 0.5 mM EDTA. The samples were then centrifuged at 15,000 rpm for 15 minutes. The final assay mixture was prepared with a 50 mM phosphate buffer (pH 7.8), 13 mM methionine, 75 μM NBT, 60 μM riboflavin, 0.1 mM EDTA, 200 μl enzyme extract and 2 μM riboflavin was used for spectrophotometric calculations recorded at 560 nm.

CAT Activity

Catalase (CAT; EC: 1.11.1.6) activity was measured following the protocol as suggested by McKersie et al. (1990). The leaf tissues 200 mg from each of the treated samples was homogenized in a 5 ml of 50 mM Tris NaOH buffer (pH 8.0) that comprised of 0.5 mM EDTA, 2 % (w/v) PVP, 0.5% (v/v) Triton X-100. The absorption of the final assay mixture having 200 μL enzymic extract, 50 mM H_2O_2 and 100 mM phosphate buffer (pH 7.0) was recorded for 5 min at 240 nm and the activity was expressed as μmol of H_2O_2 oxidized min^{-1} mg^{-1} protein (extinction coefficient of 0.036 mM^{-1} cm^{-1}).

Histochemical Staining for Assessment of Lignification

The histochemical analysis for detection of the lignified tissue was done following the protocol as suggested by Guo et al. (2001). The transverse section (TS) of stem tissues from the second internode region was cut with Leica VT1000 Semiautomatic Vibrating Blade Microtome used for cutting the thin sections with thickness of 150 micron collected from all the treated tissues, and further, mounted in 1% phloroglucinol solution made with 95% alcohol. The mounted samples were covered with 0.1 mL concentrated HCl and was placed on a clean glass slide covered with the coverslip (Guo et al., 2001). The stained samples were visualized under a compound light microscope (Nikon, Japan). The lignified tissues were identified as intense pink coloration of deposited lignified material.

Statistical Analysis

Statistical analysis was done using the statistical package SPSS (SPSS Inc., Version 16.0). All the experiments were performed in three independent biological replicates with each replicate performed in triplicates, and for statistical analysis, the mean value of each replication was used, one-way analysis of variance (ANOVA) performed for significance difference, while the mean separations were compared with Duncan's multiple range test at the $P \leq 0.05$ significance level.

RESULTS

Pathogenicity Tests

The symptoms of vascular wilt disease were initially observed 15–20 days post inoculating the *Fol* pathogen. The earlier symptom developed was yellowing of the big and lower leaves followed by their drooping at later stages. At later stages, it was found that the upper aerial leaves had shown the loss of turgidity with dried lower leaves. On the basis of root-dip inoculation test (Nirmaladevi et al., 2016) pathogen was able to cause the vascular wilt disease, and was also recovered from the diseased wilted plants. In contrast, the uninoculated tomato seedlings did not develop any diseased symptoms, and therefore, validated the Koch's postulates. The recovered *Fol* pathogen was grown in a fresh Petri-plate on PDA medium. The microscopic study was done to observe the structure of pathogenic hyphal filaments and conidial structure. The structure of fungal mycelium with oval or kidney shaped microconidia, and sickle shaped macroconidia has been shown (**Figure 1**).

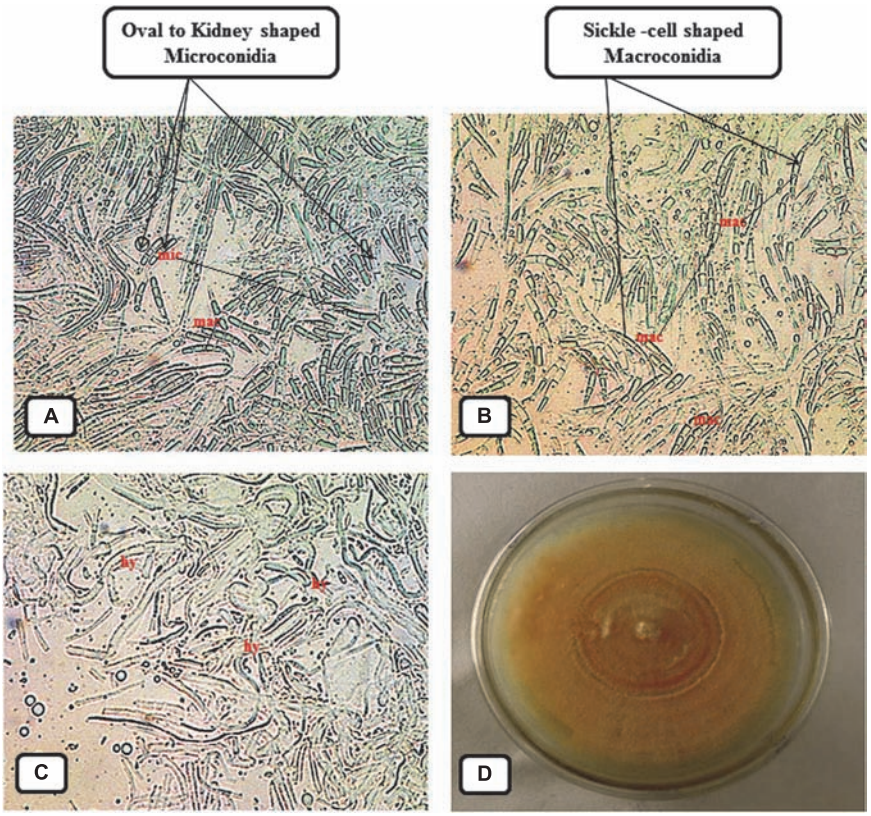


FIGURE 1 | Photograph showing morphological and cultural characteristic of the vascular wilt pathogen *Fusarium oxysporum* f. sp. *lycopersici* (*Fol*) maintained on Potato Dextrose Agar (PDA) medium. **(A)** Microscopic observation of the small, oval or kidney shaped, and one or two celled, Microconidia (Abbreviation: mic) present in between the larger sickle shaped Macroconidia. **(B)** Microscopic view of the sickle-shaped, thin walled and delicate Macroconidia (Abbreviation: mac). **(C)** Structure of the fungal mycelium composed of interwoven hyphal filaments (Abbreviation: hy). **(D)** Photograph showing cultural characteristic and growth pattern of the *Fol* pathogen grown on Petri-plate.

In vitro Antagonistic Assay

It was found that all the isolates (T_1–T_{10}) of *Trichoderma* were found to be significantly effective in inhibiting the mycelial growth of the *Fol* pathogen over their respective control. However, *T. erinaceum* (T_7) (**Figure 2I**) showed the best antagonistic activity against the *Fol* pathogen followed by *T. longibranchiatum* (T_9) and *T. asperellum* (T_6). Therefore, the *T. erinaceum* (T_7) isolate was selected for seed bio-priming and greenhouse experiments. The dual culture assay showing the mycoparasitic interaction of *T. asperellum* with the *Fol* pathogen has been shown in **Figure 2II**.

Morphological Growth Characteristic

The bio-priming of tomato plants with *T. erinaceum* resulted in the profuse growth of the tomato plants, bearing changes in several morphological attributes like an increase in plant height, the thickness of stem, root growth, root length, and number of leaves etc., and the same was compared with an untreated control sample. To observe the changes occurring in the morphological parameters, regular monitoring of the *T. erinaceum* bioprimed and unprimed (control) plants were done. The stem from *T. erinaceum* bioprimed and unprimed (control) were sectioned with a razor blade at the junction of root and stem to observe the morphological changes, particularly, measured in terms of root growth, and thickness of the stem collected at 15 and 45 day intervals and the same was compared with an unprimed (control) of the same day. The morphological changes in the root growth and the thickness of the stem in control plants compared with bioprimed plants collected at 15 days (**Figure 3I-A**) and 45 days (**Figure 3I-B**) have been shown. The photograph was taken to show the effect of the priming response of *T. erinaceum* on the morphological growth parameters of tomato plants (**Figure 3II-A**) and the unprimed plants (**Figure 3II-B**) has been shown.

Gene Prediction, Chromosomal Map and *Cis*-Acting DNA Regulatory Element Analysis

Based on Blast annotation results we predicted the genomic position of each characterized *SlWRKY* gene that comprised of "TSS", exonic coding sequences (both CDS_f and CDS_l), and the "poly A" tail region across the whole tomato genome. The complete *SlWRKY31* gene coding sequence including TSS and poly A tail have been represented (**Supplementary Figure S1A**).

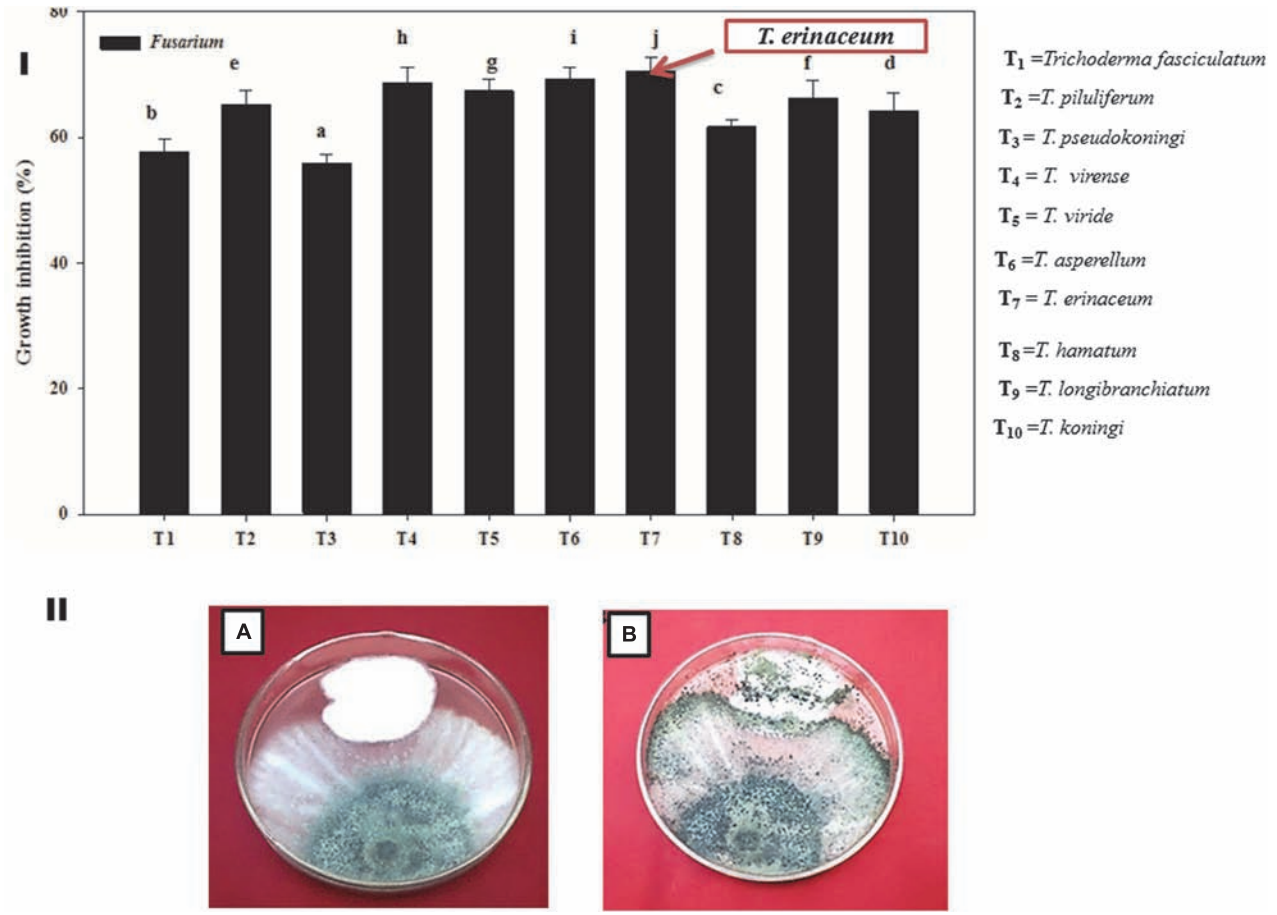


FIGURE 2 | (I) Bar graph showing the efficacy of different species of biocontrol agent *Trichoderma* spp. in inhibiting the mycelial growth of the *Fol* pathogen. The figure represents the *in vitro* mycoparasitic interaction of different isolates of *Trichoderma* spp. (T_1–T_{10}) against the *Fol* pathogen through dual culture assay. The bar graphs represent the percentage growth inhibition of the pathogenic hyphae (*Fol*) by different species of *Trichoderma* employed in the study. The *T. erinaceum* (T_7) showed the maximum inhibitory action against the *Fol* pathogen. **(II)** The Dual culture Assay showing the *in vitro* mycoparasitic and antagonistic activity of *Trichoderma* spp. against the pathogen *Fusarium oxysporum* f. sp. *lycopersici*. **(A)** Culture plate showing the mycoparasitic behavior of *Trichoderma* spp. and the *Fol* pathogen 5 days post inoculation. **(B)** The mycoparasitic activity of the *Trichoderma* showing the growth over the *Fol* pathogen and the picture was taken 10 days post inoculation of the *Trichoderma* spp. and the *Fol* pathogen (inoculated simultaneously). Mean (±SE) was calculated from three replicates for each treatment. Bars with distinct letters are significantly different at $P \leq 0.05$ using the DMRT test.

The presence of TSS and poly A in the entire coding sequence, and particularly, the TSS before the poly A region revealed that our gene of interest is present in a positive frame, and the gene prediction result is accurate. The promoter region lying upstream of the translational start site of the *SlWRKY31* gene has been shown in **Supplementary Figure S1B**. The position of CDS encoding *SlWRKY4* including TSS and poly A tail has been shown (**Supplementary Figures S2A,C**). Further, the upstream sequences lying *SlWRKY4* and *SlWRKY37* have been shown in **Supplementary Figures S2B,D**. We identified a genomic locus of 8606 bp that contains the *SlWRKY4* gene, which is unambiguously mapped to a position on Chromosome (Chr) 3. Similarly, the genomic locus of 4065 and 3674 bp accommodated the *SlWRKY31* and *SlWRKY37* genes and were mapped on "Chr 6" and "Chr 1," respectively. We found that besides W-box and other defense related elements, the promoter region of our characterized *SlWRKY* genes was flanked frequently with other abiotic stress-responsive elements like *HSE*, *ABRE* and *MBS* which clearly indicates their possible biological role in the management of abiotic stresses as well. The promoter region of tomato *SlWRKY4* and *SlWRKY37* genes have *LTR*, *ABRE*, *ERE*, *MYB* core elements. In contrast, the promoter region of tomato *SlWRKY31* gene was characterized by the presence of *TGA* motifs, *TCA element*, and *TATC box* along with other common motifs. The different promoters with their sequences and relevant functions have been shown in **Supplementary Table S2**.

Gene Expression Analysis

The qRT-PCR studies unraveled the distinct temporal and the tissue-specific expression of tomato defense-related *WRKY* transcripts. In our previous study, we reported that during the *Fol* challenged conditions in tomato, a total of 16 different Sl*WRKY* genes were involved in plant defense, of which only three *WRKYs* (*SlWRKY4*, *SlWRKY31*, and *SlWRKY37*) were

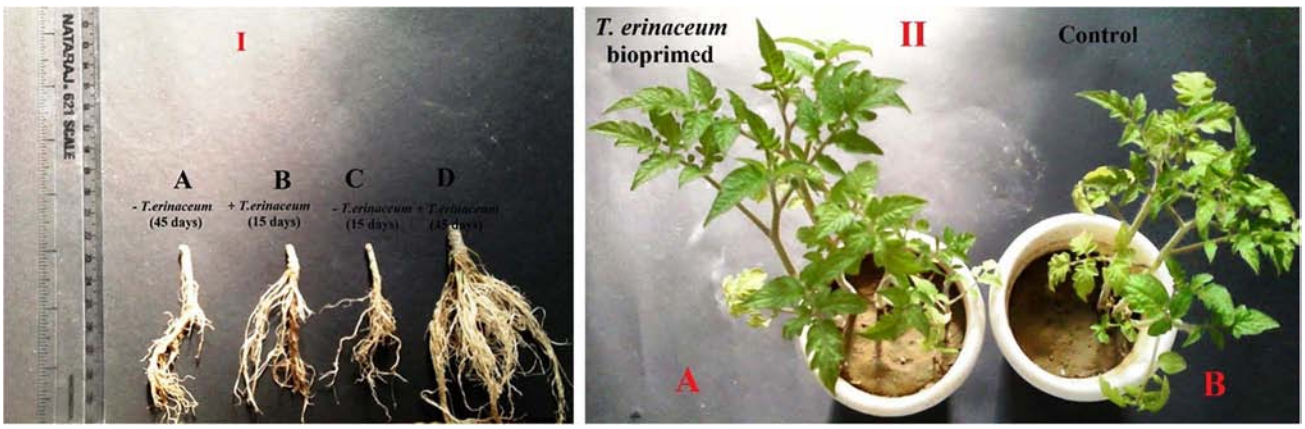


FIGURE 3 | (I) Morphological growth characteristic of the *T. erinaceum* bioprimed and unprimed plants at two different intervals 15 and 45 days. The *T. erinaceum* bioprimed samples were found to have had profuse growth with increased plant height, root growth, stem diameter and number of leaves compared to control plants. **(A)** Picture showing root growth and thickness of stem at root-shoot junction after 45 days in the unprimed plants **(B)** *T. erinaceum* bioprimed plants after 15 days **(C)** *T. erinaceum* unprimed plants after 15 days **(D)** *T. erinaceum* bioprimed plants after 45 days. **(II) (A)** Pots showing the growth pattern and morphological parameters of unprimed (control) and *T. erinaceum* bioprimed plants.

shown to have had clear-cut differential expression (Aamir et al., 2018). In the present study, we have measured the tissue-specific and time-dependent response, measured in the form of gene expression profile changes (**Figure 4**). The quantitative and semiquantitative expression of *WRKY*s were measured in terms of relative fold change in expression values compared to control tissues under the defined condition *Fol* treated (Aamir et al., 2018); *T. erinaceum* (bioprimed) and co-inoculated with both *Fol* + *T. erinaceum* challenged tissues, and the results were compared with unprimed (control) tissues. In the qPCR analysis, we found negative regulation (down regulation) of *SlWRKY4* in all the treatments in both root (**Figure 4A**) and leaf tissue samples (**Figure 4D**). However, the highest repression of the *SlWRKY4* was observed in root (24 h) and leaf (48 h) tissues with 0.03 and 0.08 fold decrease in *T. erinaceum* primed plants followed by *Fol* + *T. erinaceum* treatments. In contrast, we found increased expression of *SlWRKY31* gene in *T. erinaceum* bioprimed plants (14.83 fold) in root tissues (at 48 h) followed by *Fol* + *T. erinaceum* treatments (**Figure 4B**). In leaf tissues, a similar trend of expression was recorded, however; upregulation in both genes was comparatively less than root tissues (**Figure 4E**). Interestingly, our results showed *Fol* + *T. erinaceum* bioprimed plants had an aggravated defense response as measured from the upregulated transcript profile of *SlWRKY31* and *SlWRKY37*. At early stages of treatments (0–24 h), we found increased expression of *SlWRKY31* and *SlWRKY37* in root tissues (**Figure 4C**). However, the expression of *SlWRKY31* in leaf tissues (at 0–24 h) was recorded to be comparatively less than roots. Further, one major contrasting difference recorded was that the expression of *SlWRKY37* in root tissue was less (0–24 h) than the leaf tissue of the same duration in *T. erinaceum* primed treatments. However, the expression in bioprimed samples further increases sharply in root tissues and decreases abruptly in leaf tissues (24–48 h). The *Fol* + *T. erinaceum* challenged samples showed the greatest change at 48 h in root tissue whereas a more or less similar expression trend was observed in leaf tissues (**Figure 4F**). However, in leaf tissues, the highest upregulation was observed in the transcript profile of *SlWRKY31* and *SlWRKY37* in *T. erinaceum* bioprimed tissues, explicitly, with 21.19 fold (*SlWRKY31*) and 14.07 fold (*SlWRKY37*) respectively. In this way, the results suggested that *SlWRKY31* and *SlWRKY37* function as a positive regulator and *SlWRKY4* as a negative regulator of plant defense programmed against the *Fol* challenged condition with a more aggressive defense response in *Trichoderma* pre-primed plants compared to non-primed plants.

We also checked the expression profile changes of the genes encoding transcripts involved in the cellular anti-oxidative defense mechanism. It was found that an upregulated expression of the *SOD* gene was recorded in *Fol* + *T. erinaceum* challenged root tissues (**Figure 5A**) compared to leaf tissues of the same time interval (**Figure 5C**). However, the expression of the *SOD* gene was more pronounced in *Fol* + *T. erinaceum* treated plants, and highest expression with 6.84 fold (leaves) and 5.88 fold (root) increase was recorded in *Fol* + *T. erinaceum* challenged samples analyzed at 48 h. We found upregulated transcripts of *SlGPX1* in all treatments in root tissues measured at a different time interval (0, 24, and 48 h). However, the highest expression of *SlGPX1* was reported in the *Fol* + *T. erinaceum* treated root tissues (**Figure 5B**) and to some extent in leaf tissues. Comparatively, *T. erinaceum* bioprimed leaf tissues were found to have more or less similar expression profiles at 24–48 h (**Figure 5D**). Further, the increased expression of PR-3 protein (chitinases) was found at initial hours (0–24 h) in *T. erinaceum* bioprimed followed by *Fol* + *T. erinaceum* challenged root tissues (**Figure 6A**). In contrast, the highest upregulation transcript profile of chitinases in leaf tissues was recorded in *T. erinaceum* bioprimed samples with 16.39 and 27.57 fold increase at 24 and 48 h, respectively (**Figure 6C**). Further, an increasing trend similar to chitinases in expression profile of PR-2 (glucanases) was reported in *T. erinaceum* and *Fol* + *T. erinaceum* bioprimed

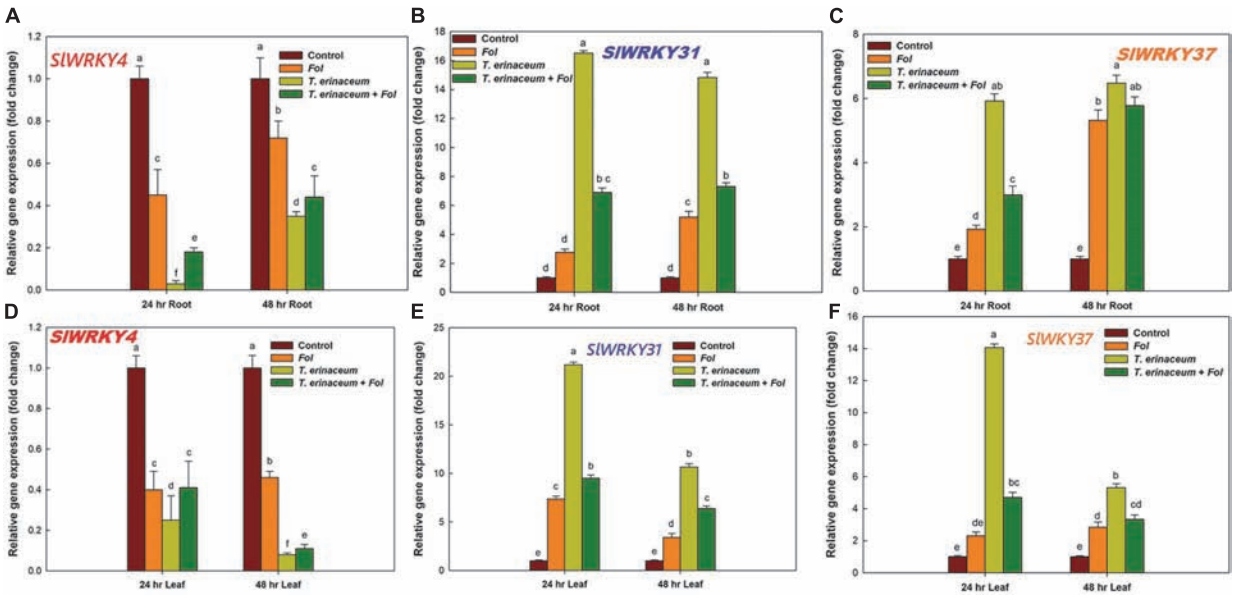


FIGURE 4 | Quantitative PCR results showing the tissue specific and time bound differential expression of tomato *WRKY* genes in all the four defined conditions measured at different time intervals (0, 24, and 48 h). **(A)** The quantitative expression of *SlWRKY4* gene in root tissues data showing the relative change in expression values (fold change). **(B)** The relative fold change in expression value for *SlWRKY31* (previously named as *SlWRKY33A*; *SlDRW1)* expressed in tomato roots. **(C)** The relative fold change in expression value for *SlWRKY37* gene expressed in root tissues. **(D)** Relative fold change in expression values for *SlWRKY4* expressed in tomato leaves. **(E)** The expression data for *SlWRKY31* in leaves. **(F)** The expression data for *SlWRKY37* in leaf tissues. The *SlWRKY4* genes were found to be downregulated in both root and leaf tissues. Bars with distinct alphabetical letters are significantly different at $P \leq 0.05$ applying the DMRT test.

root tissues (**Figure 6B**). In the case of leaf tissues, decreased expression of glucanases was recorded in all the tissue samples analyzed except *T. erinaceum* bioprimed leaf tissues (**Figure 6D**).

The semi-quantitative expression of each *WRKY* gene at different time intervals and in different tissue has been shown (**Supplementary Figures S3A–D**). The semi-quantitative expression of Cu/Zn-superoxide dismutase has been shown in **Supplementary Figure S3E**. The semi-quantitative expression of *SlGPX1*, Chitinase and β-1, 3 glucanases with respect to internal constitutive *ACTIN* gene have been shown (**Supplementary Figures S4A–C**). We have further analyzed the replicative count data for leaf tissue samples at 48 h through Bioconductor R for the *Fol* challenged, *T. erinaceum* bioprimed, *Fol* + *T. erinaceum* bioprimed and unprimed control samples. The differential expression of the replicated count data was analyzed using the fold change expression values through Bioconductor R and have been represented in the form of heat map diagramme. The heat map diagramme for *WRKYs* expression profile changes in root tissue at different time intervals (0–48 h) has been shown (**Figures 7A–D**). The heat map diagramme for showing the relative expression values for *SOD*, *SlGPX1*, Chitinase and β-1, 3 glucanases in both root and leaf tissues for all the four defined conditions at different time intervals (24 and 48 h) has been shown in **Figures 8A,B**.

Quantitative Estimation of H_2O_2

The amount of H_2O_2 produced and accumulated was significantly higher in *Fol* challenged plants and was greatly reduced in the plants pretreated with *T. erinaceum* as compared to *Fol* + *T. erinaceum* treated samples. It was found that the amount of H_2O_2 produced was significantly higher at 48 h than recorded at early phases (0–24 h) of *Fol* inoculation. After 48 h post inoculation we did not find any further increase in the amount of H_2O_2 which confirmed the role of antioxidative defense enzymes under *Fol* challenged conditions. However, the amount of H_2O_2 generated and accumulated increased slowly in the leaves of all treatments but this rise was comparatively less than that observed in *Fol* + *T. erinaceum* treated samples (**Figure 9A**). Furthermore, it was found that the amount of H_2O_2 produced increased gradually from 0 to 24 h, was highest at 48 h and then declined successively in all the treatments. H_2O_2 content was 23.60% less in the *Fol* + *T. erinaceum* treated plants at 48 h when compared with *Fol* challenged plants. In *T. erinaceum* treated plants, it was 32.06% lesser than the *Fol* challenged plants.

Assessment of Antioxidative Enzymes

SOD Activity

The SOD activity was found to increase successively on increasing time interval up to 0–72 h which declined thereafter. However, the SOD activity was reported to be maximum in case of the *Fol* + *T. erinaceum* samples at each time interval followed by the *T. erinaceum* bioprimed samples. The SOD activity was reported to be higher at 48 h post inoculation in all the pre-treated samples but found with maximum increase for the *Fol* + *T. erinaceum* bioprimed samples, followed by

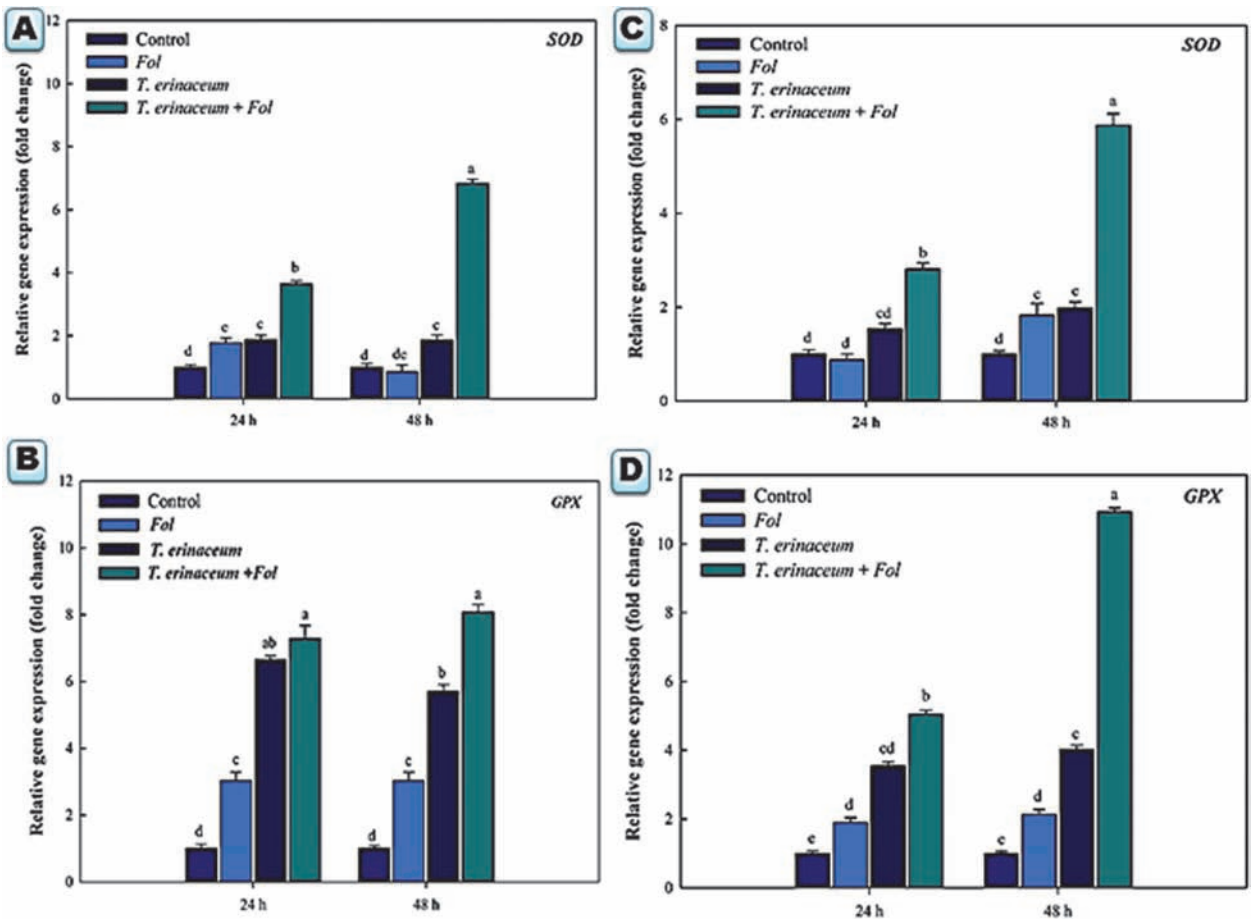


FIGURE 5 | Quantitative PCR results showing the tissue specific differential expression profile changes of the Cu-Zn SOD and glutathione peroxidase encoding gene *SlGPX1* genes expressed in the root and leaf tissues and were measured at time intervals (0, 24, and 48 h) in each of the four different treatments. The data represents the relative fold changes in the expression values for the four different treatments. **(A)** The expression profile of the *SOD* gene in the root tissues. **(B)** The expression profile of the *SlGPX1* gene in the root tissues. **(C)** The gene expression profile of the *SOD* gene in leaf tissues. **(D)** The expression profile of the *SlGPX1* gene in leaf tissues. Bars with distinct alphabetical letters are significantly different at $P \leq 0.05$ using the DMRT test.

T. erinaceum and the *Fol* challenged condition, respectively (**Figure 9B**). Overall, the reported SOD activity calculated for the *Fol* + *T. erinaceum* challenged samples were 183.05% more than the *Fol* challenged leaf tissues, whereas in the case of *T. erinaceum* bioprimed tissues the calculated percentage increase in SOD activity was 133.94% higher than the *Fol* challenged leaf tissue samples.

CAT Activity

In our results, the CAT activity was found to be increased in the *Fol* challenged leaf tissues at the initial hour of *Fol* inoculation (0–24 h) compared to control (unprimed) samples. However, the CAT activity was at it's maximum in *T. erinaceum* bioprimed samples in all the treatments followed by the *Fol* + *T. erinaceum* treated leaf tissues. One more interesting observation found that the CAT activity showed a fluctuating type pattern where it raised in the initial hour (0–24 h), decreased further, and then increased at 72 h post inoculation in all the treatments (**Figure 9C**). Furthermore, the rise in the CAT activity at later stages of treatment (48–72 h) was much more than those recorded for initial hour increment. In all the pre-treated tissues we found that the maximum rise in the CAT activity for *T. erinaceum* challenged plants 159.79% greater than the *Fol* challenged samples, followed by the *Fol* + *T. erinaceum* challenged samples. The CAT activity was comparatively 136.14% greater for the *Fol* + *T. erinaceum* challenged (compared to the *Fol* treated leaf tissues). It has been well demonstrated that pre-treatment of biocontrol *Trichoderma* causes transcriptional reprogramming of the oxidative stress response, and accumulation of *ROS* gene network (SOD, CAT, GPx, APx) (Singh et al., 2011; Brotman et al., 2013) which results in increased activities of the antioxidant enzymatic pool.

Histochemical Analysis for Lignin Assessment

The amount of lignified tissues was found to be greater in *T. erinaceum* plants co-inoculated with *Fol* (*Fol* + *T. erinaceum*) followed by *T. erinaceum* bioprimed samples. We have measured

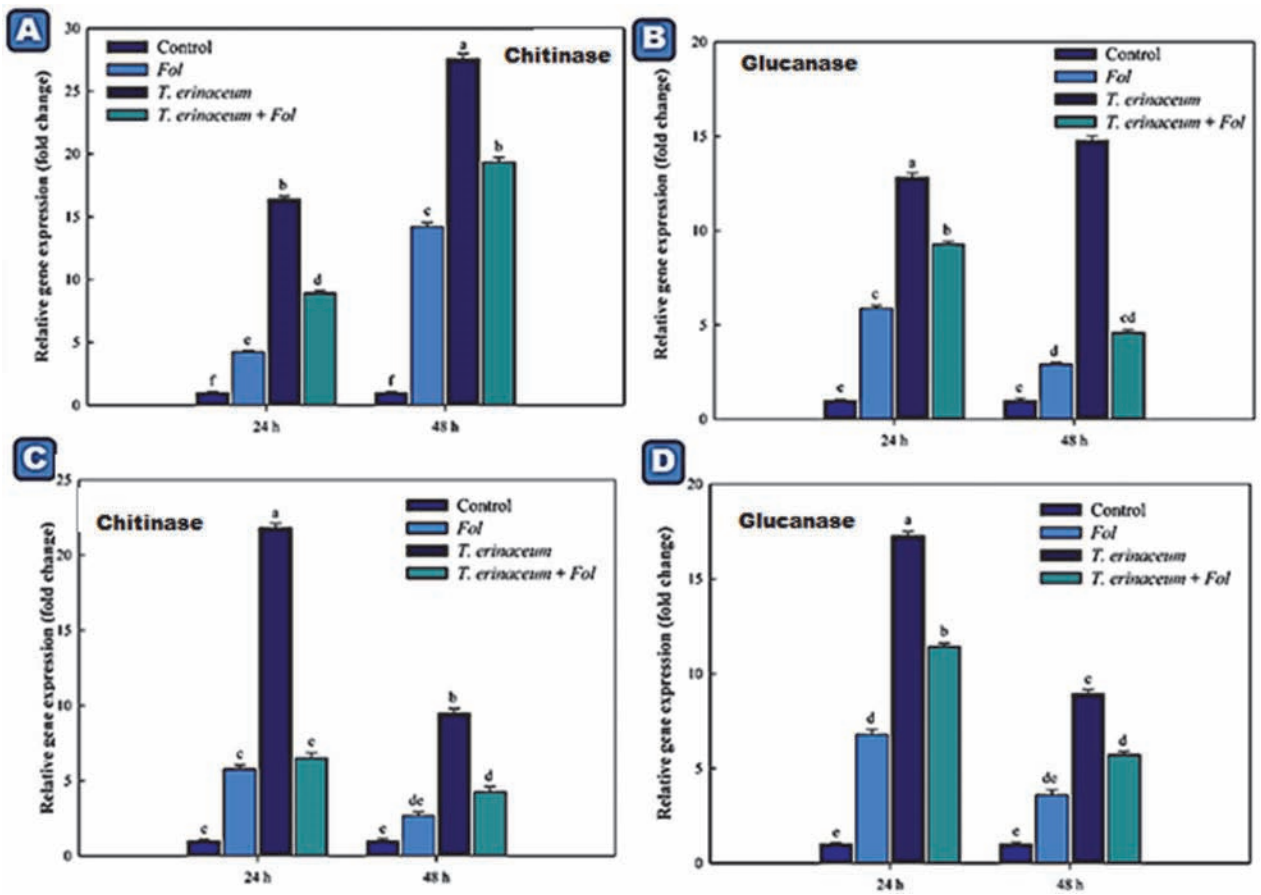


FIGURE 6 | Quantitative PCR results showing the tissue specific differential expression of the genes encoding PR proteins (Chitinases and Glucanases) expressed in the root and leaf tissues and were measured at different time intervals (0, 24, and 48 h). The data represents the relative fold changes in the expression values for the four different treatments. **(A)** The expression profile of chitinase encoding gene (*CHI1*) expressed in the leaf tissues. **(B)** The expression profile of the glucanases encoding gene expressed in the leaf tissues. **(C)** The expression profile of the chitinase encoding gene in the root tissues. **(D)** The expression profile of the glucanase encoding gene in the root tissues and were measured at different time intervals. Bars with distinct alphabetical letters are significantly different at $P \leq 0.05$ using the DMRT test.

the deposited lignified tissues at two different time intervals i.e., on 15 days and later on, 45 days post inoculating the pathogen in both bioprimed and unprimed plants. The pink colored staining of Phloroglucinol-HCl stained lignified tissues, was more pronounced and was shown to have intense coloration. The *Fol* challenged sample had a comparatively higher amount of lignified tissue than uninoculated control as visualized under the compound microscope. The lignified tissue in the *Fol* challenged stem on 15 days (**Figure 10C**) showed less lignified tissues compared to stem tissues observed on 45 days (**Figure 10D**) whereas the control plant observed at 15 days (**Figure 10A**) and 45 days (**Figure 10B**) was found to have a comparatively lesser amount of lignified tissues. However, in all the histochemical tissue sections analyzed the deposited lignin was quantitatively more in *T. erinaceum* bio-primed tissue sections. The histochemical tissue sections from the *T. erinaceum* bioprimed plants at 15 days (**Figure 10E**) and 45 days (**Figure 10F**), respectively. The *Fol* + *T. erinaceum* tissues were found to have comparatively more lignified tissues on 15 and 45 days, respectively (**Figures 10G,H**) which confirmed that the defense programming of the host was more pronounced in the presence of *T. erinaceum* induced microbial bio-priming under the *Fol* challenged conditions. Moreover, *T. erinaceum* pretreated plants co-inoculated with pathogen *Fol* + *T. erinaceum* had the maximum amount of deposited lignified tissues which confirmed that the defense response in the presence of beneficial microbe (*T. erinaceum*) become more robust when plants further encounter the pathogen.

DISCUSSION

In their natural habitat, plants are continuously exposed to various abiotic and biotic stresses. However, plants adapt to such changes by acquiring a great degree of phenotypic plasticity that is mainly determined by the plant's genome (Aamir et al., 2017). The integration of a multitude of partly synergistic and/or partly antagonistic signals enables plants to respond well against such extreme conditions (Pandey and Somssich, 2009).

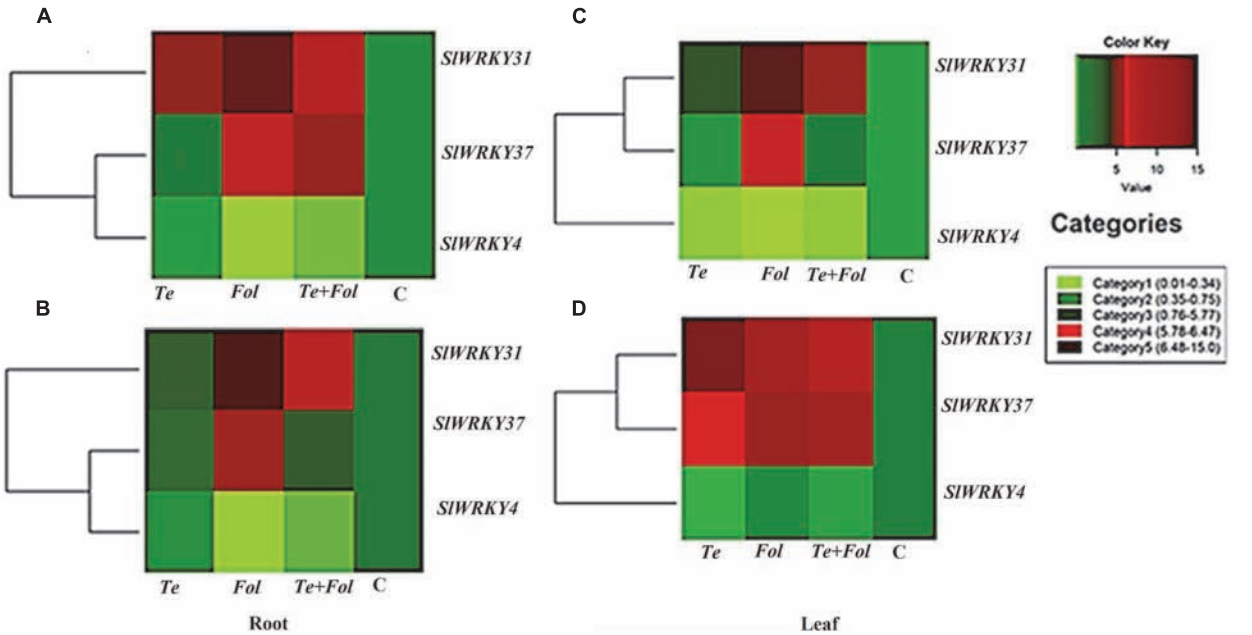


FIGURE 7 | Heat map and clustering analysis of tomato *WRKY* genes expressed under the four different treatment conditions. The figure includes a "heat map" which is the part of the figure having different colors (red, green, and maroon) along with dendrogram. The rows represent genes while the treatments are shown as columns. The row dendrogram represents the gene clusters with similar pattern. The expression levels are mapped on the color scale provided at the right top of the figure along with the categories scale on the right. **(A)** The heat map showing the expression profile changes of the *SlWRKY4*, *SlWRKY31*, and the *SlWRKY37* genes in each of the four different treatments at 24 h in root tissues. **(B)** The heat map showing the expression profile changes of the *SlWRKY4*, *SlWRKY31*, and the *SlWRKY37* genes root tissues at 48 h. **(C)** The heat map showing the expression profile changes of the *SlWRKY4*, *SlWRKY31*, and the *SlWRKY37* genes in the leaf tissues at 24 h. **(D)** The heat map showing the expression profile changes of the *SlWRKY4*, *SlWRKY31*, and *SlWRKY37* genes in the leaf tissues at 48 h.

In many studies, it has been reported that members of the *WRKY* gene family contribute multiple biological processes involved in plant growth and development. Apart from this, the role of *WRKY* genes in the regulation of the stress response against the abiotic and biotic damages has also been demonstrated (Huang et al., 2012; Shankar et al., 2013; Bakshi and Oelmüller, 2014; Bai et al., 2018). Moreover, it has been reported that some WRKY Tfs function in combined stresses (both abiotic and biotic), and have been found active at crossroads of plant responses to both biotic and abiotic stresses (Qiu and Yu, 2009; Gao et al., 2011; Huang et al., 2012). For example, the tomato homologs (*SlWRKY31* and *SlWRKY33*) of *AtWRKY33* are activators of plant defense against several pathogens (Lippok et al., 2007; Liu et al., 2015; Li et al., 2016). In addition, the role of *SlWRKY31* and its homolog *SlWRKY33* was found to be involved in plant defense against drought and/or salt stresses (Huang et al., 2012). In tomato, many WRKY members have been demonstrated to act as a positive regulator of plant defense response against biotic stresses. Interestingly, different researchers redundantly annotated the tomato *WRKY* genes, and it is hard to follow the identical genes through different publications. For example, SlWRKY33 was published as SlWRKY33B and SlWRKY33A (Zhou et al., 2015) and SlWRKY31 was described as SlDRW1 (Liu et al., 2015). Likewise, *Arabidopsis* WRKY33 protein homolog in tomato SlWRKY31 (previously named as SlWRKY33A and SlWRKY33B; Zhou et al., 2015) was found to complement the function of the *atwrky33* mutant that was unable to provide tolerance against *B. cinerea* (Zheng et al., 2006). Overexpression of *S. pimpinellifolium* (closest wild relative of domestic tomato; The Tomato Genome Consortium, 2012) allele of *SlWRKY33* was found to provide resistance against hemi-biotrophic oomycetes *Phytophthora infestans* in tomato and *Phytophthora nicotianae* in tobacco. Similarly, in Group I, the closest homologs of *AtWRKY33* including *SlWRKY31* and *SlWRKY33* have been reported to provide defense against several soil-borne pathogens (Zheng et al., 2006; Lippok et al., 2007; Liu et al., 2015; Li et al., 2016). In contrast, the induction of *SlWRKY31* and *SlWRKY33* was observed under drought and/or salt stresses (Huang et al., 2012). In our previous report, we analyzed the Gene Expression Omnibus (GEO) datasets (GEO accession: GSE52336) to analyze the transcriptional profile of tomato defense-related *WRKY* genes. The bioinformatics analysis revealed that during *Fol* pathogenesis in tomato, a total 16 EST transcripts belonging to the *WRKY* gene family (based on Blast-x results and the presence of functional domain analysis) were found to be differentially expressed (Aamir et al., 2018). However, of these totally expressed transcripts, only three transcripts i.e., *SlWRKY4*, *SlWRKY31*, and *SlWRKY37* were found to have a clear-cut upregulated expression profile in all the *Fol* challenged samples (compared to control samples). The *in-silico* results were further confirmed through real time PCR and qRT-PCR studies. Apart from *WRKYs* role in plant-microbe interaction, Wenke et al. (2012) reported the role of the *WRKY* gene-mediated regulatory

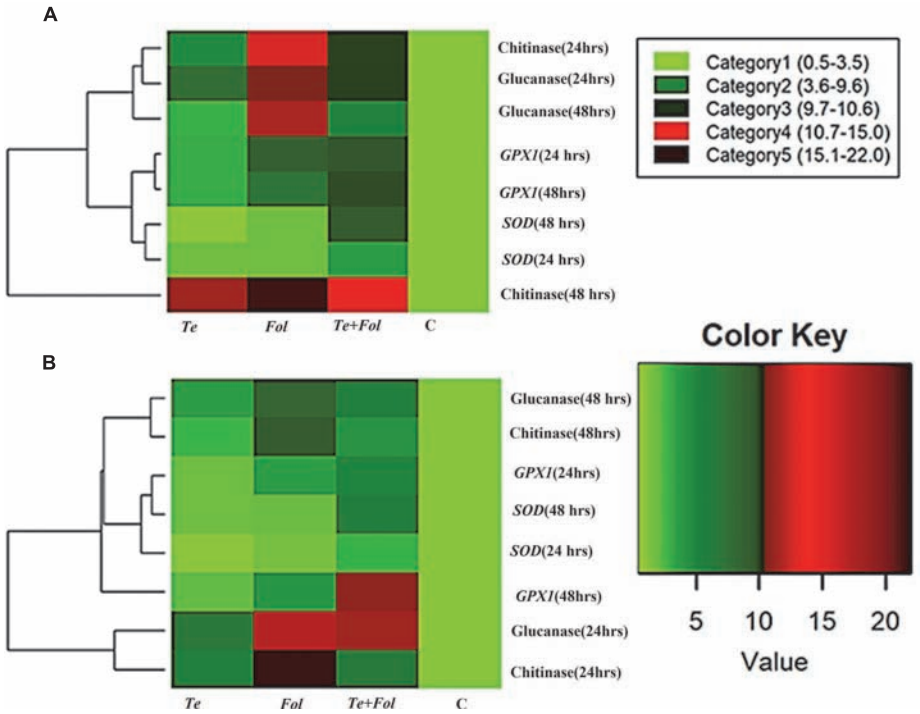


FIGURE 8 | Heat map and clustering analysis of PR proteins and antioxidative defense genes in tomato. The rows represent the expression profile of defense related antioxidative genes and those encoding for PR proteins (chitinase and glucanase) that were expressed in a time-dependent and tissue-specific manner while the treatments are shown as columns. The row dendrogram represents the gene clusters with similar pattern. **(A)** The heat map showing the expression profile changes of the genes encoding for the PR proteins (chitinases and the glucanases) and antioxidative enzymes including *SOD* and *SlGPX1* in each of the four different treatments at 0, 24, and 48 h in the root tissues. **(B)** The heat map showing the expression profile changes at 0, 24, and 48 h in leaf tissues.

network in plant-biotic interaction, influenced through secretory volatile compounds (without having direct surface−to−surface contact) and that played, a significant role in plant's fitness and vitality. De Palma et al. (2019) investigated the transcriptional response of tomato roots when colonized with endophytic *T. harzianum* T_{22}. The root transcriptomes collected at different time intervals (0, 24, 48, and 72 h) post inoculation with beneficial fungus revealed the epigenetic and post-transcriptional regulation mechanisms (De Palma et al., 2019). *Arabidopsis* shares the highest evolutionary conservation with tomatoes (Yue et al., 2016) and the close phylogenetic relationship between tomato and *Arabidopsis* genes is well supported and exemplified by the fact that both species partially employ similar proteins for their developmental programming such as epidermal cell differentiation, development of root hairs, initiation of trichomes and accumulation of anthocyanins (Tominaga-Wada et al., 2013; Wada et al., 2014, 2015). The differential expression of tomato *SlWRKY2*, *SlWRKY3*, *SlWRKY4*, *SlWRKY6*, *SlWRKY7*, *SlWRKY23*, *SlWRKY51*, *SlWRKY53*, *SlWRKY80*, and *SlWRKY71* has been reported following the attack of *Cladosporium fulvum* in tomato plants (van Esse et al., 2009). In our results, we found that increased expression of the tomato *WRKY31* and *WRKY37* genes in the *Fol* challenged root and leaf tissues at increased time intervals (compared to control). Birkenbihl et al. (2012) demonstrated the role of *AtWRKY33* in the plant defense against the necrotrophic fungi *Alternaria brassicicola* and *Botrytis cinerea* through mutant studies. Later on, it was reported that *AtWRKY33* work as a global transcriptional regulator of metabolic and hormonal responses toward the necrotrophic pathogen, *Botrytis cinerea* (Birkenbihl et al., 2012). In a recent study, the time-dependent fluctuation in *WRKY*s gene expression was recorded following the attack of two pathogens *F. oxysporum* f. sp. *conglutinans* and *Pectobacterium carotovorum* sub. sp. *carotovorum* (Kayum et al., 2015). It was found that *Brassica rapa WRKY4* showed the highest expression on the 6th day post inoculation of the pathogen. However, *P. carotovorum* subs. sp. *carotovorum* infection revealed fluctuation in *WRKYs* expression at different time intervals (Kayum et al., 2015).

We found the highest expression (compared to the *Fol* challenged samples) of *SlWRKY* genes in *T. erinaceum* pre-treated tissues followed by *Fol* + *T. erinaceum* bioprimed tissue samples. In this context, Bakshi and Oelmüller (2014) reported that bio-priming with *Trichoderma* affects the expression of many defense-related genes involved in regulating ROS homeostasis and plant defense against stress response, particularly, *WRKY* genes. We found similar trends in our qPCR results, the highest upregulation was observed in the transcript profile of *SlWRKY31* with 16.53 fold (root) and 21.19 fold (leaves) increase in *T. erinaceum* bioprimed tissues at an initial hour (0–24 h) of treatments. These observations suggest that *Trichoderma* primed tissues reprogrammed the host defense machinery with respect to an elevated alarm state within tomato plants enrolling numerous

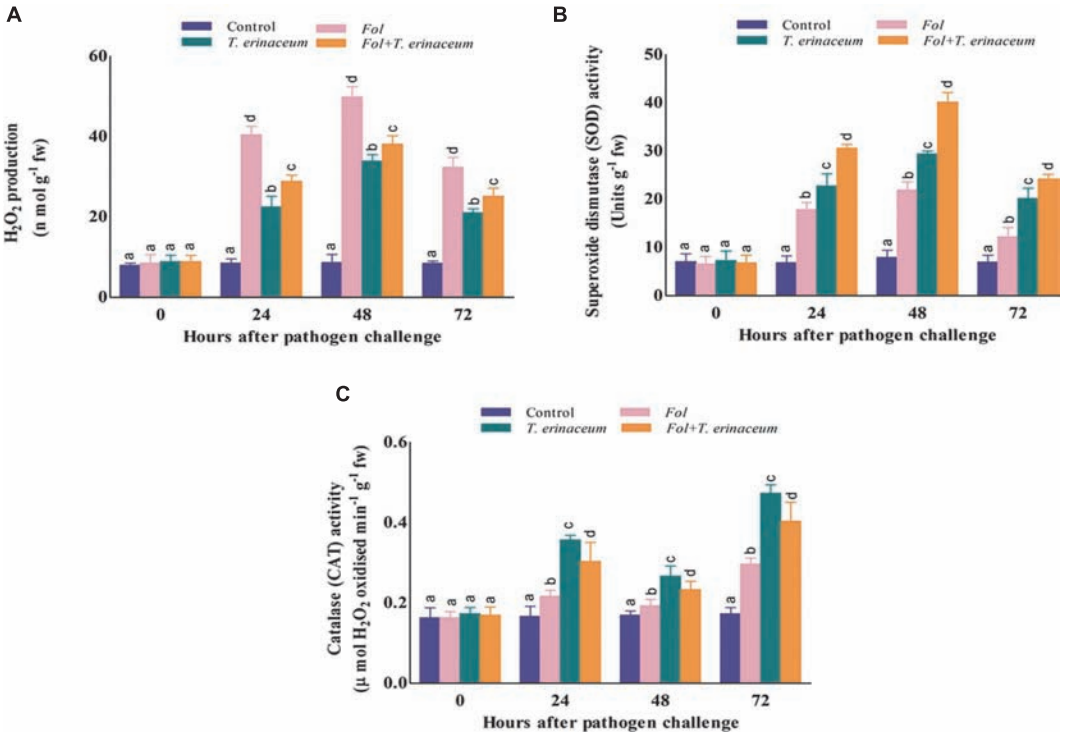


FIGURE 9 | The comparative evaluation of biochemical activities of *T. erinaceum* bioprimed and unprimed samples under the *Fol* challenged conditions. **(A)** The H_2O_2 accumulated at different time intervals (0–72 h) in all the four different treatments. **(B)** Measurement of the SOD activity. **(C)** The CAT activity. Mean (±SE) was calculated from three replicates for each treatment. Bars with distinct alphabetical letters are significantly different at $P \leq 0.05$ using the DMRT test.

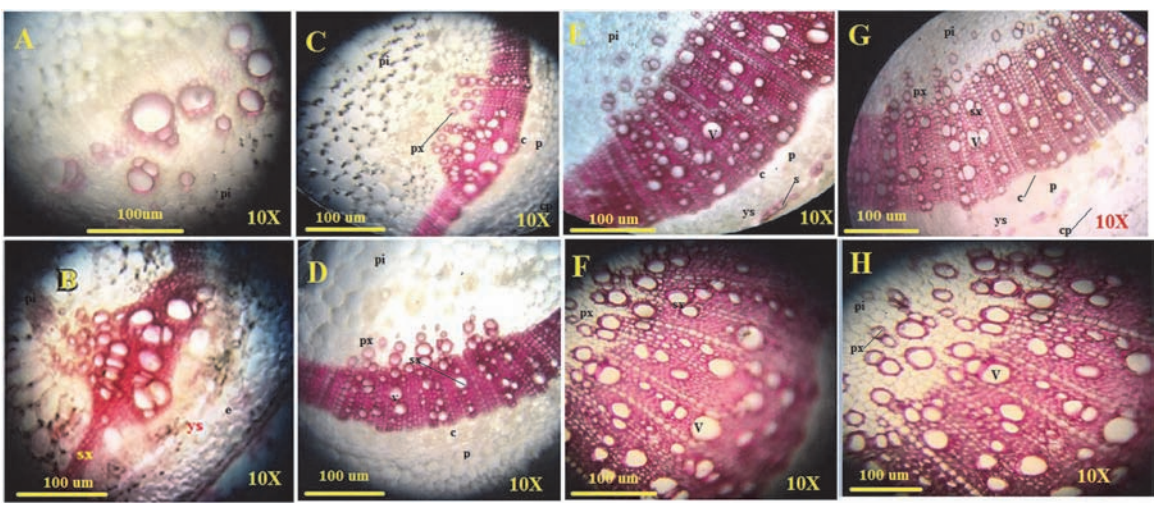


FIGURE 10 | Assessment of the plant defense response in the form of lignification. The figure shows the transverse sections of stem tissues with lignin deposition in the walls of interfascicular fibers and xylem cells collected from four different transverse section of control, *Fol* challenged, *T. erinaceum* bioprimed, and *T. erinaceum* bioprimed + *Fol* challenged and were collected at two different intervals 15 and 45 days. The stem tissue sections from 2nd internode region were dissected through Vibratome, stained with Phloroglucinol-HCl (pink or fuchsia color). The intensity of the pink color represents higher deposition of lignin. Vascular bundles with lignified primary xylem; sclerenchyma starting to differentiate with no lignified cell walls. **(A)** Microscopic view of the histochemical section (transverse) of the control stem tissue taken from an unprimed plant (control) at 15 days interval. **(B)** The control stem tissue analyzed at 45 days interval. **(C)** The shoot tissue sections from the *Fol* challenged plant observed at 15 days interval. **(D)** The *Fol* challenged shoot tissues at 45 days interval. **(E)** The shoot tissues were taken from a *T. erinaceum* bioprimed plant at 15 days interval. **(F)** *T. erinaceum* bioprimed tissues at 45 days interval. **(G)** The shoot tissues taken from *Fol* challenged sample co-inoculated with *T. erinaceum* analyzed at 15 days interval. **(H)** The *Fol* challenged + *T. erinaceum* shoot tissues analyzed after 45 days interval. All the images were observed at 10× magnification. Bar = 100 µM. px, primary xylem; sx, secondary xylem; f, xylem; pi, pith; p, phloem; c, cambium; v, vessel; cp, cortical parenchyma. The amount of lignin deposition was maximum in tissues that were *Fol* challenged and co-inoculated with *Trichoderma.*

WRKYs and other defense-related transcripts. In one report, the quantitative PCR (qRT-PCR) experiment when combined with *in-silico* results revealed the expression of defense-related genes in beans following the interaction of *T. velutinum* with *R. solani* or without *R. solani* (Mayo et al., 2016). It was found that of totally, expressed 48 genes, only *WRKY33*, *CH5b* (endochitinase precursor) and *hGS* (encoding for homoglutathione synthetase) showed upregulation in presence of *T. velutinum*. Further, it was found that treatment of *T. velutinum* resulted into downregulation of other genes or had no effect (*OSM34*) at all (Mayo et al., 2016). In contrast, *R. solani* infection caused downregulation of most of the genes analyzed, except *OSM34*, *CNGC2*, and *PR1* that were not affected. However, the presence of both *R. solani* and *T. velutinum*, showed downregulation of most of the genes analyzed, except upregulation of *hGS*, while other *CH5b* was not significantly affected (Mayo et al., 2016). In our study, we found downregulation of the *SlWRKY4* in both root and leaf tissues compared to upregulated *SlWRKY31* and *SlWRKY37* transcripts. However, the expression profile change for *SlWRKY4* was more conspicuous in root tissues at 24 h, which further decreased at 48 h.

The WRKY Tfs play an important role in the alleviation of both abiotic and biotic stresses (Bakshi and Oelmüller, 2014; Phukan et al., 2016; Bai et al., 2018). For example, *SlWRKY31* has been reported to offer defense against necrotrophic pathogens (Zheng et al., 2006) and also play a crucial role in drought and salt stress signaling (Huang et al., 2012). *SlWRKY3* provides resistance against salt stress and functions as a positive regulator against the root-knot nematode *Meloidogyne javanica* (Hichri et al., 2017; Chinnapandi et al., 2019). Similarly, downregulation of *SlWRKY4* has been reported in both abiotic (drought) and biotic stress (*Fol* pathogen) response (Aamir et al., 2018; Karkute et al., 2018). Therefore, identification and characterization of WRKY members that play dual function (managing both abiotic and biotic stresses) is an interesting approach and could be useful in crop improvement through plant breeding and/or transgenic technology. In addition, the functional relevance of WRKY proteins (having the dual function) and molecular mechanism of *WRKY* gene-mediated signaling at the point of multiple stresses is fully unexplored. Notably, *SlWRKY23* play a crucial role in the defense response against the powdery mildew fungus *Oidium neolycopersici*. However, *SlWRKY23* function under the saline stress condition when simultaneously challenged with pathogen was compromised rather than showing an additive effect (Kissoudis, 2016). With this view, we have analyzed *cis*-acting DNA regulatory element at the promoter region of characterized *SlWRKY* genes for finding their putative functions based on the presence of functional promoter elements. In a recent study, it was found that nine different *WRKY* genes in tomato showed upregulated expression under drought stress condition (Karkute et al., 2018). Further, *in silico cis*-acting DNA regulatory element analysis revealed that the promoter region of the characterized stress-responsive *WRKY* genes was flanked by various abiotic stress responsive elements like *ABRE*, *HSE*, *MBS* and Py-rich stretch, and were reported to promote high transcription levels (Karkute et al., 2018). We demonstrated the role of *SlWRKY31*, *SlWRKY37* and *SlWRKY4* in tomato defense response against the *Fol* challenged condition. However, promoter analysis results revealed the presence of different abiotic stress-responsive elements in *SlWRKY33* and *SlWRKY37* which showed their possible biological role in heat stress, drought stress response and hormonal response. In contrast, *SlWRKY4* could have a possible functional role in SA signaling and low-temperature response. In one report, Karkute et al. (2018) found that the promoter region of the drought stress-responsive *SlWRKY* genes were flanked by abiotic stress-responsive elements including *ABRE*, *HSE,* and *MBS*. 5' UTR Py-rich stretch which supports our promoter search results. In our results, we found the same *cis* acting elements at the promoter region of *SlWRKY4*, *SlWRKY31* and S*lWRKY37* (encoding SlWRKY37 isoform I). It has been suggested that expression of the gene is governed by Tfs which binds to specific protein binding sites in the promoter region of the respective gene and therefore, characterization of the promoter element could provide critical knowledge about gene function and regulation (Karkute et al., 2018). The presence of W-box sequences recognized by *WRKY* proteins in the promoter region of heat tolerance related genes like *HSP* and *HSF* genes (*HSFA2*, *HSFB1*, HSP101, and *MBF1c*) further explains the function of *HSE* elements (Li et al., 2011). In this context, Fragkostefanakis et al. (2016) identified and characterized some heat stress-responsive genes in tomato male reproductive tissues that lack the functional HSE element in their promoter region and are regulated directly by WRKY Tfs which further confirms WRKYs role in the alleviation of heat stress response. Zhou et al. (2014) characterized tomato *SlWRKY33*, homologous to *AtWRKY33* in heat induced autophagy along with tomato autophagy-related (*ATG*) genes as it was found that silencing of *SlWRKY3*3 genes compromised tomato heat tolerance and reduced heat-induced *ATG* gene expression (Zhou et al., 2014). Overall, promoter analysis results revealed that our characterized *WRKYs* could have a possible biological role in managing both biotic and abiotic stress response.

The molecular mechanism underlying the manipulation of plant defense by beneficial symbiosis with *Trichoderma* spp. is still unknown. However, *Trichoderma*, promotes plant growth and development through several mechanisms including, root growth, increased nutrient uptake, and expression of plant defense-related genes (Mayo et al., 2016). Fungal interaction with plant roots could result in large-scale transcriptional re-programming events, in host tissues as well as fungus, and leads to transcriptional changes, therefore allowing the successful colonization of fungus to host tissues through transient repression of host immune responses (Morán-Diez et al., 2012; Brotman et al., 2013). In this context, Brotman et al. (2013) reported the increased expression of A*tWRKY40* and *AtWRKY18* after the colonization of *T. asperelloides* 203 to *Arabidopsis* root. Sáenz-Mata et al. (2014) reported the quantitative expression of 8 *WRKY* genes during its interaction with beneficial fungus *T. atroviridae*. The study concluded the existence of complex signaling route during *Trichoderma*-plant interaction involving both JA and SA signaling cascades and regulated by differential *WRKY* gene expression in *Arabidopsis* at the molecular level. Similarly, *T. harzianum* T_{34} colonization with *Arabidopsis* roots resulted into downregulation of defense-related genes and other

Tfs including pathogenesis-related protein 1 (PR-1), *AtWRKY54*, flavin monooxygenase1 (*FMO1*), and glutathione transferases (Morán-Diez et al., 2012). Since *Trichoderma*, elicits induced systemic resistance (ISR) by the JA/ET-dependent pathway, and triggers a priming response in the plant (Korolev et al., 2008) the induced resistance provoked after a pathogen attack in unprimed tissues regulates through SA dependent signaling. Further, SA mediated signaling activates several jasmonate ZIM-domain genes and leads into downregulation of JA mediated responses. The final response of the signaling cascades results in the encoding of the JA responsive repressors along with other SA dependent WRKY Tfs, which causes susceptibility of mutant plants toward necrotrophic pathogens (Birkenbihl et al., 2012). This concludes into that *WRKY33*, a positive regulator of JA-related genes is a repressor of the SA pathway (Bakshi and Oelmüller, 2014).

The molecular mechanism that results in the modulation of defense transcriptome of plant tissues during *Trichoderma*-plant interaction is not fully explored. However, *Trichoderma* colonization to plant roots results into the secretion of secondary metabolites, proteins or other structural components that function as microbe associated molecular patterns (MAMPs). Further, plant-specific receptors use certain molecules (hydrolytic enzymes that may function on both pathogens and plant cell wall) could be employed as damage-associated molecular patterns (DAMPs) (Hermosa et al., 2013). These MAMPs and DAMPs stimulate the various signaling cascades, required for plant defense responses against phytopathogens, and mediated through multiple hormonal responses in cross-communication signaling pathways (Hermosa et al., 2013). In addition, plants have developed a plethora of defense strategies to combat pathogenic challenges. The most common mechanisms include the accumulation of ROS, the synthesis of PR proteins and phytoalexins, alteration in cell walls and enhanced activities of plant defense-related enzymes (Jothi and Babu, 2002; Bindschedler et al., 2006). Furthermore, the role of plant peroxidases (POs) has been well reported in defense responses including lignification, hypersensitive response, phenolics cross-linking and production of phytoalexins (Wojtaszek, 1997). The SAR regulated through SA signaling could result in direct activation and expression of the genes encoding PR proteins and in low dosages do not activate the defense genes in a direct way, but prime the tissues for potentiated defense-gene expression upon next *Fol* infection (Zehra et al., 2017). The *Fol* induced oxidative stress could be regulated by antioxidative defense enzymes including SOD and CAT that function along with other antioxidative enzymes to promote the oxidative damage by scavenging ROS (Saed-Moucheshi et al., 2014; Zehra et al., 2017). Moreover, *SOD* gene upregulation could result in diminished ROS activity and therefore higher accumulation of H_2O_2, and lead into the activation of the phenylpropanoid signaling. Additionally, the ROS intermediates play an important role in plant defense activation through cell-wall reinforcement, lignin biosynthesis, synthesis of secondary metabolites toxic to pathogen, activation of genes involved in plant defense, development of SAR against the targeted pathogen and/or exposure of the hypersensitive response (De Palma et al., 2016; Zehra et al., 2017).

In our results, root tissues had more expression of *SOD* gene compared to leaf tissues after the *Fol* infection at increasing time interval (0–48 h). Further, *T. erinaceum* bioprimed root tissues (0–48 h) were found to have an increased *SOD* expression compared to leaf tissues (24–48 h). In contrast, *Fol* + *T. erinaceum* treated leaf tissues showed a drastic change in *SOD* expression followed by the root tissues at later stages (24–48 h). Comparatively, it was found that in root tissues *SlGPX1* expression was more pronounced, and the *Fol* + *T. erinaceum* treated root tissues showed the highest expression of *SlGPX1* than the leaf tissues of the same duration. In this context, Mastouri et al. (2012) reported an increased expression of the *SOD* gene after the tomato root colonization by *T. harzianum* T_{22}, with a drastic rise in the relative expression of chloroplastic F-SOD_p and CZ-SOD_p but not cytosolic SOD_c. Furthermore, it was found that roots maintained a relatively higher *SOD* activity under stress compared to control (declined *SOD* levels at later stages). The study concluded that T_{22} root colonization induced the *SOD* expression in chloroplasts and appreciably up-regulated the tomato *SOD* gene (Mastouri et al., 2012).

The biosynthesis of the lignin and other phenolic compounds are due to activation of the phenylpropanoid pathway (Patel et al., 2017; Zhou et al., 2018). Phloroglucinol-HCl staining (pink or fuchsia color) is a common method for lignin determination, and it is not a true lignin stain as it stains only cinnamaldehyde end-groups (Mitra and Loqué, 2014). Staining with Phloroglucinol-HCl yields a characteristic cherry pink or fuchsia color in the xylem and interfascicular fibers where these aldehyde groups are present. In our study, we found better illustrations of the lignified cellular layers at their early stages of development than at late stages because at late stages the clear-cut demarcation between the cells having actual lignified cellular layer might not be possible due to tissue differentiation. Lignin estimation through phloroglucinol staining specifically stains metaxylem and not sclerenchymatous tissues (Stafford, 1962). The tissue sectioning with Vibratome allows thin sectioning of equal thickness that assists in generating sharp images and reduces much the risk of producing inaccuracies observed due to differences in thickness. The core concept behind the thickness differences and image generation is that tissues having unequal thicknesses would transmit different intensities of light, and therefore, producing blurred images that generally occurred due to bad sample preparation. Although clear-cut demarcation of the observed differences between the lignified tissues are harder to visualize (Mitra and Loqué, 2014) even differences could be determined based on intake of pink coloration determining the amount of lignin deposited (variation in lignin content) by various tissue types.

CONCLUSION

Microbial priming with *Trichoderma* spp. results into transcriptional regulation of defense-related genes with altered expression level. The modulated plant defense under primed

condition is characterized by accumulation of defense-related transcripts and ROS molecules, activation of phenylpropanoid metabolism and accumulation of specific isoforms of hydrolytic enzymes such as chitinases and glucanases.

The differential tissue-specific and temporal expression of *WRKY* genes provided the evidence of diverse and complex signaling and transcriptional networks of plant response to beneficial interaction establishment. The highest expression of accumulated *WRKY* transcripts in *T. erinaceum* bioprimed tissues predicted a distinct signaling mechanism of the host (tomato) with *WRKY* genes, in the pre-stage bioprimed condition. Since the defense priming is clearly expressed at transcriptional level, research on mechanisms underlying the primed state has focussed on expression on signaling intermediates in transcriptional networks. Further, enhanced accumulation of *SlWRKY* transcripts in both bioprimed and *T. erinaceum* primed and co-inoculated with *Fol* pathogen plants revealed the transcriptional reprogramming of host defense during its colonization with *T. erinaceum* and during its antagonism/interaction made with the *Fol* pathogen. The data from our study provide sufficient evidence for the existence of a complex signaling network involving *WRKY* genes along with other signaling cascades during *T. erinaceum* induced bioprimed conditions. Further, bio-priming with *T. erinaceum* resulted into robust antioxidative enzyme profile changes, strong biochemical (accumulation of defense-related compounds) and structural changes (lignification).

AUTHOR CONTRIBUTIONS

MA conceived the idea, planned the experiments, performed all the experiments, did computational analysis of the results, and finally prepared and wrote the manuscript. VS assisted in computational analysis of the results. SK performed the quantitative and semi-quantitative PCR work. MD and AZ helped in some experimental sections, and also helped in writing, reviewing, and editing the manuscript. WA assisted in performing statistical calculations. RU and SS supervised the whole work. All authors read and approved the final version of manuscript for publication.

ACKNOWLEDGMENTS

MA is thankful to the Indian Council of Medical Research (ICMR), New Delhi for research facilities in the form of ICMR-JRF and further as ICMR-SRT. MA is highly grateful to Prof. Madhoolika Agarwal, Coordinator, Interdisciplinary School of Life Sciences (ISLS), Banaras Hindu University, for providing microtome and vibratome facilities. We also acknowledge Centre for Bioinformatics, School of Biotechnology, Banaras Hindu University (BHU) for *in silico* work. All the authors are thankful to the Head, Department of Botany, Institute of Science, Banaras Hindu University for providing infrastructure facilities, and also acknowledge DST-FIST for instrumentation facilities for this work.

SUPPLEMENTARY MATERIAL

The Supplementary Material for this article can be found online at: https://www.frontiersin.org/articles/10.3389/fpls.2019.00911/full#supplementary-material

FIGURE S1 | (A) The position of CDS encoding *WRKY31* gene including the TSS and Poly A tail region as predicted through Fgenesh gene prediction tool. We have selected 1500 bp nucleotide upstream from the translational start site for promoter search for each *WRKY* gene. The CDSf represent the first type or First (Starting with Start codon), CDSi - internal (internal exon), CDSl - last coding segment, (ending with stop codon); TSS- represent the position of transcription start site (TATA-box position); Poly A represent the 3′ polyadenylation site. The presence of TSS before the first coding sequence CDSf and the Poly A tail after the last coding sequence (CDSl) predicted the complete coding sequence of *SlWRKY31* gene. Further, presence of TSS and Poly A in the entire coding sequence revealed that the gene of interest is in positive frame. **(B)** The structural organization of the *WRKY31*gene showing the intron-exon boundary and the upstream region with the position of CDS region that encodes each of the *SlWRKY31* transcription factor. The figure also show the other *cis*-regulatory element surrounding the promoter region of tomato *WRKY31* including the *TGA* and *TCA element*.

FIGURE S2 | (A) The position of CDS encoded by *SlWRKY4* gene along with the TSS and the Poly A tail region. **(B)** The identified *cis*-acting regulatory elements (*ABRE*, *ERE*, *MYB*, and *LTR* elements) in the promoter region of the *SlWRKY4* gene with intron-exon boundaries including TSS (position of transcriptional start site; TATA box and poly A tail). **(C)** Position of the CDS encoded by the *SlWRKY37* including the TSS and the Poly A tail. **(D)** The location of different *cis*-regulatory elements surrounding the promoter region of the Sl*WRKY37* gene.

FIGURE S3 | (A) The semi-quantitative expression profile changes in *SlWRKY4*, *SlWRKY31*, and *SlWRKY37* genes in four different treatments in both root and leaf tissues measured at different time intervals, and revealed through semi-quantitative PCR experiments. The expression of each gene was compared with a housekeeping *ACTIN* gene, having constitutive and stable expression pattern. **(A)** The expression of *SlWRKY4*, *SlWRKY31*, and *SlWRKY37* genes at 48 h interval in the leaf tissues. **(B)** The expression of *SlWRKY4*, *SlWRKY31* and *SlWRKY37* at 48 h interval in the root tissues. **(C)** The expression of the *SlWRKY4*, *SlWRKY31*, and *SlWRKY37* in the leaf tissues at 24 h. **(D)** The expression of the *SlWRKY4*, *SlWRKY33*, and *SlWRKY37* in the root tissues at 24 h. **(E)** The expression of the *CZn SOD* gene in both root and leaf tissues measured at different time intervals (0, 24, and 48 h).

FIGURE S4 | The expression profile changes in genes that encodes for the tomato Glutathione peroxidase (SlGPX1), Chitinase and Glucanases in both root and leaf tissues at different time intervals, and revealed through the semi-quantitative PCR experiments. **(A)** The expression of the genes that encodes for GPX1 at 0, 24, and 48 h intervals in both root and leaf tissues. **(B)** The expression of the Chitinases in both leaf and root tissues at different time intervals. **(C)** The expression of the Glucanases in root and leaf tissues at 0, 24, and 48 h.

TABLE S1 | List of different species of *Trichoderma* spp. used in this study along with their isolation source, isolate code and gene accession identity.

TABLE S2 | List of different *cis* regulatory DNA elements that were present in the promoter region of *WRKY* genes in tomato along with their sequence and assigned functions.

REFERENCES

Aamir, M., Singh, V. K., Dubey, M. K., Kashyap, S. P., Zehra, A., Upadhyay, R. S., et al. (2018). Structural and functional dissection of differentially expressed tomato WRKY transcripts in host defense response against the vascular wilt pathogen (*Fusarium oxysporum* f. sp. Lycopersici). *PLoS One* 13:e0193922. doi: 10.1371/journal.pone.0193922

Aamir, M., Singh, V. K., Meena, M., Upadhyay, R. S., Gupta, V. K., and Singh, S. (2017). Structural and functional insights into WRKY3 and WRKY4 transcription factors to unravel the WRKY–DNA (W-Box) complex interaction in tomato (*Solanum lycopersicum* L.). A computational approach. *Front. Plant Sci.* 8:819. doi: 10.3389/fpls.2017.00819

Ahmad, P., Hashem, A., Abd-Allah, E. F., Alqarawi, A. A., John, R., Egamberdieva, D., et al. (2015). Role of *Trichoderma harzianum* in mitigating NaCl stress in indian mustard (*Brassica juncea* L) through antioxidative defense system. *Front. Plant Sci.* 6:868. doi: 10.3389/fpls.2015.00868

Bai, Y., Sunarti, S., Kissoudis, C., Visser, R. G. F., and van der Linden, C. G. (2018). The role of tomato WRKY genes in plant responses to combined abiotic and biotic stresses. *Front. Plant Sci.* 9:801. doi: 10.3389/fpls.2018.00801

Bakshi, M., and Oelmüller, R. (2014). WRKY transcription factors: jack of many trades in plants. *Plant Signal. Behav.* 9:e27700. doi: 10.4161/psb.27700

Balasubramanian, V., Vashisht, D., Cletus, J., and Sakthivel, N. (2012). Plant β-1, 3-glucanases: their biological functions and transgenic expression against phytopathogenic fungi. *Biotechnol. Lett.* 4, 1983–1990. doi: 10.1007/s10529-012-1012-6

Beauchamp, C., and Fridovich, I. (1971). Superoxide dismutase: improved assays and an assay applicable to acrylamide gels. *Anal. Biochem.* 4, 276–287. doi: 10.1016/0003-2697(71)90370-8

Bindschedler, L. V., Dewdney, J., Blee, K. A., Stone, J. M., Asai, T., Plotnikov, J., et al. (2006). Peroxidase-dependent apoplastic oxidative burst in *Arabidopsis* required for pathogen resistance. *Plant J.* 47, 851–863. doi: 10.1111/j.1365-313x.2006.02837.x

Birkenbihl, R. P., Diezel, C., and Somssich, I. E. (2012). Arabidopsis WRKY33 is a key transcriptional regulator of hormonal and metabolic responses toward *Botrytis cinerea* infection. *Plant Physiol.* 159, 266–285. doi: 10.1104/pp.111.192641

Brand, L. H., Fischer, N. M., Harter, K., Kohlbacher, O., and Wanke, D. (2013). Elucidating the evolutionary conserved DNA-binding specificities of wrky transcription factors by molecular dynamics and *in vitro* binding assays. *Nucleic Acids Res.* 41, 9764–9778. doi: 10.1093/nar/gkt732

Brand, L. H., Kirchler, T., Hummel, S., Chaban, C., and Wanke, D. (2010). DPI-ELISA: a fast and versatile method to specify the binding of plant transcription factors to DNA *in vitro*. *Plant Methods* 6:25. doi: 10.1186/1746-4811-6-25

Brotman, Y., Landau, U., Cuadros-Inostroza, A., Takayuki, T., Fernie, A. R., Chet, I., et al. (2013). *Trichoderma*-plant root colonization: escaping early plant defense responses and activation of the antioxidant machinery for saline stress tolerance. *PLoS Pathog.* 9:e1003221. doi: 10.1371/journal.ppat.1003221

Chen, L., Song, Y., Li, S., Zhang, L., Zou, C., and Yu, D. (2012). The role of WRKY transcription factors in plant abiotic stresses. *Biochim. Biophys. Acta* 1819, 120–128. doi: 10.1016/j.bbagrm.2011.09.002

Chi, Y., Yang, Y., Zhou, Y., Zhou, J., Fan, B., Yu, J. Q., et al. (2013). Protein–protein interactions in the regulation of WRKY transcription factors. *Mol. Plant* 6, 287–300. doi: 10.1093/mp/sst026

Chinnapandi, B., Bucki, P., Fitoussi, N., Kolomiets, M., Borrego, E., and Braun Miyara, S. (2019). Tomato SlWRKY3 acts as a positive regulator for resistance against the root-knot nematode *Meloidogyne javanica* by activating lipids and hormone-mediated defense-signaling pathways. *Plant Signal Behav.* 14:1601951. doi: 10.1080/15592324.2019.1601951

Chopada, G. B., Singh, P., and Korat, C. (2014). Pathogenic variation among *Fusarium oxysporum* f. sp. *lycopersici* isolates and varietal screening of tomato against wilt under South Gujarat. *Bioscan* 9, 351–354.

Ciolkowski, I., Wanke, D., Birkenbihl, R., and Somssich, I. (2008). Studies on DNA-binding selectivity of WRKY transcription factors lend structural clues into WRKY-domain function. *Plant Mol. Biol.* 68, 81–92. doi: 10.1007/s11103-008-9353-1

De Palma, M., D'Agostino, N., Proietti, S., Bertini, L., Lorito, M., Ruocco, M., et al. (2016). Suppression subtractive hybridization analysis provides new insights into the tomato (*Solanum lycopersicum* L.) response to the plant probiotic microorganism *Trichoderma longibrachiatum* MK1. *J. Plant Physiol.* 190, 79–94. doi: 10.1016/j.jplph.2015.11.005

De Palma, M., Salzano, M., Villano, C., Aversano, R., Lorito, M., Ruocco, M., et al. (2019). Transcriptome reprogramming, epigenetic modifications and alternative splicing orchestrate the tomato root response to the beneficial fungus *Trichoderma harzianum*. *Hortic. Res.* 6:5. doi: 10.1038/s41438-018-0079-1

de Pater, S., Greco, V., Pham, K., Memelink, J., and Kijne, J. (1996). Characterization of a zinc-dependent transcriptional activator from *Arabidopsis*. *Nucleic Acids Res.* 24, 4624–4631. doi: 10.1093/nar/24.23.4624

Ebrahim, S., Usha, K., and Singh, B. (2011). Pathogenesis related (PR) proteins in plant defense mechanism. *Sci. Against Microb. Pathog.* 2, 1043–1054.

Eulgem, T., Rushton, P. J., Robatzek, S., and Somssich, I. E. (2000). The WRKY superfamily of plant transcription factors. *Trends Plant Sci.* 5, 199–206. doi: 10.1016/S1360-1385(00)01600-9

Fragkostefanakis, S., Mesihovic, A., Simm, S., Paupière, M. J., Hu, Y., Paul, P., et al. (2016). HsfA2 controls the activity of developmentally and stress-regulated heat stress protection mechanisms in tomato male reproductive tissues. *Plant Physiol.* 170, 2461–2477. doi: 10.1104/pp.15.01913

Fu, J., Liu, Z., Li, Z., Wang, Y., and Yang, K. (2017). Alleviation of the effects of saline- alkaline stress on maize seedlings by regulation of active oxygen metabolism by *Trichoderma asperellum*. *PLoS One* 12:e0179617. doi: 10.1371/journal.pone.0179617

Gao, Q. M., Venugopal, S., Navarre, D., and Kachroo, A. (2011). Low oleic acid-derived repression of jasmonic acid-inducible defense responses requires the WRKY50 and WRKY51 proteins. *Plant Physiol.* 155, 464–476. doi: 10.1104/pp.110.166876

Garrett, S. D. (1956). *Biology of Root-Infecting Fungi*. Cambridge: Cambridge University Press.

Gharbi, Y., Barkallah, M., Bouazizi, E., Gdoura, R., and Triki, M. A. (2017). Differential biochemical and physiological responses of two olive cultivars differing by their susceptibility to the hemibiotrophic pathogen *Verticillium dahliae*. *Physiol. Mol. Plant Pathol.* 97, 30–39. doi: 10.1016/j.pmpp.2016.12.001

Guo, D., Chen, F., Inoue, K., Blount, J. W., and Dixon, R. A. (2001). Downregulation of caffeic acid 3-O-methyltransferase and caffeoyl CoA 3-O-methyltransferase in transgenic alfalfa: impacts on lignin structure and implications for the biosynthesis of G and S lignin. *Plant Cell* 13, 73–88. doi: 10.1105/tpc.13.1.73

Hermosa, R., Rubio, M. B., Cardoza, R. E., Nicolás, C., Monte, E., and Gutiérrez, S. (2013). The contribution of *Trichoderma* to balancing the costs of plant growth and defense. *Int. Microbiol.* 16, 69–80.

Hermosa, R., Viterbo, A., Chet, I., and Monte, E. (2012). Plant-beneficial effects of *Trichoderma* and of its genes. *Microbiology* 158, 17–25. doi: 10.1099/mic.0.052274-0

Hichri, I., Muhovski, Y., Žižková, E., Dobrev, P. I., Gharbi, E., Franco-Zorrilla, J. M., et al. (2017). The *Solanum lycopersicum* WRKY3 transcription factor SlWRKY3 is involved in salt stress tolerance in tomato. *Front. Plant Sci.* 8:1343. doi: 10.3389/fpls.2017.01343

Hu, B., Jin, J., Guo, A. Y., Zhang, H., Luo, J., and Gao, G. (2015). GSDS 2.0: an upgraded gene feature visualization server. *Bioinformatics* 31, 1296–1297. doi: 10.1093/bioinformatics/btu817

Huang, S., Gao, Y., Liu, J., Peng, X., Niu, X., Fei, Z., et al. (2012). Genome-wide analysis of WRKY transcription factors in *Solanum lycopersicum*. *Mol. Genet. Genomics* 287, 495–513. doi: 10.1007/s00438-012-0696-6

Jain, A., Singh, S., Kumar Sarma, B., and Bahadur Singh, H. (2012). Microbial consortium–mediated reprogramming of defence network in pea to enhance tolerance against *Sclerotinia sclerotiorum*. *J. Appl. Microbiol.* 112, 537–550. doi: 10.1111/j.1365-2672.2011.05220.x

Jana, S., and Choudhuri, M. A. (1981). Glycolate metabolism of three submersed aquatic angiosperms: effect of heavy metals. *Aquat. Bot.* 11, 67–77. doi: 10.1016/0304-3770(81)90047-4

Jaskiewicz, M., Conrath, U., and Peterhänsel, C. (2011). Chromatin modification acts as a memory for systemic acquired resistance in the plant stress response. *EMBO Rep.* 12, 50–55. doi: 10.1038/embor.2010.186

Jensen, B., Knudsen, I. M., Madsen, M., and Jensen, D. F. (2004). Biopriming of infected carrot seed with an antagonist, *Clonostachys rosea*, selected for control of seed borne *Alternaria* spp. *Phytopathology* 94, 551–560. doi: 10.1094/PHYTO.2004.94.6.551

Jothi, G., and Babu, R. S. (2002). Peroxidase and chitinase activities in brinjal inoculated with *Meloidogyne incognita* (Kofoid & White) chitwood and endomycorrhiza. *J. Biol. Control.* 16, 161–164.

Karkute, S. G., Gujjar, R. S., Rai, A., Akhtar, M., and Singh, M. (2018). Genome wide expression analysis of WRKY genes in tomato (*Solanum lycopersicum*) under drought stress. *Plant Gene* 13, 8–17. doi: 10.1016/j.plgene.2017.11.002

Kayum, M. A., Jung, H. J., Park, J. I., Ahmed, N. U., Saha, G., Yang, T. J., et al. (2015). Identification and expression analysis of WRKY family genes under biotic and abiotic stresses in *Brassica rapa*. *Mol. Genet. Genomics* 290, 79–95. doi: 10.1007/s00438-014-0898-1

Kiranmai, K., Lokanadha Rao, G., Pandurangaiah, M., Nareshkumar, A., Amaranatha Reddy, V., Lokesh, U., et al. (2018). A novel WRKY transcription factor, MuWRKY3 (*Macrotyloma uniflorum* Lam. Verdc.) enhances drought stress tolerance in transgenic groundnut *(Arachis hypogaea* L.) plants. *Front. Plant Sci.* 9:346. doi: 10.3389/fpls.2018.00346

Kissoudis, C. (2016). *Genetics and Regulation of Combined Abiotic and Biotic Stress Tolerance in Tomato*. Doctoral dissertation, Wageningen University.

Korolev, N., David, D. R., and Elad, Y. (2008). The role of phytohormones in basal resistance and trichoderma-induced systemic resistance to *Botrytis cinerea* in *Arabidopsis thaliana*. *Biocontrol* 53, 667–683. doi: 10.1007/s10526-007-9103-3

Lai, Z. B., Vinod, K. M., Zheng, Z. Y., Fan, B. F., and Chen, Z. X. (2008). Roles of *Arabidopsis* WRKY3 and WRKY4 transcription factors in plant responses to pathogens. *BMC Plant Biol.* 8:68. doi: 10.1186/1471-2229-8-68

Lescot, M., Déhais, P., Thijs, G., Marchal, K., Moreau, Y., Van de Peer, Y., et al. (2002). Plant CARE, a database of plant cis-acting regulatory elements and a portal to tools for *in silico* analysis of promoter sequences. *Nucleic Acids Res.* 30, 325–327. doi: 10.1093/nar/30.1.325

Li, S., Fu, Q., Chen, L., Huang, W., and Yu, D. (2011). *Arabidopsis thaliana* WRKY25, WRKY26, and WRKY33 coordinate induction of plant thermotolerance. *Planta*, 233, 1237–1252. doi: 10.1007/s00425-011-1375-2

Li, B., Meng, X., Shan, L., and He, P. (2016). Transcriptional regulation of pattern-triggered immunity in plants. *Cell Host Microbe* 19, 641–650. doi: 10.1016/j.chom.2016.04.011

Lippok, B., Birkenbihl, R. P., Rivory, G., Brümmer, J., Schmelzer, E., Logemann, E., et al. (2007). Expression of AtWRKY33 encoding a pathogen-or PAMP-responsive WRKY transcription factor is regulated by a composite DNA motif containing W box elements. *Mol. Plant Microbe Interact.* 20, 420–429. doi: 10.1094/mpmi-20-4-0420

Liu, S., Kracher, B., Ziegler, J., Birkenbihl, R. P., and Somssich, I. E. (2015). Negative regulation of ABA signaling by WRKY33 is critical for *Arabidopsis* immunity towards *Botrytis cinerea* 2100. *eLife* 4:e07295. doi: 10.7554/eLife.07295

Livak, K. J., and Schmittgen, T. D. (2001). Analysis of relative gene expression data using real-time quantitative PCR and the 2- ΔΔCT method. *Methods* 25, 402–408. doi: 10.1006/meth.2001.1262

Mangelsen, E., Kilian, J., Berendzen, K. W., Kolukisaoglu, ÜH., Harter, K., Jansson, C., et al. (2008). Phylogenetic and comparative gene expression analysis of barley (*Hordeum vulgare*) WRKY transcription factor family reveals putatively retained functions between monocots and dicots. *BMC Genomics* 9:194. doi: 10.1186/1471-2164-9-194

Martínez-Medina, A., Fernández, I., Sánchez-Guzmán, M. J., Jung, S. C., Pascual, J. A., and Pozo, M. J. (2013). Deciphering the hormonal signalling network behind the systemic resistance induced by *Trichoderma harzianum* in tomato. *Front. Plant Sci.* 4:206. doi: 10.3389/fpls.2013.00206

Martínez-Medina, A., Fernandez, I., Lok, G. B., Pozo, M. J., Pieterse, C. M., and Van Wees, S. (2017). Shifting from priming of salicylic acid-to jasmonic acid-regulated defences by *Trichoderma* protects tomato against the root knot nematode *Meloidogyne incognita*. *New Phytol.* 213, 1363–1377. doi: 10.1111/nph.14251

Mastouri, F., Björkman, T., and Harman, G. E. (2012). *Trichoderma harzianum* enhances antioxidant defense of tomato seedlings and resistance to water deficit. *Mol. Plant Microbe Interact.* 25, 1264–1271. doi: 10.1094/MPMI-09-11-0240

Mayo, S., Cominelli, E., Sparvoli, F., González-López, O., Rodríguez-González, A., Gutiérrez, S., et al. (2016). Development of a qPCR strategy to select bean genes involved in plant defense response and regulated by the *Trichoderma velutinum Rhizoctonia solani* interaction. *Front. Plant Sci.* 7:1109. doi: 10.3389/fpls.2016.01109

McKersie, B. D., Hoekstra, F., and Krieg, L. (1990). Differences in the susceptibility of plant membrane lipids to peroxidation. *Biochim. Biophys. Acta* 1030, 119–126. doi: 10.1016/0005-2736(90)90246-k

Meshram, S., Patel, J. S., Yadav, S. K., Kumar, G., Singh, D. P., Singh, H. B., et al. (2019). Trichoderma mediate early and enhanced lignifications in chickpea during *Fusarium oxysporum* f. sp. ciceris infection. *J. Basic Microbiol.* 59, 74–86. doi: 10.1002/jobm.201800212

Mitra, P. P., and Loqué, D. (2014). Histochemical staining of *Arabidopsis thaliana* secondary cell wall elements. *J. Vis. Exp.* 87:e51381. doi: 10.3791/51381

Morán-Diez, E., Rubio, M. B., Domínguez, S., Hermosa, R., Monte, E., and Nicolás, C. (2012). Transcriptomic response of *Arabidopsis thaliana* after root colonization by *Trichoderma harzianum*. *J. Plant Physiol.* 169, 614–620. doi: 10.1016/j.jplph.2011.12.016

Nirmaladevi, D., Venkataramana, M., Srivastava, R. K., Uppalapati, S. R., Gupta, V. K., Yli-Mattila, T., et al. (2016). Molecular phylogeny, pathogenicity and toxigenicity of *Fusarium oxysporum* f. sp. lycopersici. *Sci. Rep.* 6:21367. doi: 10.1038/srep21367

Pandey, S. P., and Somssich, I. E. (2009). The role of WRKY transcription factors in plant immunity. *Plant Physiol.* 150, 1648–1655. doi: 10.1104/pp.109.138990

Patel, J. S., Kharwar, R. N., Singh, H. B., Upadhyay, R. S., and Sarma, B. K. (2017). *Trichoderma asperellum* (T42) and *Pseudomonas fluorescens* (OKC)-enhances resistance of pea against *Erysiphe pisi* through enhanced ROS generation and lignifications. *Front. Microbiol.* 8:306. doi: 10.3389/fmicb.2017.00306

Phukan, U. J., Jeena, G. S., and Shukla, R. K. (2016). WRKY transcription factors: molecular regulation and stress responses in plants. *Front. Plant Sci.* 7:760. doi: 10.3389/fpls.2016.00760

Pieterse, C. M., Van der Does, D., Zamioudis, C., Leon-Reyes, A., and Van Wees, S. C. (2012). Hormonal modulation of plant immunity. *Annu. Rev. Cell Dev. Biol.* 28, 489–521. doi: 10.1146/annurev-cellbio-092910-154055

Pieterse, C. M., Zamioudis, C., Berendsen, R. L., Weller, D. M., Van Wees, S. C., and Bakker, P. A. (2014). Induced systemic resistance by beneficial microbes. *Annu. Rev. Phytopathol.* 52, 347–375. doi: 10.1146/annurev-phyto-082712-102340

Pusztahelyi, T., Holb, I. J., and Pócsi, I. (2015). Secondary metabolites in fungus-plant interactions. *Front. Plant. Sci.* 6:573. doi: 10.3389/fpls.2015.00573

Qiu, Y., and Yu, D. (2009). Over-expression of the stress-induced OsWRKY45 enhances disease resistance and drought tolerance in *Arabidopsis*. *Environ. Exp. Bot.* 65, 35–47. doi: 10.1016/j.envexpbot.2008.07.002

Robert-Seilaniantz, A., Grant, M., and Jones, J. D. (2011). Hormone crosstalk in plant disease and defense: more than just jasmonate-salicylate antagonism. *Annu. Rev. Phytopathol.* 49, 317–343. doi: 10.1146/annurev-phyto-073009-114447

Ruocco, M., Lanzuise, S., Lombardi, N., Woo, S. L., Vinale, F., Marra, R., et al. (2015). Multiple roles and effects of a novel *Trichoderma* hydrophobin. *Mol. Plant Microbe Interact.* 28, 167–179. doi: 10.1094/MPMI-07-14-0194-R

Rushton, P. J., Torres, J. T., Parniske, M., Wernert, P., Hahlbrock, K., and Somssich, I. E. (1996). Interaction of elicitor-inducing DNA-binding proteins with elicitor response elements in the promoters of parsley PR1 genes. *EMBO J.* 15, 5690–5700. doi: 10.1002/j.1460-2075.1996.tb00953.x

Saed-Moucheshi, A., Shekoofa, A., and Pessarakli, M. (2014). Reactive oxygen species (ROS) generation and detoxifying in plants. *J. Plant Nutr.* 37, 1573–1585. doi: 10.1080/01904167.2013.868483

Sáenz-Mata, J., Salazar-Badillo, F. B., and Jiménez-Bremont, J. F. (2014). Transcriptional regulation of *Arabidopsis thaliana WRKY* genes under interaction with beneficial fungus *Trichoderma atroviride*. *Acta Physiol. Plant* 36, 1085–1093. doi: 10.1007/s11738-013-1483-7

Shankar, A., Pandey, A., and Pandey, G. K. (2013). WRKY transcription factor: role in abiotic and biotic stress. *Plant Stress* 7, 26–34.

Shaw, S., Le Cocq, K., Paszkiewicz, K., Moore, K., Winsbury, R., de Torres Zabala, M., et al. (2016). Transcriptional reprogramming underpins enhanced plant growth promotion by the biocontrol fungus *Trichoderma hamatum* GD12 during antagonistic interactions with *Sclerotinia sclerotiorum* in soil. *Mol. Plant Pathol.* 17, 1425–1441. doi: 10.1111/mpp.12429

Shen, Q.-H., Saijo, Y., Mauch, S., Biskup, C., Bieri, S., Keller, B., et al. (2007). Nuclear activity of MLA immune receptors links isolate-specific and basal disease-resistance responses. *Science* 315, 1098–1103. doi: 10.1126/science.1136372

Singh, B. N., Singh, A., Singh, S. P., and Singh, H. B. (2011). Trichoderma harzianum-mediated reprogramming of oxidative stress response in root apoplast of sunflower enhances defence against *Rhizoctonia solani*. *Eur. J. Plant Pathol.* 131, 121–134. doi: 10.1007/s10658-011-9792-4

Singh, P., Shekhar, S., Rustagi, A., Sharma, V., and Kumar, D. (2018). "Insights into the role of WRKY superfamily of protein transcription factor in defense response" in *Molecular Aspects of Plant-Pathogen Interaction*, eds A. Singh, I. K. Singh (Singapore: Springer), 185–202. doi: 10.1007/978-981-10-7371-7_8

Spence, C., Alff, E., Johnson, C., Ramos, C., Donofrio, N., Sundaresan, V., et al. (2014). Natural rice rhizospheric microbes suppress rice blast infections. *BMC Plant Biol.* 14:130. doi: 10.1186/1471-2229-14-130

Stafford, H. A. (1962). Histochemical & biochemical differences between lignin-like materials in *Phleum pratense* L. *Plant Physiol.* 37:643.

Sun, C., Palmqvist, S., Olsson, H., Borén, M., Ahlandsberg, S., and Jansson, C. (2003). A novel WRKY transcription factor, SUSIBA2, participates in sugar signaling in barley by binding to the sugar-responsive elements of the iso1 promoter. *Plant Cell* 15, 2076–2092. doi: 10.1105/tpc.014597

Taylor, A., Vagany, V., Barbara, D. J., Thomas, B., Pink, D. A. C., Jones, J. E., et al. (2013). Identification of differential resistance to six *Fusarium oxysporum* f. sp. cepae isolates in commercial onion cultivars through the development of a rapid seedling assay. *Plant Pathol.* 62, 103–111. doi: 10.1111/j.1365-3059.2012.02624.x

Tomato Genome Consortium. (2012). The tomato genome sequence provides insights into fleshy fruit evolution. *Nature* 485:635. doi: 10.1038/nature11119

Tominaga-Wada, R., Nukumizu, Y., and Wada, T. (2013). Tomato (*Solanum lycopersicum*) homologs of TRIPTYCHON (SlTRY) and GLABRA3 (SlGL3) are involved in anthocyanin accumulation. *Plant Signal. Behav.* 8:e24575. doi: 10.4161/psb.24575

Tucci, M., Ruocco, M., De Masi, L., De Palma, M., and Lorito, M. (2011). The beneficial effect of *Trichoderma* spp. on tomato is modulated by the plant genotype. *Mol. Plant Pathol.* 12, 341–354. doi: 10.1111/j.1364-3703.2010.00674.x

Untergasser, A., Cutcutache, I., Koressaar, T., Ye, J., Faircloth, B. C., Remm, M., et al. (2012). Primer3—new capabilities and interfaces. *Nucleic Acids Res.* 40:e115. doi: 10.1093/nar/gks596

van Esse, H. P., Fradin, E. F., de Groot, P. J., de Wit, P. J., and Thomma, B. P. (2009). Tomato transcriptional responses to a foliar and a vascular fungal pathogen are distinct. *Mol. Plant Microbe Interact.* 22, 245–258. doi: 10.1094/MPMI-22-3-0245

Vergne, E., Ballini, E., Marques, S., Sidi Mammar, B., Droc, G., Gaillard, S., et al. (2007). Early and specific gene expression triggered by rice resistance gene Pi33 in response to infection by ACE1 avirulent blast fungus. *New Phytol.* 174, 159–171. doi: 10.1111/j.1469-8137.2007.01971.x

Vives-Peris, V., Marmaneu, D., Gómez-Cadenas, A., and Pérez-Clemente, R. M. (2018). Characterization of Citrus WRKY transcription factors and their responses to phytohormones and abiotic stresses. *Biol. Plant.* 62, 33–44. doi: 10.1007/s10535-017-0737-4

Wada, T., Kunihiro, A., and Tominaga-Wada, R. (2014). Arabidopsis CAPRICE (MYB) and GLABRA3 (bHLH) control tomato (*Solanum lycopersicum*) anthocyanin biosynthesis. *PLoS One* 9:e109093. doi: 10.1371/journal.pone.0109093

Wada, T., Onishi, M., Kunihiro, A., and Tominaga-Wada, R. (2015). Overexpressing CAPRICE and GLABRA3 did not change the anthocyanin content of tomato (*Solanum lycopersicum*) fruit peel. *Plant Signal. Behav.* 10:e1000131. doi: 10.1080/15592324.2014.1000131

Wenke, K., Wanke, D., Kilian, J., Berendzen, K., Harter, K., and Piechulla, B. (2012). Volatiles of two growth-inhibiting rhizobacteria commonly engage AtWRKY18 function. *Plant J.* 70, 445–459. doi: 10.1111/j.1365-313X.2011.04891.x

Wojtaszek, P. (1997). Oxidative burst: an early plant response to pathogen infection. *Biochem. J.* 322, 681–692. doi: 10.1042/bj3220681

Ye, J., Coulouris, G., Zaretskaya, I., Cutcutache, I., Rozen, S., and Madden, T. L. (2012). Primer-BLAST: a tool to design target-specific primers for polymerase chain reaction. *BMC Bioinformatics* 13:134. doi: 10.1186/1471-2105-13-134

Yedidia, I., Benhamou, N., Kapulnik, Y., and Chet, I. (2000). Induction and accumulation of PR proteins activityduring early stages of root colonizationby the mycoparasite *Trichoderma harzianum* strain T-203. *Plant Physiol. Biochem.* 38, 863–873. doi: 10.1016/s0981-9428(00)01198-0

Yoshioka, Y., Ichikawa, H., Naznin, H. A., Kogure, A., and Hyakumachi, M. (2012). Systemic resistance induced in *Arabidopsis thaliana* by *Trichoderma asperellum* SKT-1, a microbial pesticide of seedborne diseases of rice. *Pest Manag. Sci.* 68, 60–66. doi: 10.1002/ps.2220

Yue, J., Xu, W., Ban, R., Hunag, S., Miao, M., Tang, X., et al. (2016). PTIR: predicted tomato interactome resource. *Sci. Rep.* 6:25047. doi: 10.1038/srep25047

Zehra, A., Meena, M., Dubey, M. K., Aamir, M., and Upadhyay, R. S. (2017). Synergistic effects of plant defense elicitors and *Trichoderma harzianum* on enhanced induction of antioxidant defense system in tomato against Fusarium wilt disease. *Bot. Stud.* 58:44. doi: 10.1186/s40529-017-0198-2

Zhang, S., Gan, Y., and Xu, B. (2016). Application of plant-growth-promoting fungi *Trichoderma longibrachiatum* T6 enhances tolerance of wheat to salt stress through improvement of antioxidative defense system and gene expression. *Front. Plant Sci.* 7:1405. doi: 10.3389/fpls.2016.01405

Zheng, Z., Qamar, S. A., Chen, Z., and Mengiste, T. (2006). *Arabidopsis* WRKY33 transcription factor is required for resistance to necrotrophic fungal pathogens. *Plant J.* 48, 592–605. doi: 10.1111/j.1365-313x.2006.02901.x

Zhou, J., Wang, J., Yu, J. Q., and Chen, Z. (2014). Role and regulation of autophagy in heat stress responses of tomato plants. *Front. Plant Sci.* 5:174. doi: 10.3389/fpls.2014.00174

Zhou, J., Wang, J., Zheng, Z., Fan, B., Yu, J. Q., and Chen, Z. (2015). Characterization of the promoter and extended C-terminal domain of *Arabidopsis* WRKY33 and functional analysis of tomato WRKY33 homologues in plant stress responses. *J. Exp. Bot.* 66, 4567–4583. doi: 10.1093/jxb/erv221

Zhou, Y., Ma, J., Xie, J., Deng, L., Yao, S., and Zeng, K. (2018). Transcriptomic and biochemical analysis of highlighted induction of phenylpropanoid pathway metabolism of citrus fruit in response to salicylic acid, *Pichia membranaefaciens* and oligochitosan. *Postharvest Biol. Technol.* 142, 81–92. doi: 10.1016/j.postharvbio.2018.01.021

Conflict of Interest Statement: The authors declare that the research was conducted in the absence of any commercial or financial relationships that could be construed as a potential conflict of interest.

Advantages of publishing in Frontiers

OPEN ACCESS
Articles are free to read for greatest visibility and readership

FAST PUBLICATION
Around 90 days from submission to decision

HIGH QUALITY PEER-REVIEW
Rigorous, collaborative, and constructive peer-review

TRANSPARENT PEER-REVIEW
Editors and reviewers acknowledged by name on published articles

Frontiers
Avenue du Tribunal-Fédéral 34
1005 Lausanne | Switzerland

Visit us: www.frontiersin.org
Contact us: info@frontiersin.org | +41 21 510 17 00

REPRODUCIBILITY OF RESEARCH
Support open data and methods to enhance research reproducibility

DIGITAL PUBLISHING
Articles designed for optimal readership across devices

FOLLOW US
@frontiersin

IMPACT METRICS
Advanced article metrics track visibility across digital media

EXTENSIVE PROMOTION
Marketing and promotion of impactful research

LOOP RESEARCH NETWORK
Our network increases your article's readership