BENEFICIAL MICROBIOTA INTERACTING WITH THE PLANT IMMUNE SYSTEM

EDITED BY: Christos Zamioudis, Corné M. J. Pieterse, Roeland Lucas Berendsen, Paulo José Pereira Lima Teixeira and Ioannis Stringlis

PUBLISHED IN: Frontiers in Plant Science and Frontiers in Microbiology

frontiers Research Topics

frontiers

ISSN 1664-8714
ISBN 978-2-88971-139-0
DOI 10.3389/978-2-88971-139-0

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: frontiersin.org/about/contact

BENEFICIAL MICROBIOTA INTERACTING WITH THE PLANT IMMUNE SYSTEM

Topic Editors:
Christos Zamioudis, Democritus University of Thrace, Greece
Corné M. J. Pieterse, Utrecht University, Netherlands
Roeland Lucas Berendsen, Utrecht University, Netherlands
Paulo José Pereira Lima Teixeira, University of São Paulo, Brazil
Ioannis Stringlis, Utrecht University, Netherlands

Citation: Zamioudis, C., Pieterse, C. M. J., Berendsen, R. L., Teixeira, P. J. P. L., Stringlis, I., eds. (2021). Beneficial Microbiota Interacting with the Plant Immune System. Lausanne: Frontiers Media SA. doi: 10.3389/978-2-88971-139-0

Table of Contents

frontiers in Plant Science

EDITORIAL
published: 22 June 2021
doi: 10.3389/fpls.2021.698902

Editorial: Beneficial Microbiota Interacting With the Plant Immune System

*Ioannis A. Stringlis[1], Paulo J. P. L. Teixeira[2], Roeland L. Berendsen[1], Corné M. J. Pieterse[1] and Christos Zamioudis[3]**

[1] Plant-Microbe Interactions, Department of Biology, Science for Life, Utrecht University, Utrecht, Netherlands, [2] Department of Biology, 'Luiz de Queiroz' College of Agriculture (ESALQ), University of São Paulo (USP), Piracicaba, Brazil, [3] Laboratory of Plant Pathology, Department of Agricultural Development, Democritus University of Thrace, Orestiada, Greece

Keywords: microbiome, beneficial microbes, crop protection, rhizosphere, phyllosphere, plant immune system, induced systemic resistance, plant pathogens

Editorial on the Research Topic

Beneficial Microbiota Interacting With the Plant Immune System

INTRODUCTION

The Green Revolution during the 50s and 60s was a milestone in the history of mankind. Based on the principles "higher yields, more food, less poverty and hunger," it radically transformed agriculture and dramatically increased global food production (Khush, 2001). Despite the success, intensive agricultural practices that include the exhaustive use of synthetic fertilizers and agrochemicals and the overexploitation of natural resources, eventually came with serious environmental costs (Tang et al., 2021). Today, more and more farmers around the world realize that the soils used to cultivate monocultures for many years are rapidly degrading (Banwart, 2011). In addition, the withdrawal of agrochemicals from the market that are effective but unsafe for the environment and the consumer health created additional difficulties in the control of devastating pathogens and pests. With the advent of a rapidly growing human population, anticipated to reach about 10 billion people by the year 2050 (FAO, 2009), a new revolution in agriculture seems to be more timely than ever in order to sustain and further increase food production (Evans and Lawson, 2020).

Plants are massively colonized by communities of microbes that are referred to as the plant microbiota. Plant-associated beneficial microbes have long been known to provide important ecosystem services and promote plant health by enhancing growth, suppressing pathogens and training plant immunity (Berendsen et al., 2012; Trivedi et al., 2020). Lorenz Hiltner, a pioneer of microbial ecology, was the first to recognize the important role of beneficial bacteria that colonize the rhizosphere (Hartmann et al., 2008). Since then, our understanding regarding the structure and the function of the plant microbiomes has been greatly improved (Tian et al., 2020). Towards a new Green Revolution that is protective to the environment and safe to humans, the enhanced interest in the plant microbiome clearly stems from its strong potential to provide eco-friendly solutions in plant disease protection and novel tools to promote sustainability in agroecosystems (Qiu et al., 2019).

Understanding the complexity of plant-microbiome interactions is essential to transform fundamental knowledge to microbiome-informed innovations in modern agriculture. We host here in this Research Topic, "Beneficial Microbiota Interacting with the Plant Immune System," 16 articles that enhance our knowledge on the supportive functions of beneficial microbes in plant disease resistance. In particular, the Topic contains research articles focusing on the protective functions of individual biological control agents (BCAs) against important diseases in agricultural

OPEN ACCESS

Edited by:
Andrea Chini, Centro Nacional de Biotecnología, Spain

Reviewed by:
Víctor Flors, University of Jaume I, Spain
Andrea Genre, University of Turin, Italy

***Correspondence:**
Christos Zamioudis
czamioud@agro.duth.gr

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

Received: *22 April 2021*
Accepted: *14 May 2021*
Published: *22 June 2021*

Citation:
Stringlis IA, Teixeira PJPL, Berendsen RL, Pieterse CMJ and Zamioudis C (2021) Editorial: Beneficial Microbiota Interacting With the Plant Immune System. Front. Plant Sci. 12:698902. doi: 10.3389/fpls.2021.698902

and forest ecosystems, but also metagenomic studies that provide a more holistic view on the way microbiota interact with plant immunity. In addition, one method paper presents a pipeline to dissect selected plant responses to bacteria with different lifestyles and 5 review articles summarize our current knowledge on the mechanisms by which beneficial bacteria and fungi promote host defenses and plant health in below- and aboveground plant tissues. New experimental platforms and integrated approaches that combine (meta)-omics with functional analyses are needed in future research in order to obtain a comprehensive understanding of the mechanisms by which beneficial microbes interact with phytopathogens and plant immunity.

CONTENT COLLECTION

Original Research Articles

By testing more than 500 bacterial isolates, Park et al. identified *Bacillus thuringiensis* strain JCK-1233 and a specific diketopiperazine produced by JCK-1233 to induce resistance in pine trees thereby suppressing the wilt disease caused by the nematode *Bursaphelenchus xylophilus*, one of the most important pests affecting pine forests worldwide. Thus, also in forest ecosystems where the application of synthetic pesticides against devastating pests is costly and often complicated by diverse ecological risks, BCAs could be implemented as eco-friendly and cost-effective alternatives.

Chen et al. studied the mechanisms by which another Bacillus strain, *Bacillus velezensis* CLA178, suppresses the tumors caused by Agrobacterium infection in the ornamental plant *Rosa multiflora*. Combined with work in Arabidopsis, the authors found that, when applied to the roots, CLA178 promoted plant growth and further primed the expression of defense genes regulated by the salicylic acid and ethylene signaling pathways. The sequenced genome of CLA178 included in the study pave the way for genome-centered future analyses in order to reveal the bacterial genetics involved in both phenomena.

Wu et al. utilized pot experiments to demonstrate that the antagonism between the biocontrol strain *Bacillus velezensis* HN03 and the wilt pathogen of banana *Fusarium oxysporum* f. sp. *cubense*, depends on the nutritional content of the soil. In particular, the authors found that synchronous application of HN03 and compost potentiated the biocontrol outcome of individual treatments through reciprocal interactions, which the authors summarize in a conceptual model. Considering that the application of BCAs in the field often fails to deliver the anticipated outcomes, this study provides means to enhance the biocontrol activity of selected BCAs under agricultural settings.

In their genomics study, Samaras et al. provide insights into the mechanism underpinning the protective functions of *Bacillus subtilis* MBI 600, one of the many commercialized *Bacillus* strains. The authors sequenced the MBI 600 genome and through comparative genomics identified common genes to other Bacilli, but also unique genomic features related to root colonization, plant growth promotion and biocontrol activity. Interestingly, by generating an *yfp*-tagged strain, the authors were able to study the colonization potential of MBI 600 in the roots of cucumber in different growth substrates. Overall, this interesting study demonstrates the power of microbial genetics in dissecting the mechanisms by which beneficial microbes train immunity and improve host health.

Sacristán-Pérez-Minayo et al. characterized the effects of two *Pseudomonas* strains on sugar beet productivity. When used as soil inoculants in the field, both beneficials improved the yield and the quality of the tubers, however, they failed to provide protection against two important sugar beet pathogens. The study clearly suggests that delivering multiple traits in the field could be a difficult task; rather than the application of single microbes, the application of microbials at the community level may be a more reasonable approach.

In their study, Chen et al. demonstrated that prolonged monoculture with the medicinal herb *Radix pseudostellariae* reduced the diversity of antagonistic *Trichoderma* communities in the rhizosphere, consequently increasing the abundance of pathogenic *Fusarium oxysporum*. Interestingly, the authors found that the application of the *Trichoderma* strain *T. harzianum* ZC51 could improve plant resistance and reduce the growth inhibitory effect stemming from the consecutive monoculture. This study clearly shows the impact of monoculture on the rhizosphere microbiome and further provides means to improve soil health through the application of soil beneficials.

The interesting work of Anguita-Maeso et al. reveals that a wild olive variety that is otherwise resistant to the wilt fungus *Verticillium dahliae* becomes susceptible to the pathogen when propagated *in vitro*. The authors provide evidence that *in vitro* micropropagation of this particular olive accession alters community structure resulting in the breakdown of resistance to *Verticillium*. Thus, the xylem microbiomes could be exploited as a reliable resistance source to devastating root-infecting pathogens.

The seedling stage is the most vulnerable time in the life cycle of a plant, and the role of seed-derived microbiota in promoting seedling health is well-established. Focusing on rice, Wang et al. utilized an axenic growth system and carried out metagenomic analyses to demonstrate that during seed germination, the pool of microbes that colonize the seeds are separated to distinct assemblages in the different plant tissues. Interestingly, the authors found that functions related to plant growth and pathogen suppression are enriched in the core microbiomes transferred from the seed to the newly established plants.

Invasive alien plant species (IAPS) may cause severe damage to natural ecosystems by reducing the richness and abundance of native plant species. *Mikania micrantha*, a fast-growing vine, is ranked amongst the top 100 worst IAPS in the world. Having the authors previously published the *M. micrantha* genome, the exciting metagenomics study of Yin et al. in this collection indicates that the roots of *M. micrantha* host more phosphate-solubilizing and pathogen-suppressive rhizobacteria than the roots of two coexisting native plant species. Thus, rhizosphere microbes seem to play important roles in the establishment of invasive plants and may even act as drivers of plant invasion.

The leaves of the perennial herb *Tricyrtis macropoda* have an unusual phenotype with spots covering the leaf surface.

Wang et al. found that the composition of the fungal microbiome in the spots differs from the fungal communities in the green parts. By analyzing the metabolome of spotted and non-spotted leaf parts, a significant correlation between the endophytic fungal communities and the production of metabolites has been established. Overall, this study provides new insights into the relationship between microbes and plant phenotypes and further demonstrates the value of -omics toward understanding the molecular cues driving microbiome assembly in the host.

Methods Articles

Hydrogen peroxide (H_2O_2) functions as an important signaling molecule in plants during biotic interactions. Carril et al. developed a protocol to visualize and quantify H_2O_2 production in wheat leaves after infection with a pathogenic bacterium or after co-inoculation of the pathogen with a beneficial bacterium. DAB staining combined with an imaging analysis pipeline revealed that co-inoculation yielded less H_2O_2 accumulation and less visible disease symptoms compared to the pathogen infection alone. Therefore, this protocol can successfully determine the H_2O_2 levels accumulating in response to bacteria with different lifestyles.

Review Articles

A fast-growing field of research focuses on microbial biocontrol in the phyllosphere. Legein et al. review the different factors influencing microbial adaptation in the phyllosphere. These factors range from environmental stresses to microbe-microbe and plant-microbe interactions. The authors present the current knowledge on the interplay between these factors and dissect the mechanisms involved in the biocontrol activity of microbial inoculants in the phyllosphere. Demonstrating examples from *in vitro* and field experiments, the authors suggest the integration of experimental data coming from both sources to design successful and sustainable biocontrol strategies against leaf pathogens.

Our knowledge on the plant microbiome and the diverse ways it affects plant fitness is expanding continuously. This is also demonstrated in the review by Lee and Ryu, where algae are presented as new members of the beneficial plant microbiome. The authors discuss their presence on plant tissues and in the soil and their occurrence in plant microbiome datasets. They further provide examples highlighting the beneficial effects of algae on plant growth and disease suppression as well as the mechanisms involved. Application of algae as biofertilizer to modulate microbiome activity and improve crop yields is suggested as an extra alternative to conventional agricultural practices.

Focusing on the three major classes of soil-borne beneficials, plant growth promoting rhizobacteria, biological control agents and root nodulating rhizobia, Lucke et al. provide an excellent overview of the mechanisms by which these classes of microbes deliver plant-growth and disease-suppressive compounds. Toward identifying potent biologicals, the authors point to the need for genome-centered studies in order to identify the microbial genes responsible for the beneficial functions.

Ectomycorrhizal fungi (EMF) are soil-borne microbes that form mutualistic associations with forest trees. The review of Dreischhoff et al. brings into the light a thus-far unexplored function of the EMF related to plant immunity. The authors review the evidence supporting that EMF activate local and systemic immune responses with the latter sharing characteristics of both induced and systemic acquired resistance. Toward enhancing our understanding of the mechanisms by which EMF interact with the immune system of host trees, the authors provide a guide to future research that will help to reveal aspects related to EMF-induced resistance.

The ability of beneficial microbes to modulate plant immunity largely relies on the secretion of a diverse array of low-molecular weight metabolites. The molecular determinants specifically involved in the phenomenon of rhizobacteria-mediated induced systemic resistance is reviewed in this article collection by Pršić and Ongena. The authors provide an updated overview of ISR elicitors originating from diverse rhizosphere bacterial species, such as acyl-homoserine lactones, cyclic lipopeptides, rhamnolipids, N-alkylated benzylamine derivatives, siderophores, antibiotics, and volatile organic compounds. They further emphasize on the necessity to reveal in future studies how these molecules are sensed by the host and what type of defense responses are manifested upon elicitor perception.

AUTHOR CONTRIBUTIONS

The authors jointly defined the content of this Research Topic and participated in the editing process. All authors made substantial, direct and intellectual contribution to the composing of this editorial, and approved it for publication.

FUNDING

IAS, RLB, and CMJP were supported by the Dutch Research Council (NWO/OCW), as part of the MiCRop Consortium programme Harnessing the second genome of plants (grant number 024.004.014).

ACKNOWLEDGMENTS

The editors would like to thank all authors who selected for this Topic to publish their work and all reviewers who evaluated manuscripts for this Research Topic.

REFERENCES

Banwart, S. (2011). Save our soils. *Nature* 474, 151–152. doi: 10.1038/474151a

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

Evans, J. R., and Lawson, T. (2020). From green to gold: agricultural revolution for food security. *J. Exp. Bot.* 71, 2211–2215. doi: 10.1093/jxb/eraa110

FAO (2009). *How to Feed the World in 2050*. High Level Expert Forum. FAO, Rome.

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

Khush, G. S. (2001). Green revolution: the way forward. *Nat. Rev. Genet.* 2, 815–822. doi: 10.1038/35093585

Qiu, Z., Egidi, E., Liu, H., Kaur, S., and Singh, B. K. (2019). New frontiers in agriculture productivity: optimised microbial inoculants and *in situ* microbiome engineering. *Biotechnol. Adv.* 37:107371. doi: 10.1016/j.biotechadv.2019.03.010

Tang, F. H. M., Lenzen, M., McBratney, A., and Maggi, F. (2021). Risk of pesticide pollution at the global scale. *Nat. Geosci.* 14, 206–210. doi: 10.1038/s41561-021-00712-5

Tian, L., Lin, X., Tian, J., Ji, L., Chen, Y., Tran, L.-S. P., et al. (2020). Research advances of beneficial microbiota associated with crop plants. *Int. J. Mol. Sci.* 21:1792. doi: 10.3390/ijms21051792

Trivedi, P., Leach, J. E., Tringe, S. G., Sa, T., and Singh, B. K. (2020). Plant–microbiome interactions: from community assembly to plant health. *Nat. Rev. Microbiol.* 18, 607–621. doi: 10.1038/s41579-020-0412-1

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

METHODS
published: 23 June 2020
doi: 10.3389/fpls.2020.00889

An Optimized *in situ* Quantification Method of Leaf H_2O_2 Unveils Interaction Dynamics of Pathogenic and Beneficial Bacteria in Wheat

*Pablo Carril[1,2], Anabela Bernardes da Silva[2], Rogério Tenreiro[2] and Cristina Cruz[1]**

[1] Plant-Soil Ecology Laboratory, Center for Ecology, Evolution and Environmental Changes (CE3C), Faculty of Sciences, University of Lisbon, Lisbon, Portugal, [2] BioISI – Biosystems and Integrative Sciences Institute, Faculty of Sciences, University of Lisbon, Lisbon, Portugal

Hydrogen peroxide (H_2O_2) functions as an important signaling molecule in plants during biotic interactions. However, the extent to which H_2O_2 accumulates during these interactions and its implications in the development of disease symptoms is unclear. In this work, we provide a step-by-step optimized protocol for *in situ* quantification of relative H_2O_2 concentrations in wheat leaves infected with the pathogenic bacterium *Pseudomonas syringae* pv. *atrofaciens* (*Psa*), either alone or in the presence of the beneficial bacterium *Herbaspirillum seropedicae* (RAM10). This protocol involved the use of 3-3′diaminobenzidine (DAB) staining method combined with image processing to conduct deconvolution and downstream analysis of the digitalized leaf image. The application of a linear regression model allowed to relate the intensity of the pixels resulting from DAB staining with a given concentration of H_2O_2. Decreasing H_2O_2 accumulation patterns were detected at increasing distances from the site of pathogen infection, and H_2O_2 concentrations were different depending on the bacterial combinations tested. Notably, *Psa*-challenged plants in presence of RAM10 accumulated less H_2O_2 in the leaf and showed reduced necrotic symptoms, pointing to a potential role of RAM10 in reducing pathogen-triggered H_2O_2 levels in young wheat plants.

Keywords: hydrogen peroxide (H_2O_2), biotic interactions, image processing, color deconvolution, 3-3′diaminobenzidine (DAB)

OPEN ACCESS

Edited by:
Ioannis Stringlis, Utrecht University, Netherlands

Reviewed by:
Silvia Proietti, University of Tuscia, Italy
Brian H. Kvitko, University of Georgia, United States

****Correspondence:***
Cristina Cruz
ccruz@fc.ul.pt

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

Received: *23 April 2020*
Accepted: *29 May 2020*
Published: *23 June 2020*

Citation:
Carril P, da Silva AB, Tenreiro R and Cruz C (2020) An Optimized in situ Quantification Method of Leaf H_2O_2 Unveils Interaction Dynamics of Pathogenic and Beneficial Bacteria in Wheat. Front. Plant Sci. 11:889. doi: 10.3389/fpls.2020.00889

INTRODUCTION

Accumulation of reactive oxygen species (ROS) is a common plant response to pathogens, having many and often contrasting functions depending on the plant-pathogen system under study (González-Bosch, 2018). Any type of ROS has specific biochemical characteristics and most of them are extremely unstable (Mittler, 2017). However, hydrogen peroxide (H_2O_2)

Abbreviations: AUC, area under curve; DAB, 3,3′-diaminobenzidine; *Psa*, *Pseudomonas syringae* pv. *atrofaciens* strain 2213; RAM10, *Herbaspirillum seropedicae* strain RAM10; ROI, region of interest; SDW, sterile deionized water.

is relatively more stable having a half-life time of more than 1 ms, and is considered the predominant ROS involved in cellular signaling (Černý et al., 2018). ROS regulate numerous immune responses to invading microorganisms, including both the hypersensitive and programmed cell death responses, the cross-linking of cell wall proteins, the deposition of callose or the activation of redox-sensitive genes. Furthermore, ROS, participate in cell-to cell signal transduction to systemic tissues, where localized ROS bursts can induce defenses to prepare (or "prime") plants for future challenges (Torres et al., 2006; Noctor et al., 2018).

Changes in ROS levels also occur during beneficial interactions. Upon contact with plant growth promoting rhizobacteria (PGPR), plant H_2O_2 levels often increase, and H_2O_2 accumulation can be primed for enhanced resistance against pathogens (Ahn et al., 2007). Notably, PGPR can alleviate oxidative stress by modifying the activity of antioxidant enzymes and by modulating H_2O_2 concentrations in the leaf (Lucas et al., 2014; García-Cristobal et al., 2015; Singh and Jha, 2017). As a consequence, PGPR have emerged as a promising alternative to increase oxidative stress tolerance and disease resistance in plants (Islam et al., 2014; Pieterse et al., 2014). Wheat (*Triticum aestivum*) is challenged by several bacterial pathogens, which can cause severe diseases and pests (Valencia-Botín and Cisneros-López, 2012). The pathogen *Pseudomonas syringae* pv. *atrofaciens* (*Psa*) can infect wheat leaves and cause longitudinal brown necrotic-like lesions in the in the site of pathogen entrance resembling those occurring during oxidative stress as a result of high accumulation of ROS in the infection point (Duveiller, 1997).

Despite oxidative stress and pathogen responses are well-studied processes involving H_2O_2 in various ways, it is unclear how H_2O_2 signaling operates in the presence of both pathogenic and beneficial bacteria. This study aims to provide an optimized protocol for *in situ* detection and quantification of relative H_2O_2 concentrations in wheat leaves bacterized with pathogenic and beneficial bacteria, both individually or in combination. This was achieved by combining the 3-3′diaminobenzidine (DAB) staining method previously used for plant material (Wang et al., 2007) and image processing with Fiji/ImageJ software. The combination of these techniques enabled the application of a linear regression model correlating the intensity of the pixels resulting from DAB staining with a given concentration of H_2O_2. This model was suitable for detection and quantification of relative H_2O_2 accumulation in different leaf areas upon infiltration with *Psa* and root-inoculation with the PGPR *Herbaspirillum seropedicae* strain RAM10, either individually or in combination. Furthermore, the area of the lesion caused by *Psa* was measured in presence or absence of previous root-inoculation with RAM10.

This method was suitable to analyze and compare the differential H_2O_2 induction effect between the experimental conditions tested. Our results show that H_2O_2 accumulates at different degrees depending on the leaf region or the different plant-bacteria interactions. Notably, *Psa*-challenged plants in presence of RAM10 showed reduced H_2O_2 accumulation as well as less necrotic symptoms in the leaf, suggesting a PGPR-mediated reduction in oxidative stress levels upon pathogen challenge.

MATERIALS AND METHODS

Bacterial Growth Conditions

Herbaspirillum seropedicae strain RAM10 (RAM10), isolated from *Graminaceae* plants (Olivares et al., 1996), was grown in DYGS medium (composition g L^{-1}: 2.0 glucose; 2.0 malic acid; 2.0 yeast extract; 1.5 peptone; 0.5 K_2HPO_4; 0.5 $MgSO_4$ $7H_2O$; 1.5 L-glutamic acid; pH 6.5) at 28°C and 120 rpm, under dark conditions overnight. Bacterial cells collected by centrifugation (2374 × *g*, 10 min) were washed twice with sterile deionized water (SDW) and resuspended in 1/4 Hoagland solution (Hoagland and Arnon, 1950) to a final OD_{600} = 1 (10^9 CFU/mL) for root inoculation of seedlings.

Pseudomonas syringae pv. *atrofaciens* strain 2213 (*Psa*), isolated from *T. aestivum* plants (McCulloch, 1920) was grown in NB medium (composition g L^{-1}: 10.0 tryptone; 5.0 meat extract; 5.0 NaCl) at 28°C and 120 rpm, under dark conditions overnight. Bacterial cells collected by centrifugation (2374 × *g*, 10 min) were washed twice and resuspended in SDW to a final density of 10^9 CFU/mL for pressure infiltration into the leaves.

Plant Growth Conditions

Wheat (*Triticum aestivum* cultivar "Trigo mole") seeds were surface-sterilized (1.5 min 70% ethanol; 1× wash in SDW; 3 min NaOCl; 10× wash in SDW), soaked for 12 h in SDW and heat-treated (10 min, 50°C; 1 mL/seed). Seeds were then aseptically transferred to square Petri dishes (20 seeds per dish) containing 1.5% water agar and incubated at 30°C in dark conditions for 48 h and kept in a growing chamber with a 16/8 h light/dark photoperiod, temperature 25/20°C and relative humidity (RH) 70%/50%), for 48 h. Four day-old seedlings were then transferred to empty tip boxes containing 225 mL of 1/4 Hoagland solution, with the leaves emerging from the holes of the rack and the Hoagland solution bathing the roots.

Measurement of Leaf Symptoms

Four day-old seedlings were divided in four groups (four tip boxes) composed of 7 seedlings each, with three replicates per group: control, non-bacterized (C); RAM10-inoculated (RAM10); *Psa*-infiltrated (*Psa*) and both RAM10-inoculated and *Psa*-infiltrated (RAM10 + *Psa*). In both RAM10 and RAM10 + *Psa* groups, 25 mL of RAM10 suspension was added to the Hoagland solution bathing the roots to a final density of 10^8 CFU/mL (250 mL final Hoagland volume). Each box was then sealed in plastic gas exchange bags. Four days after RAM10 inoculation, *Psa* and RAM10 + *Psa* groups were pressure infiltrated in the central part of the leaf with 1 mL of *Psa* culture using a needless syringe. Leaves of C and RAM10 groups were pressure infiltrated with 1 mL of SDW. Five days after infiltration, leaves were cut, mounted in transparent plastic slides and pictures were taken. The area of both necrotic and chlorotic symptoms was quantified from the digitalized images using the image processing software Fiji/ImageJ (ImageJ, RRID:SCR_003070).

For this, the affected area was manually defined using both the "polygon" selection tool and the "brush" tool to adjust the size of the selection to the shape of the affected area. The size of the affected area was expressed as mm^2 of both necrotic and chlorotic symptoms.

Construction of DAB-H_2O_2 Calibration Curve: Step-by-Step Protocol

(1) Prepare several H_2O_2 dilutions $\leq$ 47 mM from stock solution at 30% (w/w), that is 9.8 M, with ultra-pure water or sterile deionized water (SDW).

- Note: H_2O_2 Molar mass = 34.01468 g mol^{-1}, density = 1.11 g mL^{-1}.

(2) Measure the absorbance of the H_2O_2 dilutions at 240 nm in a quartz cuvette, after adjusting zero absorbance with the water used for dilutions.

(3) Calculate the exact H_2O_2 concentration of the different solutions using Lambert-Beer law, considering the molar attenuation coefficient or absorptivity (ε) for H_2O_2 at 240 nm equal to 42.3 M^{-1} cm^{-1} and pathlength (l) = 1 cm.

Note: Lambert-Beer law is valid up to an absorbance $\leq$2.

(4) Prepare paper filter disks with an area $\leq$internal area of a 2 mL microtube. Place the disk inside de microtube in horizontal position.

Impregnate all the disk surfaces with adequate volume of H_2O_2 solution, without overloading, and add the DAB solution (1 mg/mL). Make this in triplicate for each [H_2O_2]. Additionally, place 3 disks with DAB only for later background subtraction.

Note: It is important to avoid overloading of paper filter disks, since the precipitated formed by H_2O_2 reaction with DAB may sediment in the bottom of the microtube, underestimating [H_2O_2] and subsequent analysis. We used filter disks with a diameter of 55 mm, 16.6 µL of H_2O_2 solution and 150 µL DAB per disk.

(5) Incubate the microtubes at room temperature and in dark conditions, overnight.

(6) Take out the disks with clean tweezers and mount the disks in a transparent plastic slide.

(7) Digitalize the disks with a scanner and open the image with Fiji/ImageJ software.

- Apply the color deconvolution plugin in order to unmix the color vectors of the digitalized disks. From the resulting panel containing DAB color only, select each disk (region of interest, ROI) using the "oval" selection tool and measure the initial average DAB pixel intensity (*ii*), expressed as:
- $ii = \frac{\sum \text{vpx}}{\text{n°px}}$
- where *ii* is the initial average pixel intensity; $\sum$vpx is the sum of the values of the pixels composing the selected area; and n°px is the number of pixels composing the selected area.

(8) Invert the initial average pixel intensity values by using the formula:

- $iinv = 255 - ii$
- being *iinv* the inverted average pixel intensity. Note that, for 8-bit images, *i* ranges from 0 (zero = deep brown, highest expression), to 255 (total white).

(9) Subtract the background DAB intensity to the *iinv* values, according to the formula:

- $ifinal = iinv - iblank$
- where *ifinal* is the final intensity of the disk and *iblank* is the average intensity value of 3 filters drenched with DAB only.

(10) Construct a calibration curve correlating the *ifinal* values with the corresponding H_2O_2 concentration (µmol H_2O_2/cm^2). Calibration curves with average values (based on triplicates) are presented in **Figure 2A**.

(11) Quantify average pixel intensity also in the complimentary image and represent in a graph the values of average pixel intensity with the corresponding H_2O_2 concentration (µmol H_2O_2/cm^2) (**Supplementary Figures 1A,B**).

Quantification of Relative H_2O_2 Concentration in Leaves Using 3,3-Diaminobenzidine (DAB)

Ten seedlings from each of the four treatments (C, *Psa*, RAM10 + *Psa*) were grown to detect H_2O_2 accumulation in the 1st leaf. Detection of H_2O_2 in leaves was carried out using the 3,3-diaminobenzidine (DAB) staining method already used for barley and wheat plants (Thordal-Christensen et al., 1997; Wang et al., 2007), with slight modifications: 1, 2, 6, 24, and 48 h post-inoculation (hpi), leaves were cut and the cut ends were immersed in 1 mL of a solution containing 1 mg/mL DAB dissolved in HCl-acidified (pH 3.8) SDW. Leaves were incubated in a growing chamber overnight to allow DAB uptake and reaction with H_2O_2. Solutions were kept under dark conditions.

After incubation, leaves were decolorized in boiling (~78°C) 95% ethanol for 20 min and transferred into a solution containing water and 20% glycerol.

Leaf segments were placed in filter paper to remove the excess of glycerol solution, mounted in transparent plastic slides, scanned (Epson XP-235) and the images opened with Fiji/ImageJ software. Initial settings of the software were applied to measure area (mm^2) and mean pixel intensity. Global scale of the image analysis was set as 46.5 pixels = 1 mm. Then, the image was submitted to the plug-in "color deconvolution" using the built-in vector HDAB in order to limit to the DAB dye image. Three different areas (regions of interest, ROIs) were selected for analysis in the DAB only image: from 0 to 4, from 4 to 8 and from 8 to 12 mm from the *Psa* infiltration site. Selection of ROIs was performed using the "rectangle" selection tool. Once the three areas were selected, the "brush" tool was used to adjust the size of the rectangle according to the leaf shape. Then, the area in mm^2 and the mean intensity of DAB was calculated. Intensity values ranged from 0 (deep brown) to 255 (total white).

The average DAB intensity was calculated according to the formula: i_{DAB} = 255-i, being i_{DAB} = final DAB intensity of the

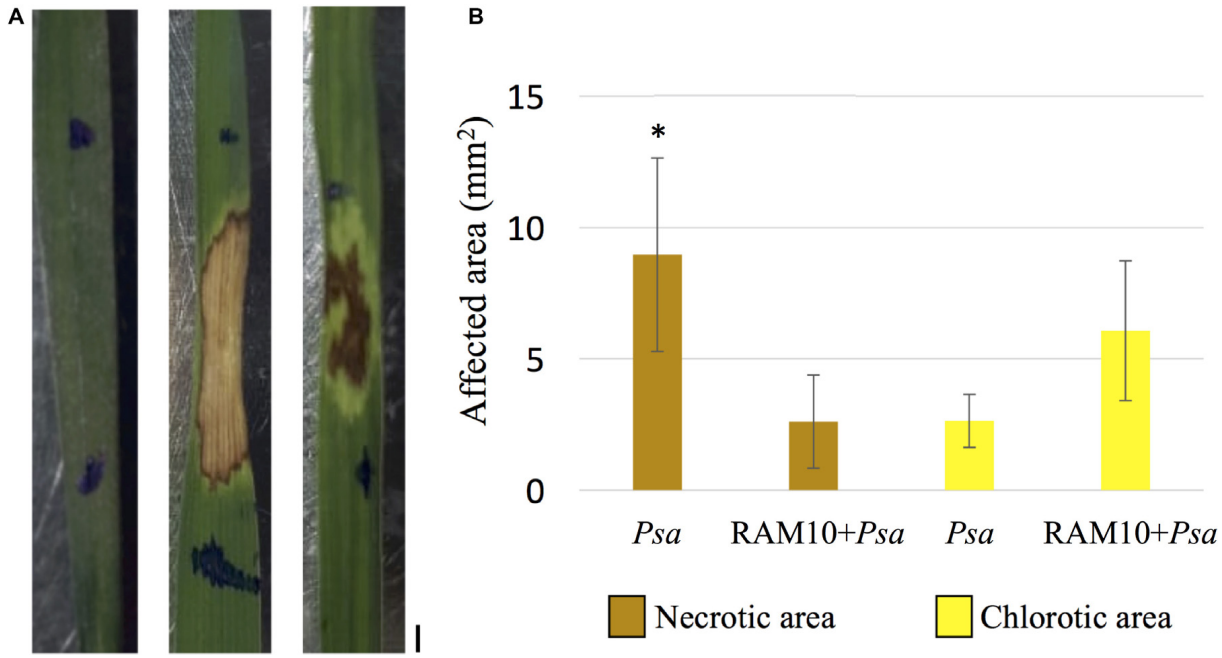

FIGURE 1 | (A) Disease symptoms in *Psa* (center) and RAM10 + *Psa* plants (right) 24 h post-infiltration of *Psa* in the center of the 1st leaf; control plant (C) is shown at left; scale bar = 1 mm. **(B)** Necrotic and chlorotic areas composing the affected area in *Psa* and RAM10 + *Psa* plants (mean ± 95% CI, corrected for multiple comparisons; asterisk indicates significant differences in necrotic area between *Psa* and RAM10 + *Psa* leaves).

ROI compared to average intensity of total white of the ROI, i = the mean DAB intensity of the ROI. In order to subtract the background of the leaf tissue, the average intensity of 20 leaves pressure-infiltrated with water, incubated in water without DAB, and then destained (blank leaves) was measured and subtracted to the i_{DAB} value calculated for each ROI, according to the formula: $f_{DAB} = i_{DAB} - i_{blank}$, being f_{DAB} = final DAB intensity and i_{blank} the average intensity of the blank leaves.

RESULTS

Psa-Triggered Disease Symptoms in Absence or Presence of RAM10

Infiltration with *Psa* in wheat leaves caused the development of dried brown, necrotic lesions surrounded by chlorosis after 24 h both in *Psa* and RAM10 + *Psa* seedlings (**Figure 1A**). Despite both diseased leaf areas being similar in size, the proportion of chlorotic and necrotic symptoms was different between the two treatments (**Figure 1B**). Around 75% of the diseased leaf area in *Psa* leaves was composed of necrotic tissue, which appeared in form of a dried brown area, presumably as a result of the onset a hypersensitive response at the site of pathogen entrance. This necrotic area often reached the borders of the leaf and was surrounded by a thin layer of chlorotic symptoms. Average necrotic area in RAM10 + *Psa* leaves was significantly reduced compared to *Psa* ones, with 30% of the total diseased leaf area showing necrosis, and with chlorosis representing most of the total diseased area.

Regression Model for H_2O_2 Quantification

A linear regression model to quantify H_2O_2 was applied by combining the DAB staining method with image processing using Fiji/ImageJ software. This was done by relating average DAB intensity values to a given amount of H_2O_2. A DAB color gradient was generated by incubating filter disks in separate microtubes containing DAB + increasing H_2O_2 concentrations. Disks ranged from light to dark-brown stained disks, relative to low to high H_2O_2 concentrations (or low to high intensities), respectively (**Figure 2A**). Disks were digitalized and subjected to the color deconvolution plugin, allowing the separation of the initial RGB image into three 8-bit images, which corresponded to the three vector colors composing the image, being: (1) the brown vector, used for subsequent H_2O_2 quantification; (2) the blue vector, not present in the DAB stained disk and (3) a residual channel, also referred to as the complimentary vector, containing the complementary of the other color(s).

Two calibration curves were constructed relating the average DAB intensity values obtained in the DAB only stained section with the corresponding H_2O_2 concentration (μmol H_2O_2/cm^2) applied. The first curve ranged from 0 to 104 DAB intensity values and the second one from 104 to 147 values. Both curves showed a linear relationship between the two variables ($R^2 \geq 0.97$; **Figure 2A**).

Previous authors have stressed the importance of taking into account the intensity values of the pixels in the complimentary image, since they may contain shades of DAB, leading to false positive stain separation (Ruifrok and Johnston, 2001;

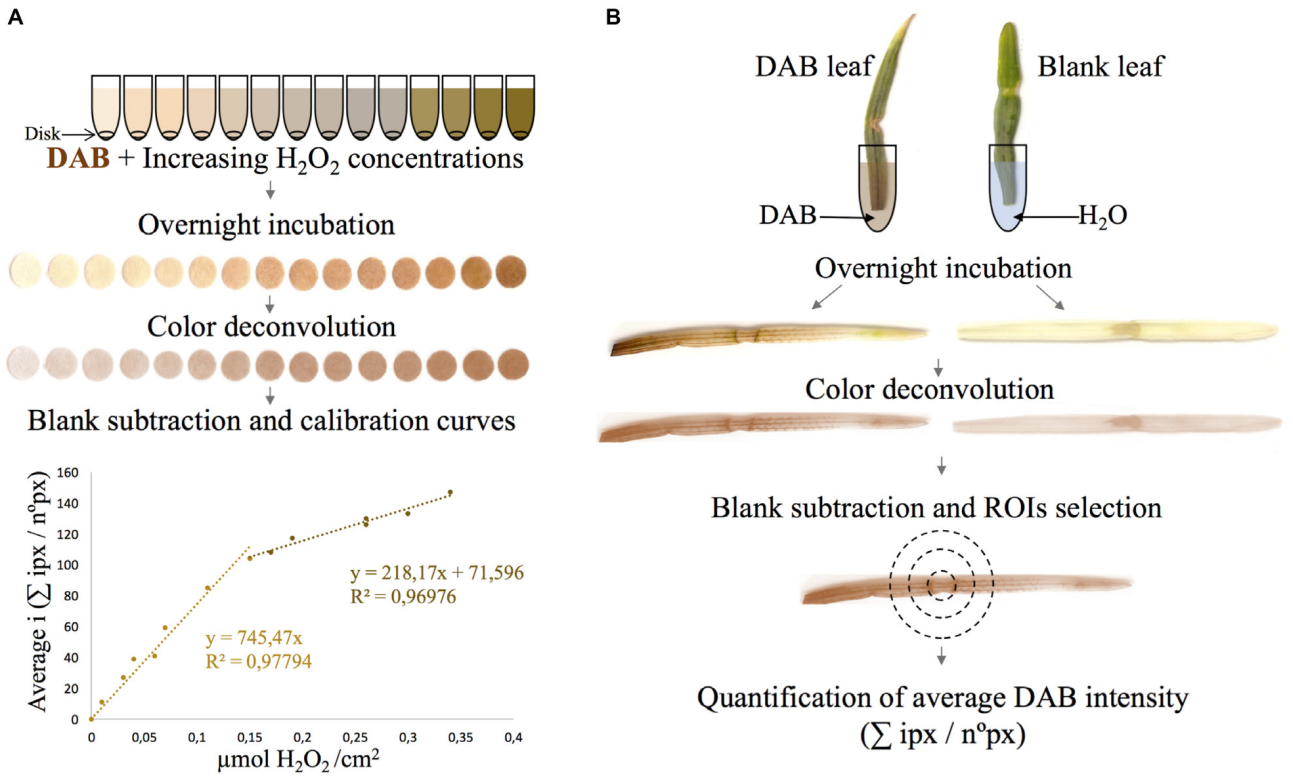

FIGURE 2 | (A) Steps for the generation of H_2O_2-DAB intensity calibration curve. Disks are put in microtubes, drenched at different H_2O_2 concentrations and 1 mg/mL DAB solution is added. After incubation, disks are digitalized and average DAB intensity in the deconvoluted image is determined. Finally, average DAB intensity values are related with those of the several H_2O_2 concentrations (µmol H_2O_2/cm^2). **(B)** Leaf incubation in 1 mg/ml DAB solution and processing of the initial leaf RGB image resulting in the deconvoluted 8-bit DAB image. Leaf background average intensity (blank) was subtracted and ROIs were selected in leaf for quantification of the final DAB average pixel intensity of specific ROIs.

Varghese et al., 2014). In order to correct the intensity values in the DAB only vector, the pixel intensity of the same disks in the complimentary vector was quantified (**Supplementary Figure 1A**), and a curve relating the average green intensity values versus µmol H_2O_2/cm^2 was plotted (**Supplementary Figure 1B**). However, contrary to the DAB only vector, the average intensity values of each disk were not proportional to the applied H_2O_2 concentration. In fact, several filters with higher H_2O_2 concentrations were less stained compared to those drenched with DAB solution only, indicating that average intensity in the complimentary image did not depend on H_2O_2 concentration. Because summing these values would decrease the accuracy of H_2O_2 quantification, average intensity values in the complimentary image were not considered.

Determination of H_2O_2 Accumulation in Leaves

In parallel, wheat leaves were incubated in the DAB solution, digitalized and subjected to the color deconvolution plugin (**Figure 2B**). DAB color was visualized, and different ROIs were selected to measure the average DAB intensity and determine relative H_2O_2 concentration using the calibration curve. Accumulation of H_2O_2 was visualized as dark-brown precipitates resulting from the oxidation of DAB by H_2O_2 present in the leaf. A more intense staining was observed in the vascular beams (**Figure 3**).

Infiltration of *Psa* induced an active production of H_2O_2 in the leaf, both upwards and downwards from the site of infection. Pathogen-infiltrated treatments (*Psa* and RAM10 + *Psa*) showed increased H_2O_2 accumulation in the whole selected leaf area (∑ROIs, **Figure 4**) compared to both C and RAM10 ones. This increase was always more pronounced in *Psa* treatment, compared to which RAM10 + *Psa* plants accumulated significantly less H_2O_2. RAM10 treatments showed similar H_2O_2 accumulation relative to C ones, indicating that root inoculation of RAM10 did not have a significant H_2O_2 induction effect in aboveground tissues.

The analysis of each independent ROI (**Figure 4**) showed that H_2O_2 is produced at different degrees in the leaf, decreasing its accumulation at increasing distances from the infiltration point. After 6 h of *Psa* inoculation, both challenged treatments reached maximum H_2O_2 accumulation in ROI1 (1.39 and 1.18 µmol/cm^2 H_2O_2 in *Psa* and RAM10 + *Psa* plants, respectively), which was covered by dried-brown necrotic tissue 24 hpi. Also, in ROI2 and ROI3, H_2O_2 levels were always higher in *Psa* plants compared to RAM10 + *Psa* ones, showing significant differences at 24 and 48 hpi.

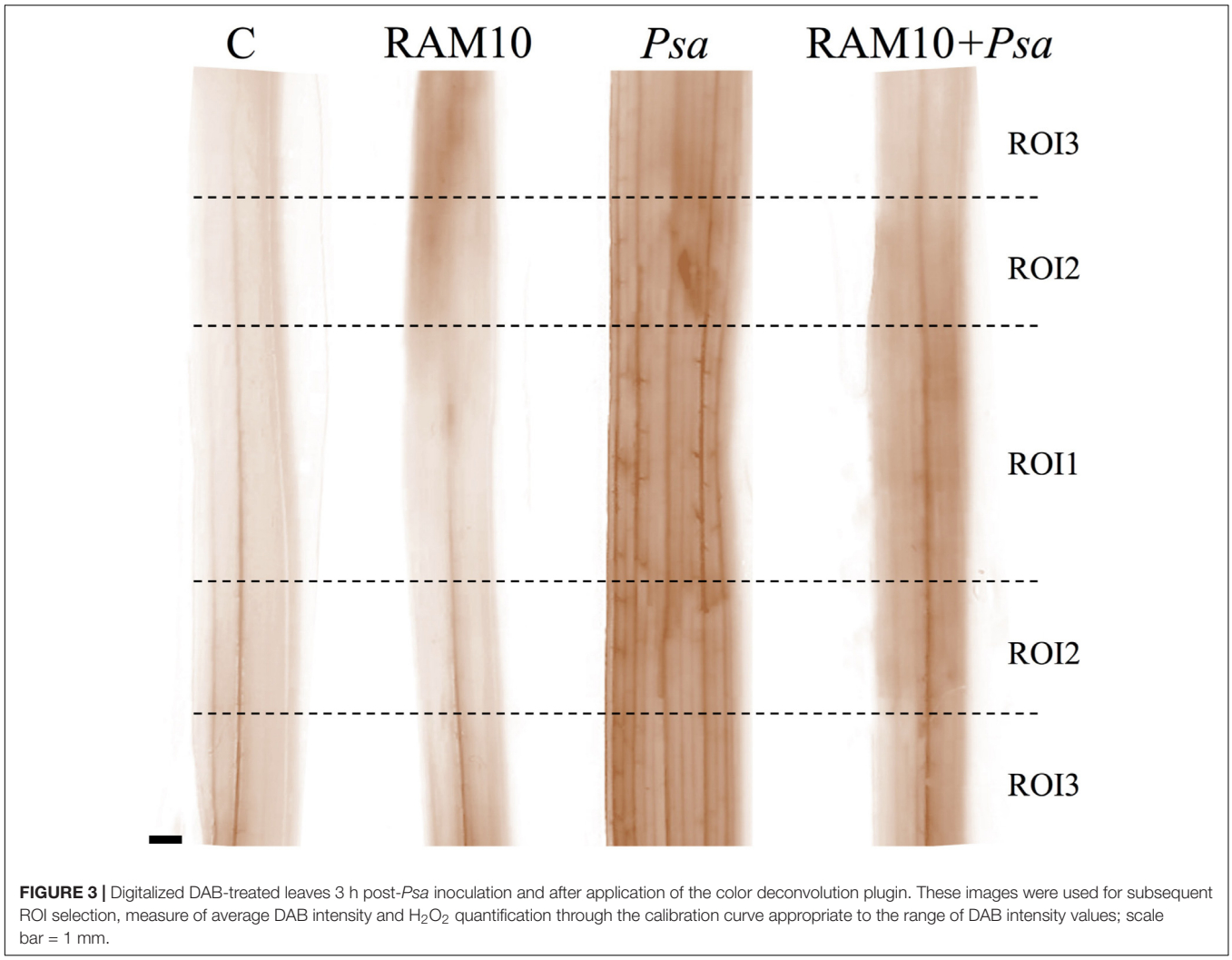

FIGURE 3 | Digitalized DAB-treated leaves 3 h post-*Psa* inoculation and after application of the color deconvolution plugin. These images were used for subsequent ROI selection, measure of average DAB intensity and H_2O_2 quantification through the calibration curve appropriate to the range of DAB intensity values; scale bar = 1 mm.

In order to assess the RAM10-mediated alleviation effect, the area under curve (AUC) of H_2O_2 accumulation was calculated at each time post-infection in the different treatments, and the evolution of cumulative H_2O_2 fold induction triggered by *Psa* was analyzed for the AUC ratios *Psa*/RAM10 and (RAM10 + *Psa*)/RAM10 (i.e., the H_2O_2 induction effect of *Psa* in presence or absence of RAM10 relative to RAM10-only inoculated seedlings; **Figure 5**, bars), as well as for the *Psa*/(RAM10 + *Psa*) AUC ratio (i.e., the H_2O_2 induction effect of *Psa* in absence of RAM10 relative to RAM10 + *Psa* seedlings; **Figure 5**, black lines).

Psa-triggered H_2O_2 induction effect was always reduced when the pathogen was inoculated in presence of RAM10. This RAM10-mediated alleviation of H_2O_2 accumulation was evident in all timepoints, where *Psa* plants accumulated between 1.5 and 2-fold more H_2O_2 relative to RAM10 + *Psa* ones along timepoints. In ROI3, while H_2O_2 accumulation in *Psa* plants increased at 24 and 48 hpi, it remained unchanged in RAM10 + *Psa* ones, highlighting a statistically significant alleviation effect of H_2O_2 accumulation in this region at 24 and 48 hpi (black line, **Figure 5**). These results point that PGPR-inoculated plants may be more sensitive to H_2O_2 signaling, not requiring its massive accumulation upon a challenge.

DISCUSSION

Detection of H_2O_2 by DAB Staining Coupled With Imaging Software Analysis

There exist numerous functions accounted to H_2O_2 in response to pathogens. Despite its crucial role in plant metabolism, there is little consensus regarding the amount of H_2O_2 dynamics in plants challenged with pathogens and pre-treated with PGPR. This is mainly due to both biological variability and technical inaccuracies during its quantification (Queval et al., 2008). Various techniques can quantify H_2O_2 contents in plant tissue extracts, such as those relying on absorbance of oxidized products with altered spectral characteristics (Junglee et al., 2014) or on light emission as fluorescence or luminescence (Miller et al., 2012). Enzymatic assays or the use of metal catalysts of H_2O_2-dependent reactions are also widely used, both of which can

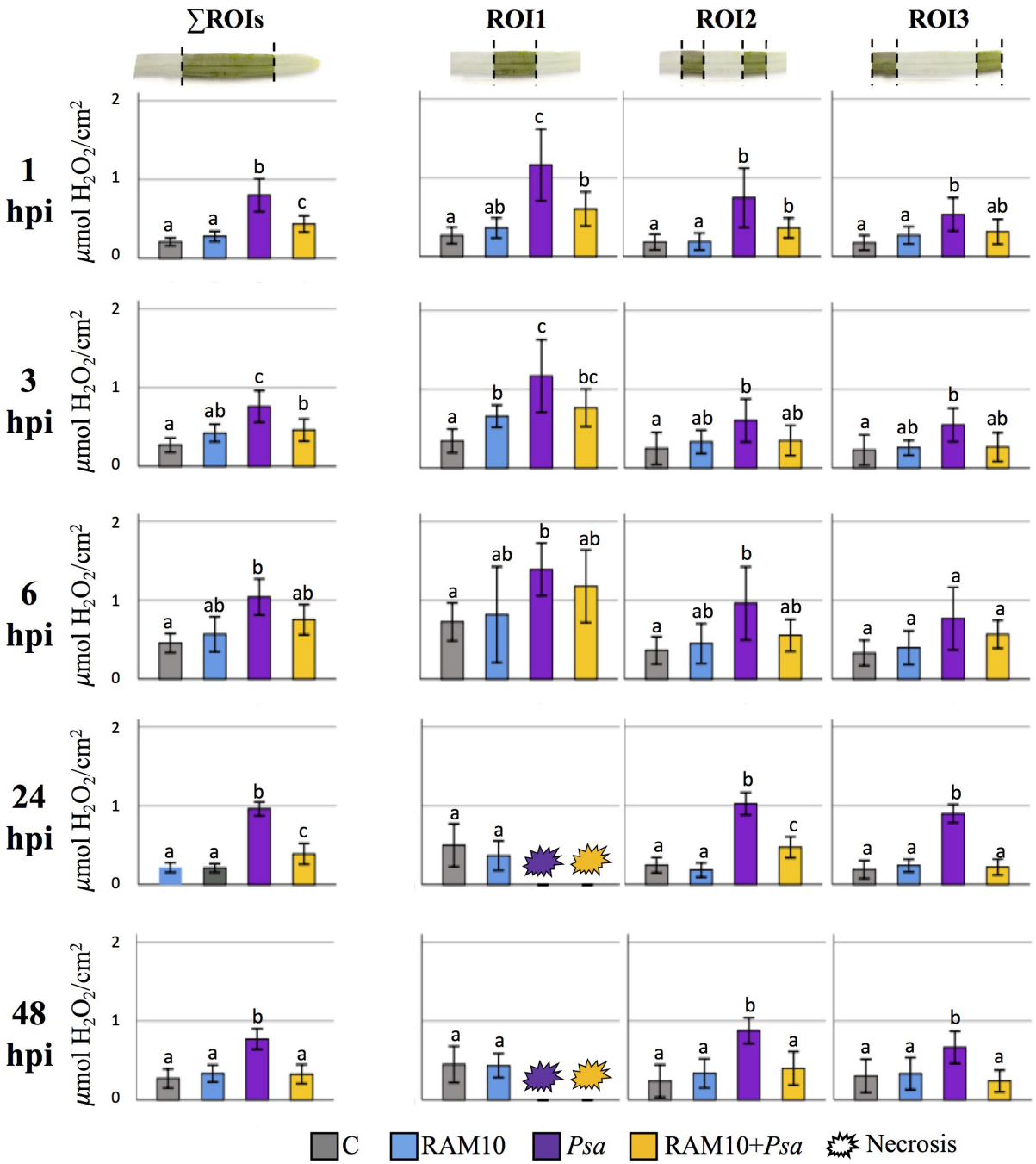

FIGURE 4 | Relative H_2O_2 concentration in the entire selected area (∑ROIs) in C, RAM10, *Psa* and RAM10 + *Psa* plants from 1 to 48 h post *Psa* inoculation. The three graphs next to each ∑ROIs represent H_2O_2 concentration at increasing distances from the inoculation point (ROI1, ROI2 and ROI3) in C, RAM10, *Psa* and RAM10 + *Psa* plants from 1 to 48 h post *Psa* infiltration. Different letters indicate statistically significant differences between groups (mean ± 95% CI, corrected for multiple comparisons; Games-Howell non-parametric test, $p < 0.05$).

overcome problems of H_2O_2 specificity (Queval et al., 2008; Nagaraja et al., 2009). However, some of the challenges that these techniques face include (1) complex interactions between metals or enzymes that may occur during the extraction rather than in the intact tissue, (2) H_2O_2 dilution effects at increasing amount of leaf tissue extracted, which may often mask the real H_2O_2 response (Queval et al., 2008), (3) manipulation effects during sample preparation.

Image analysis for *in situ* quantification of DAB staining has the advantage over biochemical assays that it is non-destructive and minimizes the manipulation of plant material. Furthermore, DAB staining method relies on the activity of peroxidases present

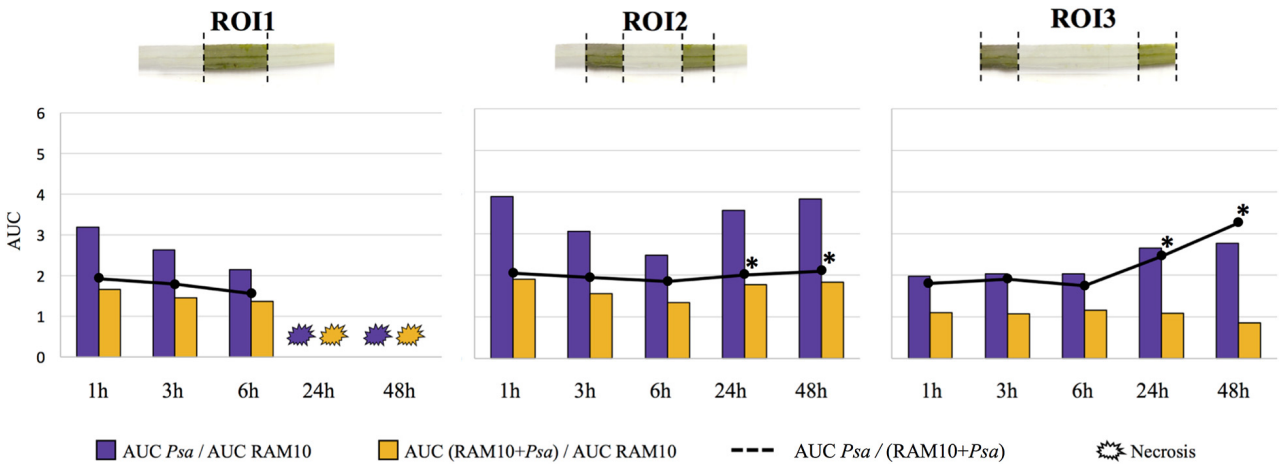

FIGURE 5 | Evolution of cumulative H_2O_2 fold induction triggered by *P. syringae* pv. *atrofaciens* in leaves of *Psa* and RAM10 + *Psa* plants relatively to RAM10 ones (bars), expressed as the ratio between the respective area under curves (AUC) at each time post-infection. The degree of RAM10-mediated alleviation of H_2O_2 accumulation in leaf tissue, calculated as the AUC ratio between *Psa*-triggered H_2O_2 production in *Psa* plants relatively to RAM10 + *Psa* ones, is represented by black lines. Asterisks show statistically significant differences ($p < 0.05$) of the ratio AUC(*Psa*)/AUC(RAM10 + *Psa*).

in the leaf, not requiring addition of external peroxidases, which may be another factor affecting ideal *in vivo* conditions. In this work, we presented an optimized *in situ* detection method using DAB staining coupled to image processing to both detect and quantify H_2O_2 in leaves.

DAB stained leaves can be digitalized, opened in Fiji/ImageJ and subjected to the color deconvolution plugin, an algorithm developed by Ruifrok and Johnston (2001), which unmixes the color information of the digitalized leaf. The color deconvolution plugin has been previously used for human tissue microscopy analysis (Lessey and Savaris, 2013; Varghese et al., 2014). In this work, this method was adapted for young wheat leaves and used to detect the spatial distribution of the DAB intensity at increasing distances from the infection site. As a result, an image with DAB only staining is generated, and the average intensity of its pixels can be quantified after the selection of specific ROIs. Having DAB stained leaves digitalized, other ROIs can be defined anytime.

In previous studies using DAB staining, leaf H_2O_2 content was estimated as the percentage of dark brown DAB pixels relative to the pixels composing the leaf area. In order to express H_2O_2 content in concentration units, these studies relied on parallel spectrophotometric assays for H_2O_2 quantification (Luna et al., 2011; Liu et al., 2014; Wu et al., 2019). However, these analyses require the involvement of different plant samples for both the DAB staining and the spectrophotometry, since the DAB signal could not directly correlate with specific H_2O_2 concentration units. In the present study, the application of a linear model combining average pixel intensity values with known H_2O_2 concentrations allowed to quantify relative H_2O_2 concentrations in the leaf according to its DAB staining intensity values. The generation of this curve avoided the manual setting of a maximum and minimum threshold intensities in the images, which can itself be subjective, leading to misinterpretations in tissue sample scoring (Varghese et al., 2014). It was neither necessary to linearize the intensity values to OD values, as indicated by previous articles (Ruifrok and Johnston, 2001; Varghese et al., 2014), since the values of DAB intensity were linearly related with the concentrations of H_2O_2 and can therefore be used for extrapolating H_2O_2 concentration from DAB intensity values. Furthermore, the H_2O_2 dilution effects were minimized by selecting small (8 mm) ROIs in intact (stressed or non-stressed) leaves after image processing. Manipulation of the plant material was almost inexistent, since the only stress applied to the plant was an initial cut at the base of the cotyledon, immediately prior to DAB incubation. Considering this, specific ROIs were selected excluding both the basal part of the leaf and its apex, which, in few cases, started to senesce (data not shown).

Application of the New Method for Studying Biotic Interactions

Leaves infiltrated with *Psa* accumulated H_2O_2 both locally and at further distances from the infection point, where dark brown DAB precipitates were found to be more intense in the vascular beams. Tissue-specific localization of H_2O_2 associated with vascular tissues has been previously observed (Ślesak et al., 2007) and is in agreement with previous studies which suggest that vascular bundles can synthesize these ROS signals during stress for rapid autopropagation and induction of systemic stress immunity (Libik-Konieczny et al., 2015; Gaupels et al., 2017).

The method proposed in this study was applicable to analyze and compare the differential H_2O_2 induction effect of *Psa* in the presence or absence of the PGPR RAM10. H_2O_2 accumulated at higher levels in the site of pathogen entrance (ROI1), which became necrotic 24 hpi. These observations suggest that induction of hypersensitive cell death by *Psa* in the site of

infection is temporally preceded by H_2O_2 accumulation in the site of pathogen entry, while H_2O_2 accumulation, but not cell death, was induced in the tissue adjacent to the infiltration point. Since ROS participate in cell-to cell signal transduction to systemic tissues, this ROS accumulation in distant parts from the pathogen entry could be a source of signals for establishment of further defenses to prepare (or "prime") plants for future challenges (Noctor et al., 2018).

Interestingly, RAM10-treated plants showed consistently less H_2O_2 accumulation, where the most remarkable alleviation effect was observed 24 and 48 hpi in the most distal area (ROI3), which maintained a low, initial *Psa*-induced H_2O_2 accumulation overtime. These observations suggest that necrosis and H_2O_2 signal propagation occurs in both *Psa* and RAM10 + *Psa* plants, but RAM10-inoculated plants can alleviate the degree of H_2O_2 accumulation upon *Psa* challenge and maintain the basal levels of stress initially triggered by *Psa* in more distal parts of the leaf, without undergoing a further H_2O_2 accumulation. This reduction in ROS levels in challenged plants pre-inoculated with a beneficial microorganism, including bacteria and fungi, has been previously observed. For example, endophytic bacteria-primed *Abelmoschus esculentus* plants expressed lower level of H_2O_2 accumulation upon *Sclerotium rolfsii* challenge, compared to unprimed plants, probably due to the enhanced expression of antioxidant enzymes (Ray et al., 2016). In tobacco leaves, *Bacillus atrophaeus* HAB-5 inhibited ROS accumulation in leaves during TMV infection, which was related with enhanced resistance against the virus and inhibition of cell death (Rajaofera et al., 2020). Besides, the fungus *T. harzianum*–mediated biocontrol may be related to alleviating *Rhizoctonia solani*-induced oxidative stress by reducing the levels of hydroxyl radical, $O_2^{\cdot -}$ and H_2O_2 after pathogen challenge (Singh and Singh, 2011). Furthermore, the pre-treatment of alfalfa plants with lipopolysaccharides from *Sinorhizobium meliloti* suppressed the yeast elicitor-induced oxidative burst reaction (Albus et al., 2001). One hypothesis to explain this alleviation effect is that RAM10 inoculation may induce weak and transient defense-associated changes in ROS signaling upon contact with roots and this signal may be transmitted to aboveground parts of the plant. Contact with RAM10 could pre-activate H_2O_2 signaling in aboveground tissues, avoiding its massive accumulation upon pathogen challenge and increasing plant sensitivity to H_2O_2 signaling. Contrarily, without being alerted by a previous contact with RAM10, pathogen infiltration in *Psa* plants would lead to a massive, uncontrolled accumulation of H_2O_2, resulting in cellular damage and increased necrotic area (Van Breusegem and Dat, 2006). In relation to this, RAM10 could prime plants to increase antioxidant enzyme activity/production upon a future infection.

CONCLUSION

In this work, we report for the first time an integrated protocol that simultaneously allows to detect DAB distribution, to quantify amount of DAB signal in different leaf regions and to relate this signal to a given concentration of H_2O_2. The method is non-expensive and applicable to analyze and compare the differential H_2O_2 induction effects of wheat plants bacterized with both pathogenic and beneficial bacteria.

This methodology allowed to show that the pathogen *Psa* clearly increased H_2O_2 accumulation in infiltrated leaves. On the contrary, both H_2O_2 levels and disease symptoms induced by this pathogen decreased in presence of RAM10, suggesting a role for this PGPR in the alleviation of pathogen-induced oxidative stress and the progression of necrotic symptoms.

DATA AVAILABILITY STATEMENT

The raw data supporting the conclusions of this article will be made available by the authors, without undue reservation.

AUTHOR CONTRIBUTIONS

PC and AS designed the experiments and analyzed the data in the H_2O_2 quantification part. PC, RT, and CC designed the experiments and analyzed the data concerning the biotic interaction part. PC performed the experiments. All researchers contributed to the research and approved the final version of the manuscript.

ACKNOWLEDGMENTS

We thank FCT/MCTES for the scholarship PD/BD135249/2017 to PC and the financial support to cE3c (Research Unit grant number UIDB/00329/2020) and BioISI (Research Unit grant numbers UIDB/04046/2020 and UIDP/04046/2020).

SUPPLEMENTARY MATERIAL

The Supplementary Material for this article can be found online at: https://www.frontiersin.org/articles/10.3389/fpls.2020.00889/full#supplementary-material

FIGURE S1 | (A) Values of H_2O_2 concentration in the disks quantified in the complementary image, their average pixel intensity values (averages of three disk replicates) and aspect of the filter disks. **(B)** Relationship between average pixel intensity detected in the complimentary image and (±STDEV) and the amount of H_2O_2 per cm^2 of disk.

REFERENCES

Ahn, I. P., Lee, S. W., and Suh, S. C. (2007). Rhizobacteria-induced priming in *Arabidopsis* is dependent on ethylene, jasmonic acid, and NPR1. *Mol. Plant Microbe Interact.* 20, 759–768. doi: 10.1094/MPMI-20-7-0759

Albus, U., Baier, R., Holst, O., Pühler, A., and Niehaus, K. (2001). Suppression of an elicitor-induced oxidative burst reaction in Medicago sativa cell cultures by

Sinorhizobium meliloti lipopolysaccharides. *New Phytol.* 597:606. doi: 10.1046/j.0028-646x.2001.00214.x

Černý, M., Habánová, H., Berka, M., Luklová, M., and Brzobohatý, B. (2018). Hydrogen peroxide: its role in plant biology and crosstalk with signalling networks. *Int. J Mol. Sci.* 19:2812. doi: 10.3390/ijms19092812

Duveiller, E. (1997). *The Bacterial Diseases of Wheat: Concepts and Methods of Disease Management*. Mexico: CIMMYT.

García-Cristobal, J., García-Villaraco, A., Ramos, B., Gutierrez-Mañero, J., and Lucas, J. A. (2015). Priming of pathogenesis related-proteins and enzymes related to oxidative stress by plant growth promoting rhizobacteria on rice plants upon abiotic and biotic stress challenge. *J. Plant Physiol.* 188, 72–79. doi: 10.1016/j.jplph.2015.09.011

Gaupels, F., Durner, J., and Kogel, K. H. (2017). Production, amplification and systemic propagation of redox messengers in plants? The phloem can do it all! *New Phytol.* 214, 554–560. doi: 10.1111/nph.14399

González-Bosch, C. (2018). Free Radical Biology and Medicine Priming plant resistance by activation of redox-sensitive genes. *Free Radic. Biol. Med.* 122, 171–180. doi: 10.1016/j.freeradbiomed.2017.12.028

Hoagland, D., and Arnon, D. (1950). *The Water-Culture Method for Growing Plants Without Soil*. Berkeley, CA: University of California.

Islam, F., Yasmeen, T., Ali, Q., Ali, S., Arif, M. S., Hussain, S., et al. (2014). Influence of *Pseudomonas aeruginosa* as PGPR on oxidative stress tolerance in wheat under Zn stress. *Ecotoxicol. Environ. Saf.* 104, 285–293. doi: 10.1016/j.ecoenv.2014.03.008

Junglee, S., Urban, L., Sallanon, H., and Lopez-Lauri, F. (2014). Optimized assay for hydrogen peroxide determination in plant tissue using potassium iodide. *Am. J. Anal. Chem.* 5, 730–736. doi: 10.4236/ajac.2014.511081

Lessey, B. A., and Savaris, R. F. (2013). Comparison of HSCORE assessment of endometrial (3 integrin subunit expression with digital HSCORE using computerized image analysis (ImageJ). *Anal. Quant. Cytopathol. Histpathol.* 35, 210–216.

Libik-Konieczny, M., Kozieradzka-Kiszkurno, M., Desel, C., Michalec-Warzecha, Ż, Miszalski, Z., and Konieczny, R. (2015). The localization of NADPH oxidase and reactive oxygen species in in vitro-cultured Mesembryanthemum crystallinum L. hypocotyls discloses their differing roles in rhizogenesis. *Protoplasma* 252, 477–487. doi: 10.1007/s00709-014-0692-2

Liu, Y. H., Offler, C. E., and Ruan, Y. L. (2014). A simple, rapid, and reliable protocol to localize hydrogen peroxide in large plant organs by DAB-mediated tissue printing. *Front. Plant Sci.* 5:745. doi: 10.3389/fpls.2014.00745

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

Luna, E., Pastor, V., Robert, J., Flors, V., Mauch-Mani, B., and Ton, J. (2011). Callose deposition: a multifaceted plant defense response. *Mol. Plant Microbe Interact.* 24, 183–193. doi: 10.1094/MPMI-07-10-0149

McCulloch, L. (1920). Basal glumerot of wheat. *J. Agric. Res.* 18, 543–549.

Miller, G., Schlauch, K., Tam, R., Cortes, D., Torres, M. A., Shulaev, V., et al. (2012). The plant NADPH oxidase RBOHD mediates rapid systemic signaling in response to diverse stimuli. *Sci. Signal.* 2:ra45. doi: 10.1126/scisignal.2000448

Mittler, R. (2017). ROS are good. *Trends Plant Sci.* 22, 11–19. doi: 10.1016/j.tplants.2016.08.002

Nagaraja, P., Shivakumar, A., and Shrestha, A. K. (2009). Quantification of hydrogen peroxide and glucose using 3-methyl-2-benzothiazolinonehydrazone hydrochloride with 10, 11-dihydro-5 H-benz (b, f) azepine as chromogenic probe. *Anal. Biochem.* 395, 231–236. doi: 10.1016/j.ab.2009.07.053

Noctor, G., Reichheld, J. P., and Foyer, C. H. (2018). ROS-related redox regulation and signaling in plants. *Semin. Cell Dev. Biol.* 80, 3–12. doi: 10.1016/j.semcdb.2017.07.013

Olivares, F. L., Baldani, V. L., Reis, V. M., Baldani, J. I, and Döbereiner, J. (1996). Occurrence of the endophytic diazotrophs Herbaspirillum spp. in roots, stems, and leaves, predominantly of Gramineae. *Biol. Fertil. Soils* 21, 197–200. doi: 10.1007/BF00335935

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

Queval, G., Hager, J., Gakière, B., and Noctor, G. (2008). Why are literature data for H2O2 contents so variable? A discussion of potential difficulties in the quantitative assay of leaf extracts. *J. Exp. Bot.* 59, 135–146. doi: 10.1093/jxb/erm193

Rajaofera, M. J. N., Wang, Y., Jatoi, Z. A., Jin, P., Cui, H., Lin, C., et al. (2020). Bacillus atrophaeus HAB-5 secretion metabolites preventing occurrence of systemic diseases in tobacco plant. *Eur. J. Plant Pathol.* 156, 159–172. doi: 10.1007/s10658-019-01873-1

Ray, S., Singh, V., Singh, S., Sarma, B. K., and Singh, H. B. (2016). Plant Physiology and Biochemistry Biochemical and histochemical analyses revealing endophytic *Alcaligenes faecalis* mediated suppression of oxidative stress in *Abelmoschus esculentus* challenged with *Sclerotium rolfsii*. *Plant Physiol. Biochem.* 109, 430–441. doi: 10.1016/j.plaphy.2016.10.019

Ruifrok, A. C., and Johnston, D. A. (2001). Quantification of histochemical staining by color deconvolution. *Anal. Quant. Cytol. Histol.* 23, 291–299.

Singh, B. N., and Singh, A. (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, 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

Ślesak, I., Libik, M., Karpinska, B., Karpinski, S., and Miszalski, Z. (2007). The role of hydrogen peroxide in regulation of plant metabolism and cellular signalling in response to environmental stresses. *Acta Biochim. Polonica* 54, 39–50. doi: 10.18388/abp.2007_3267

Thordal-Christensen, H., Zhang, Z., Wei, Y., and Collinge, D. B. (1997). Subcellular localization of H2O2 in plants. *H*2O2 accumulation in papillae and hypersensitive response during the barley—powdery mildew interaction. *Plant J.* 11, 1187–1194. doi: 10.1046/j.1365-313X.1997.11061187.x

Torres, M. A., Jones, J. D. G., and Dangl, J. L. (2006). Reactive Oxygen Species Signaling in Response to Pathogens. *Plant Physiol.* 141, 373–378. doi: 10.1104/pp.106.079467

Valencia-Botín, A. J., and Cisneros-López, M. E. (2012). A review of the studies and interactions of *Pseudomonas syringae* pathovars on wheat. *Int. J. Agron.* 2012:692350. doi: 10.1155/2012/692350

Van Breusegem, F., and Dat, J. F. (2006). Reactive oxygen species in plant cell death. *Plant Physiol.* 141, 384–390. doi: 10.1104/pp.106.078295

Varghese, F., Bukhari, A. B., Malhotra, R., and De, A. (2014). IHC Profiler: an open source plugin for the quantitative evaluation and automated scoring of immunohistochemistry images of human tissue samples. *PLoS One* 9:e96801. doi: 10.1371/journal.pone.0096801

Wang, C. F., Huang, L. L., Buchenauer, H., Han, Q. M., Zhang, H. C., and Kang, Z. S. (2007). Histochemical studies on the accumulation of reactive oxygen species (O2- and H2O2) in the incompatible and compatible interaction of wheat—*Puccinia striiformis* f. sp. tritici. *Physiol. Mol. Plant Pathol.* 71, 230–239. doi: 10.1016/j.pmpp.2008.02.006

Wu, T.-M., Huang, J.-Z., Oung, H.-M., Hsu, Y.-T., Tsai, Y.-C., and Hong, C.-Y. (2019). "H2O2 –based method for rapid detection of transgene-free rice plants from segregating CRISPR / Cas9 genome-edited progenies. *Int. J. Mol. Sci.* 20:3885. doi: 10.3390/ijms20163885

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 Microbiology

ORIGINAL RESEARCH
published: 07 July 2020
doi: 10.3389/fmicb.2020.01462

The Rhizosphere Microbiome of *Mikania micrantha* Provides Insight Into Adaptation and Invasion

Lijuan Yin[1,2†], Bo Liu[1†], Hengchao Wang[1], Yan Zhang[1], Sen Wang[1], Fan Jiang[1], Yuwei Ren[1], Hangwei Liu[1], Conghui Liu[1], Fanghao Wan[1], Haihong Wang[2], Wanqiang Qian[1] and Wei Fan[1*]*

[1] Guangdong Laboratory for Lingnan Modern Agriculture (Shenzhen Branch), Agricultural Genomics Institute at Shenzhen, Chinese Academy of Agricultural Sciences, Shenzhen, China, [2] Key Laboratory of Protein Function and Regulation in Agricultural Organisms of Guangdong Province, College of Life Science, South China Agricultural University, Guangzhou, China

OPEN ACCESS

Edited by:
Paulo José Pereira Lima Teixeira, University of São Paulo, Brazil

Reviewed by:
Rensen Zeng, Fujian Agriculture and Forestry University, China
Connor Fitzpatrick, University of Toronto, Canada

***Correspondence:**
Wanqiang Qian
qianwanqiang@caas.cn
Wei Fan
fanwei@caas.cn

† These authors have contributed equally to this work

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

Received: *16 March 2020*
Accepted: *04 June 2020*
Published: *07 July 2020*

Citation:
Yin L, Liu B, Wang H, Zhang Y, Wang S, Jiang F, Ren Y, Liu H, Liu C, Wan F, Wang H, Qian W and Fan W (2020) The Rhizosphere Microbiome of Mikania micrantha Provides Insight Into Adaptation and Invasion. Front. Microbiol. 11:1462. doi: 10.3389/fmicb.2020.01462

Mikania micrantha is a noxious invasive plant causing enormous economic losses and ecological damage. Soil microbiome plays an important role in the invasion process of *M. micrantha*, while little is known about its rhizosphere microbiome composition and function. In this study, we identified the distinct rhizosphere microbial communities of *M. micrantha*, by comparing them with those of two coexisting native plants (*Polygonum chinense* and *Paederia scandens*) and the bulk soils, using metagenomics data from field sampling and pot experiment. As a result, the enrichment of phosphorus-solubilizing bacteria *Pseudomonas* and *Enterobacter* was consistent with the increased soil available phosphorus in *M. micrantha* rhizosphere. Furthermore, the pathogens of *Fusarium oxysporum* and *Ralstonia solanacearum* and pathogenic genes of type III secretion system (T3SS) were observed to be less abundant in *M. micrantha* rhizosphere, which might be attributed to the enrichment of biocontrol bacteria *Catenulispora*, *Pseudomonas*, and *Candidatus Entotheonella* and polyketide synthase (PKS) genes involved in synthesizing antibiotics and polyketides to inhibit pathogens. These findings collectively suggested that the enrichment of microbes involved in nutrient acquisition and pathogen suppression in the rhizosphere of *M. micrantha* largely enhances its adaptation and invasion to various environments.

Keywords: Rizosphere bacteria, *Mikania micrantha*, beneficial microbes, nutrition, pathogen

INTRODUCTION

The rhizosphere is the interface where the complex interactions among soil, microbes, and the host plant are maintained (Philippot et al., 2013). Plants selectively harbor specific microbes through root exudates that contain carbohydrates, amino acids, and organic acid ions, which act as carbon source and nutrients for microbial growth (Reinhold-Hurek et al., 2015). Rhizosphere microbes play pivotal roles in plant growth, nutrient uptakes, and disease suppression (Bulgarelli et al., 2015; Edwards et al., 2015).

Invasive alien species (IAS) could reduce the richness and abundance of native species in the invaded regions, or even dramatically change the local ecological system (Pyšek and Richardson, 2008). Compared with native plants, invasive plants generally performed higher value of inherent traits on physiology, leaf-area allocation, shoot allocation, and growth rate (Van Kleunen et al., 2010) and also exhibit higher abilities to capture and utilize resources at both above- and below-ground processes, such as photosynthesis and nutrition uptake (Van Der Heijden et al., 2008). The soil microbes play important roles in the establishment of invasive plants and might also act as drivers of plant invasions (Dawson and Schrama, 2016). Previous studies showed that invasive plants can recruit different soil microbes to promote their growth over native plants (Reinhart et al., 2003). The indigenous soil microbial communities are altered due to the exotic invading plants (Kourtev et al., 2002). For example, the *Alnus trabeculosa* increased the soil bacterial diversity in the invaded regions (Xueping et al., 2016). Another invasive plant *Centaurea maculosa* enhanced its competitive ability through enriching mycorrhizal fungi that changes soil nutrient availability (Marler et al., 1999). In addition, other studies also showed that many invasive plants have fewer pathogens in rhizosphere than native plants do, escaping from pathogenic agents in soil (Mitchell and Power, 2003). *C. maculosa* could reduce local soil pathogens in invaded regions, therefore investing less in unused defense and more into growth to increase competitiveness against natives (Callaway et al., 2004). An invasive plant may influence soil nutrient content through the soil microbial communities (Piper et al., 2015; Zhao et al., 2019). For example, the invasive tree staghorn sumac changed the structure of soil N2-fixing bacterial communities to enhance soil N availability (Wu et al., 2019). *Solidago gigantea* enhances phosphorus (P) turnover rates in soil (Chapuis-Lardy et al., 2006), and *C. maculosa* increases available P in soil (Thorpe et al., 2006). Invasive plants increased the availability of vital nutrients, thus gaining a competitive advantage, which might be an important contributor to invasion success (Castro-Díez et al., 2014).

Mikania micrantha (Asteraceae family), an extremely fast-growing vine, is one of the top 100 worst IAS in the world (Lowe et al., 2000), causing severe substantial damages to natural ecosystems (Day et al., 2016) and economic losses (Macanawai et al., 2012). Several mechanisms have been proposed to explain the success of *M. micrantha* invasion, such as rapid growth caused by high regeneration capacity of each vine node (Li X. et al., 2013) and extraordinary biological characteristics including high seed production and germination (Hu and But, 1994), the strong allelopathic effects on other native plant and soil microbes (Chen et al., 2009), and high nutrient (NPK) turnover rates in soil (Sun et al., 2019; Liu et al., 2020). Recently, we have published the genome of *M. micrantha*, as well as its rhizosphere metagenome, and also found out that the rhizosphere microbes of *M. micrantha* could increase the bioavailable nitrogen content to speed up the nitrogen cycle (Liu et al., 2020), which might contribute to its rapid growth as well as invasion. Enhancing the availability of soil P is also one of the major factors for the success of plant invasion. In recent studies on P acquisition of *M. micrantha*, it was shown that the contents of soil available P and plant tissues of *M. micrantha* were significantly higher than that of native plants. However, very few studies have explained the component and mechanism of P-solubilizing bacteria. We hypothesized that the enrichment of P-solubilizing microorganisms will contribute to the available P in *M. micrantha* rhizosphere. Except for the nutrient acquisition mechanism of plant invasion, the well-known enemy release mechanism that escapes from its natural enemies in its native ranges was also confirmed in other invasive plants. Some invasive plants were not only associated with higher ability of nutrients uptake but also harbored few known pathogens that were more abundant in the rhizosphere of native plants or accumulated pathogens in the soil that are harmful to natives. The research on the invasion mechanism of *M. micrantha* mainly focuses on inherent superiority, allelopathy, and nutrient acquisition, and there is a paucity of research on the influence of pathogenic microorganisms in the *M. micrantha* rhizosphere. We hypothesized that few known pathogens were harbored in *M. micrantha* rhizosphere because of the allelopathy of its leaves and roots. In this study, using these metagenomic data, we investigated the phosphorus solubilizing bacteria and pathogens in the rhizosphere of *M. micrantha*, to better understand the role of the rhizosphere microbiome in *M. micrantha* invasion.

MATERIALS AND METHODS

Experimental Design and Sampling Collection

In order to test the contribution of rhizosphere bacteria to *M. micrantha* invasion, we conducted a pot experiment with *M. micrantha* and its two neighboring native species, namely, *Polygonum chinense* and *Paederia scandens*. These two plants are chosen as the control species because based on the investigation from the field sample, not only are these frequently and stably present in the invasive community of *M. micrantha*, but also the reproduction strategies of these two plants are very similar to those of *M. micrantha* (Sun et al., 2019). The seeds of three plants were germinated and grew to about 10 cm for transplanting. Seedlings of three plants, respectively, planted in the pot (20 cm diameter) filled with natural field soil were collected from the non-invasive area near the invader *M. micrantha* monoculture, which is located in the dry riverbed of Liuxi River, Guangzhou City, Guangdong Province, China. Four treatments (three plants plus a blank control) were replicated six times (two plants per pot with 7 kg fresh soil) and put in a greenhouse.

Three months later, we randomly selected five replicates of each treatment and the rhizosphere soil of three plants and control soil were collected. Plants were removed carefully and shaken lightly; then, the soil remaining attached to the root surface was collected with sterile water. The separated soil solution was centrifuged at 8000 r/min for 10 min to collect soil samples. The collected soils were stored at −80°C until use for microbial community analysis. *M. micrantha* is an ecologically harmful weed in the natural environment. We chose the natural field of *M. micrantha* monoculture with the dominant two coexisting native plants (*P. chinense* and *P. scandens*) in the dry

riverbed of Liuxi River in Guangzhou City. We separated five (5 m by 5 m) sampling plots by more than 200 m and used the same method of pot experiment to collect 15 rhizosphere samples of three plants and five control samples, which is in the uninvaded area near *M. micrantha* monoculture by more than 500 m, for a total of 20 samples.

DNA Extraction and Sequencing

A combination of bacteria cell lysis steps was applied before DNA extraction. The soil microbial cells were subjected to six freeze–thaw cycles (alternating vortex for 5 min, then liquid nitrogen for 5 min, and incubation at 65°C for 5 min). Next, DNA was extracted from all samples using the PowerSoil DNA isolation kit following the manufacturer's protocol (MO BIO Laboratories, QIAGEN Inc., United States). The DNA quality and quantity were checked by the Nanodrop and Qubit device, and the DNA quantity of each sample was at least 1 μg. Then, DNA fragments (200–400 bp) were processed by ultrasonic instrument (Thermo Fisher Scientific, Covaris S220). The library was constructed using TruSeq DNA PCR-Free Library Prep Kit as per standard protocol (Illumina, United States) and then sequencing was performed on Illumina HiSeq 2500 with each sample having about 10 Gb sequencing data.

Metagenomic Analyses

The raw reads were cleaned by removing adaptor sequences, low-quality sequences, host sequences, and unpaired reads by in-house software clean_adapter, clean_lowqual, and filter_unpaired_reads.pl[1], resulting in a clean and high-quality reads data with average error rate < 0.001. Then, the clean reads from each sample and pooled for four groups (*M. micrantha*, *P. chinense*, *P. scandens*, and control) were assembled by Megahit (v1.1.3). After filtering the contig length less than 500 bp, gene prediction was performed using MetaProdigal (v2.6.3), and then we filtered out the gene models with cds length less than 102 bp. The protein models of each sample and each group were also performed using MetaProdigal (v2.6.3). The non-redundant gene catalog was obtained using the genes predicted from each sample and each group by cd-hit-est (v4.6.6) with the criteria of identity > 95%, and overlap > 90% (parameter "−c 0.95 −n 10 −G 0 −aS 0.9"). The non-redundant protein catalog was obtained from the combination of protein files of each sample and each group by in-house software fishInWinter.pl[2].

To generate the taxonomic information, the non-redundant protein sequences were aligned against the NCBI-NR database using DIAMOND (v0.8.28.90) software with the parameter "blastp –evalue 10 –max-target-seqs 250" (Buchfink et al., 2015). CARMA3 software (parameter "carma –classify-blast –type p –database p") was used to assign the taxonomic annotation of the unigenes (Gerlach and Stoye, 2011). Thus, we obtained the non-redundant genes and their corresponding species classification. To obtain functional information for the gene set, the non-redundant protein sequences were searched (*E* value < 1e-5) against the KEGG protein database (release 79) using DIAMOND software (Kanehisa et al., 2004). To calculate the relative gene abundance, the clean reads from each sample were aligned against the non-redundant gene catalog by BWA-MEM (alignment length ≥ 50 bp and identity > 95%) (Li and Durbin, 2009). The alignments were parsed to produce the reads count abundance (Huang et al., 2018). Based on the taxonomic assignments using CARMA3, the relative abundance of each phylum, genus, species, and KO was calculated by summing the abundances of corresponding genes belonging to each category per sample. Similarly, the relative abundance profile of genes was also summarized into KEGG functional profiles for the functional analysis.

Functional Bacteria and Genes Collection

The bacteria and genes involved in soil microbial P-solubilizing and mineralization, pathogen, and defense were searched based on previous publications and are shown in **Supplementary Tables S1–S6** (Weller et al., 2002; Garbeva et al., 2004; Beth Mudgett, 2005; Raaijmakers and Mazzola, 2012; Sharma et al., 2013; Raj et al., 2014; Alori et al., 2017; Han et al., 2018; Dai et al., 2019). The names, KOs, and functions of the genes associated with P solubilizing and mineralization, type III secretion/effector systems, and polyketide synthase (PKS) are shown in **Supplementary Tables S2, S4, S6**, respectively.

Microbial Composition Analysis

At the gene level, Shannon index was used to analyze microbial alpha diversity using the non-redundant genes of individual samples. The overall differences in the bacterial community structures were calculated by non-metric multidimensional scaling (NMDS) using non-redundant genes of individual samples based on Bray–Curtis dissimilarity values and implemented in in R package "Phyloseq."

Statistical Analysis

Based on the relative abundance profiles at the phyla or genera level, the significantly differential abundances in the control soil and rhizospheres of *M. micrantha*, *P. chinense*, and *P. scandens* were determined using Kruskal–Wallis test with Dunn's multiple comparison (BH methods for multiple tests adjustment). The relative abundance of microbial species and functional genes involved in P solubilization, pathogens, and defense in the control soil and rhizospheres of three plants is compared using Kruskal–Wallis test with Dunn's multiple comparison (BH methods for multiple tests adjustment).

RESULTS

Microbial Structure of the Rhizosphere Microbiome

Using the metagenomic data and non-redundant gene set of *M. micrantha* genome project, deposited in NCBI (SRR8936416–SRR8936475) and AGIS ftp-site[3], we investigated the microbial

[1]https://github.com/fanagislab/DBG_assembly/tree/master/clean_illumina

[2]https://github.com/fanagislab/bioinfo_versatiles/blob/master/fishInWinter.pl

[3]ftp://ftp.agis.org.cn/Mikania_micrantha/

structure of the rhizosphere of *M. micrantha* and two native plants (*P. chinense* and *P. scandens*). The microbial alpha diversity (Shannon index) at the gene level showed no significant difference between the control soil (bulk soil) and rhizospheres ($P > 0.05$) (**Figure 1A**). However, at the gene level, the NMDS analysis revealed the distinct microbial community differences among the rhizospheres of *M. micrantha*, two native plants, and control soil at both pot experiment and invaded site (**Figure 1B**). Moreover, the NMDS plots showed that there was a clear separation between the pot experiment and field invaded site, indicating that rhizosphere microbial community was largely influenced by environmental conditions (**Figure 1C**). The dominant prokaryotic phyla found in the control and rhizosphere community included *Proteobacteria*, *Actinobacteria*, *Acidobacteria*, *Planctomycetes*, and *Chloroflexi* (**Figure 1D**), which was consistent with previous studies (Lu-Irving et al., 2019). The community differences between the control soil and rhizospheres of *M. micrantha* and native plants were also explored. *Proteobacteria* and *Actinobacteria* occupied higher percentages than in the control soil, whereas *Acidobacteria* has lower percentages ($P < 0.05$, Dunn test) in the rhizospheres (**Figure 1D**). This suggests that some bacteria from bulk soil are selected to inhabit in the rhizospheres.

Distinctive Enrichment of Plant Microbes

The microbial compositions of the rhizosphere of *M. micrantha* and the two native plants were analyzed at the genus level, at both pot experiment and invaded site. From the metagenomic data, genes could be classified to the genus level by CARMA3 software. The relative abundance of genus in each sample was calculated according to reads count at the genus level. In total, the top 69 genera (relative abundance > 0.01%) accounted for 94.8% of the total relative abundance of classified genera, and 45 of them were enriched ($P < 0.05$, Dunn test) in

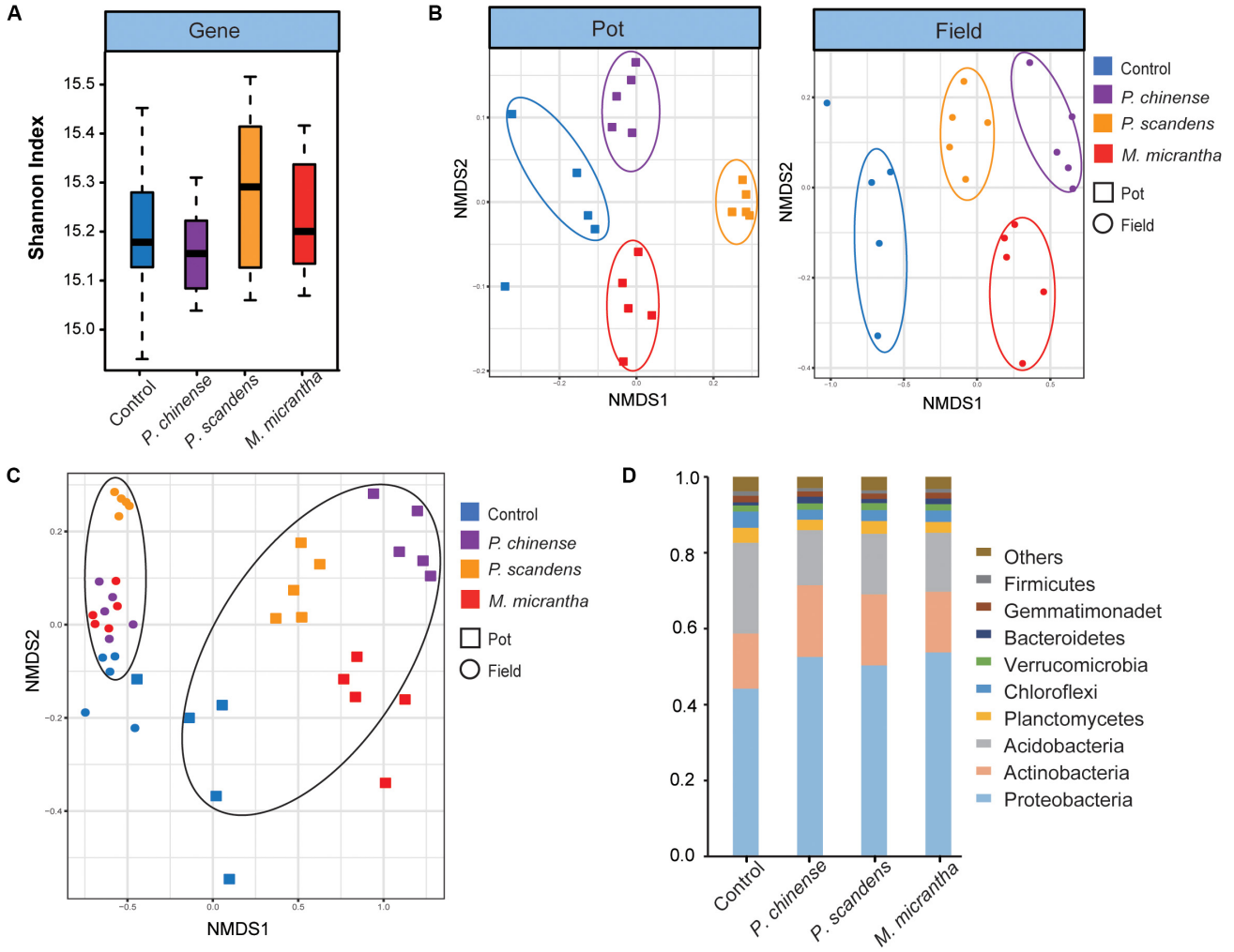

FIGURE 1 | Microbial structure in the rhizosphere of *Mikania micrantha* (*M. micrantha*), *Polygonum chinense* (*P. chinense*), and *Paederia scandens* (*P. scandens*), and control (bulk) soil. **(A)** Comparison of the microbial diversity at the gene level. **(B)** The non-metric multidimensional scaling (NMDS) plot of microbial communities at both pot experiment and invaded site. The analysis was based on Bray–Curtis dissimilarities at the gene level. **(C)** The NMDS plot of microbial communities in all samples, based on Bray–Curtis dissimilarities at the gene level. **(D)** Relative abundances at the phylum level.

rhizospheres compared to the bulk soil, most of which belong to *Proteobacteria* and *Actinobacteria* (**Figure 2**). Moreover, 30 genera were all enriched in rhizospheres of three plants, including *Bradyrhizobium*, *Mesorhizobium*, *Rhizobium*, *Burkholderia*, *Paraburkholderia*, *Methylobacterium*, *Novosphingobium*, *Pseudomonas*, *Enterobacter*, *Bacillus*, *Nocardioides*, and *Streptomyces*, many species of which were known as plant beneficial microbes that can facilitate nutrition acquisition, improve resistance to abiotic stress, and control phytopathogens (**Figure 2**) (Ahemad and Kibret, 2014; Cordovez et al., 2018; Vives-Peris et al., 2018). The enrichment of these plant microbes might facilitate plant assembling beneficial endosphere bacteria from the rhizosphere soil.

Previous studies have shown that the plant species and varieties could influence the composition of their rhizosphere (Philippot et al., 2013; Zhang et al., 2019). In our study, the microbe enrichment in rhizospheres of *M. micrantha* and native plants was also distinctive. *Enterobacter*, *Pseudomonas*, *Cupriavidus*, and *Phenylobacterium* relatively occupied higher percentages in *M. micrantha* rhizosphere compared to *P. chinense* and *P. scandens* rhizospheres (**Figure 2**). Many species belonging to *Enterobacter* and *Pseudomonas* (Meena et al., 2017; Zheng et al., 2019) are well known plant-beneficial microbes, and *Cupriavidus* and *Phenylobacterium* species were reported to participate in the mineralization of soil organic P and degrade organic material (De La Cruz-Barrón et al., 2017). In comparison, *P. chinense* rhizosphere was enriched with *Variovorax*, *Bosea*, and *Acinetobacter*, and some species of which could inhibit pathogens and supply N for plant growth (Rilling et al., 2018; Bruisson et al., 2019), and *P. scandens* rhizosphere was enriched with *Anaeromyxobacter*, *Frankia*, *Streptacidiphilus*, and *Amycolatopsis* (**Figure 2**), with nitrogen-fixing (Chaia et al., 2010) and antimicrobial activity (Buszewski et al., 2018). In summary, although many bacteria are shared among three plant species, each plant still recruits distinctive microbes in rhizosphere, possibly due to their different root exudates.

Enrichment of *Pseudomonas* and *Enterobacter* to Enhanced Phosphorus Solubilization

Phosphorus (P), is an essential element for plant growth and development (Sharma et al., 2013), playing important roles in many metabolic processes of plant, including photosynthesis, signal transduction, energy transfer, respiration, macromolecular biosynthesis (Khan et al., 2010), and nitrogen fixation (Kouas et al., 2005). Microorganisms are major members of the soil P cycle, improving available P to plants (Khan et al., 2009). We have previously reported that the available P content in *M. micrantha* rhizosphere was significantly higher than that in the rhizosphere of two native plants (Liu et al., 2020).

Phosphorus solubilizing microorganisms (PSMs), such as *Pseudomonas*, *Bacillus*, *Enterobacter*, and *Burkholderia* (Babalola and Glick, 2012), can increase soil available P via solubilization and mineralization of unavailable P in organic matter and minerals. These PSMs were enriched in the rhizospheres of *M. micrantha*, *P. chinense*, and *P. scandens* (**Figure 3A**); however, the relative abundance of PSMs is different. *Enterobacter* was most highly enriched in *M. micrantha* rhizosphere, with its

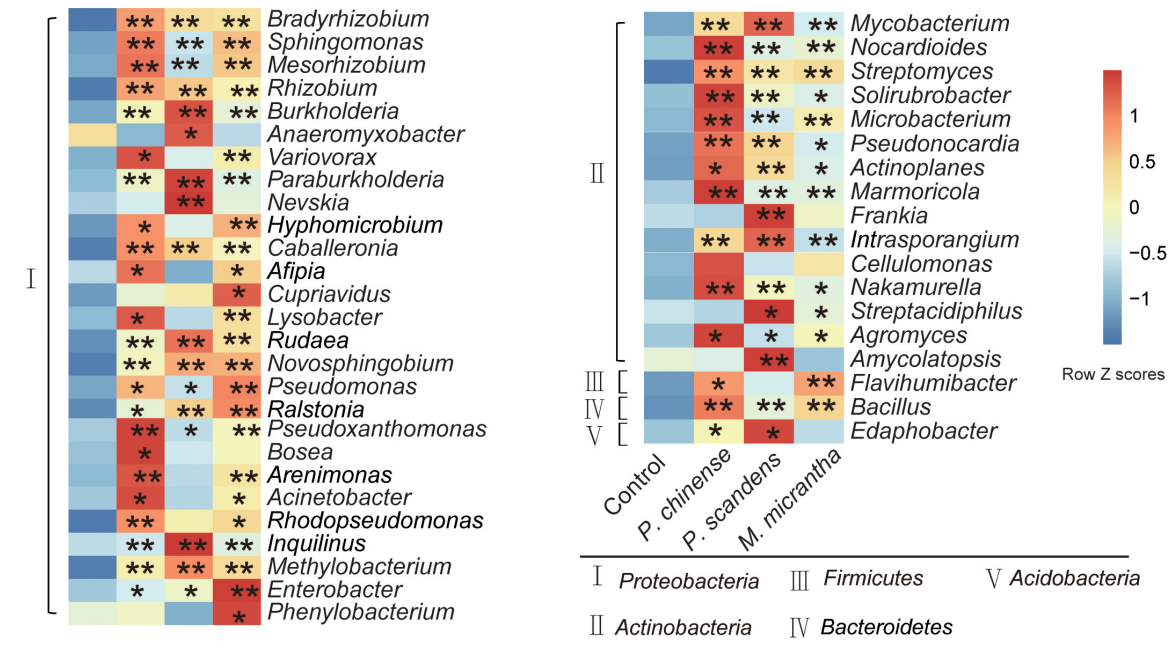

FIGURE 2 | Comparison of average relative abundance at the genus level in rhizospheres of *M. micrantha* and two native plants. The relative abundance of each genus was colored according to the row *z* score ((value - row mean)/row standard deviation). The comparisons of microbes in plant rhizospheres and control soil were compared by the Kruskal–Wallis test with Dunn's multiple comparison test (*$P < 0.05$ and **$P < 0.01$).

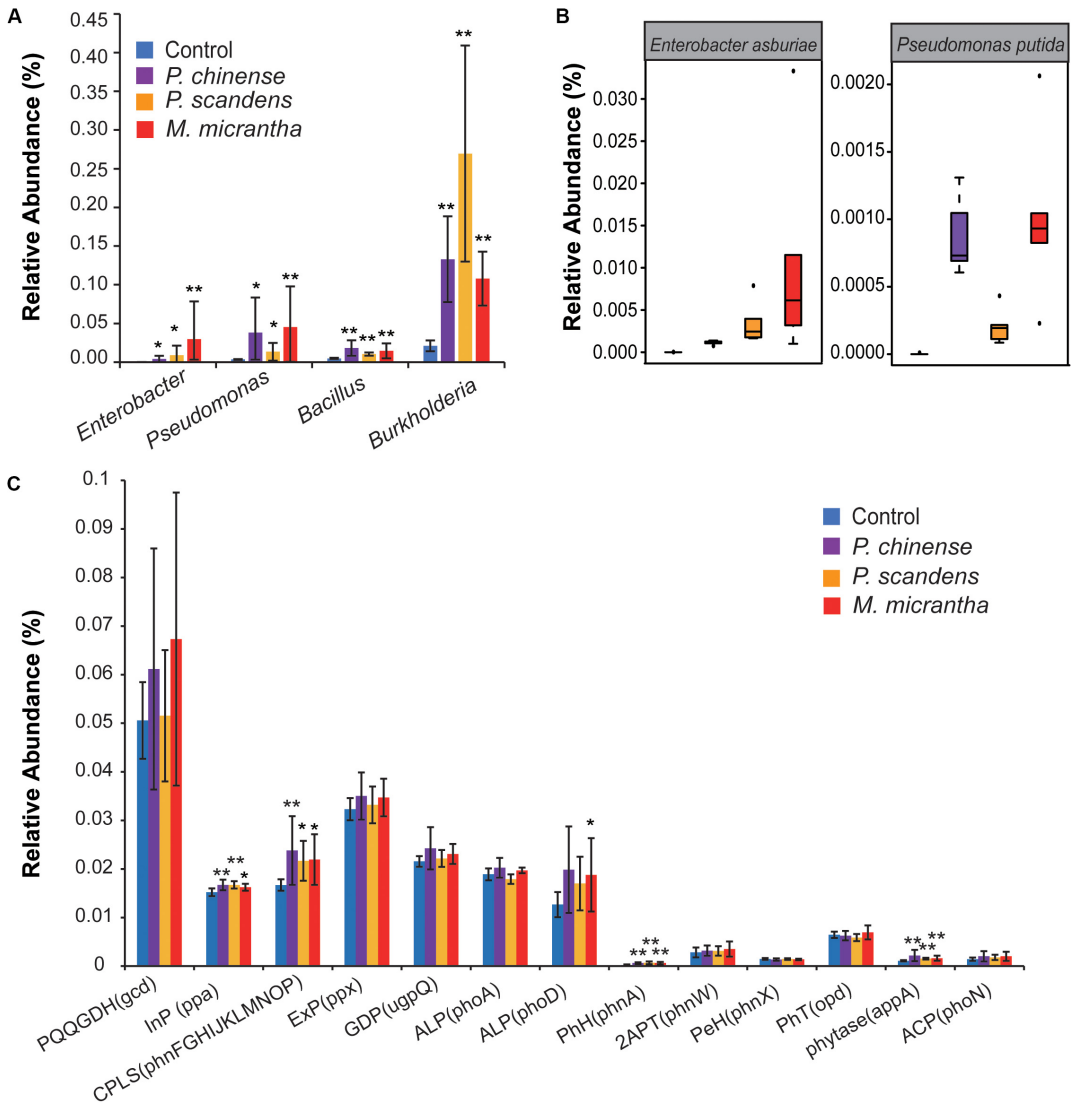

FIGURE 3 | Enhanced soil-borne available P in the rhizosphere of *M. micrantha* and two native plants. **(A)** The relative abundance of phosphate bacteria. **(B)** The relative abundance of *Enterobacter asburiae* and *Pseudomonas putida*. On each boxplot, the central mark indicates the median, the bottom and top edges of the box indicate the interquartile range (IQR), and the whiskers represent the maximum and minimum data points. **(C)** The relative abundance of genes coding for P solubilization and P mineralization. PQQGDH, quinoprotein glucose dehydrogenase; InP, inorganic pyrophosphatase; CPLS, C-P lyase subunit; ExP, exopolyphosphatase; GDP, glycerophosphoryl diester phosphodiesterase; ALP, alkaline phosphatase; PhH, phosphonoacetate hydrolase; 2APT, 2-aminoethylphosphonate-pyruvate transaminase; PeH, phosphonoacetaldehyde hydrolase; PhT, phosphotriesterase; ACP, acid phosphatase. The C-P lyase subunit was calculated as the total abundances of gene *phnF*, *phnG*, *phnH*, *phnI*, *phnJ*, *phnK*, *phnL*, *phnM*, *phnN*, *phnO*, and *phnP*. Error bars indicate average value ± SEM of indicated replicates. The pairwise comparisons of rhizosphere in each plant and control soil were used by the Kruskal–Wallis test with Dunn's multiple comparison test (*$P < 0.05$ and **$P < 0.01$).

average relative abundance 7-fold and 100-fold higher than that in *P. chinense* and *P. scandens* rhizosphere, respectively (**Figure 3A**). Similarly, the average relative abundance of *Pseudomonas* was also 1.5-fold and 13-fold higher than those in *P. chinense* and *P. scandens* rhizosphere, respectively (**Figure 3A**). In the invaded field site, the plant-growth promotion bacteria *Pseudomonas putida* (Możejko-Ciesielska and Serafim, 2019) and *Enterobacter asburiae* (Teng et al., 2019) were more abundant in *M. micrantha* rhizosphere (**Figure 3B**). On the other hand, *Bacillus* and *Burkholderia* were more enriched in rhizosphere of *P. chinense* and *P. scandens* (1.2- and 2.5-fold that in *M. micrantha* rhizosphere), which might also contribute to the solubilization of soil unavailable phosphorus. Taken together, the recruitment of these PSM would help to increase the available P content in *M. micrantha* rhizosphere.

Next, the genes generally contained in PSM were analyzed, including those coding for P mineralizing and solubilizing enzymes (Dai et al., 2019). The genes coding for organic P mineralization, such as C-P lyase, phosphonoacetate hydrolase, and phytase, as well as the genes coding for inorganic

pyrophosphatase responsible for the inorganic P solubilization, were all enriched in rhizosphere of *M. micrantha* and two native plants ($P < 0.05$) (**Figure 3C**). The genes involved in alkaline phosphatase phoD were more abundant in rhizosphere of *M. micrantha* ($P = 0.045$) and *P. chinense* ($P = 0.07$), whereas phoA showed no significant difference ($P > 0.05$) (**Figure 3C**). The relative abundance of other genes showed no significant difference ($P > 0.05$) (**Figure 3C**). These results indicated that the rhizosphere microbes in *M. micrantha* and *P. chinense* may contribute to available P through the similar P mineralization mechanism in terms of alkaline phosphatase.

Fewer Pathogens in *M. micrantha* Rhizosphere Microbiota

The plant-associated microbiome, as the second genome of the plant, has great influence on plant growth and health (Berendsen et al., 2012). To suppress the pathogen attack, plants could be able to recruit protective microorganisms in the rhizosphere, as the complement of the plant innate immune system (Mendes et al., 2014).

The aggressive soil-borne pathogens were analyzed (**Supplementary Table S3**). Although many pathogens could not be detected in our data, we found that the pathogens of *Fusarium oxysporum* (Srinivas et al., 2019) and *Ralstonia solanacearum* (Genin and Denny, 2012) were enriched in *P. chinense* (sevenfold and twofold) and *P. scandens* (sixfold and fourfold) rhizosphere compared to *M. micrantha* rhizosphere (**Figure 4A**). Besides, the genes involved in the host–pathogen interactions (**Supplementary Table S4**) [type III secretion system (T3SS)] were more abundant in the rhizosphere of *P. scandens* ($P = 0.04$) than in *M. micrantha* rhizosphere (**Figure 4B**). Plants could inhibit pathogen attack by secreting

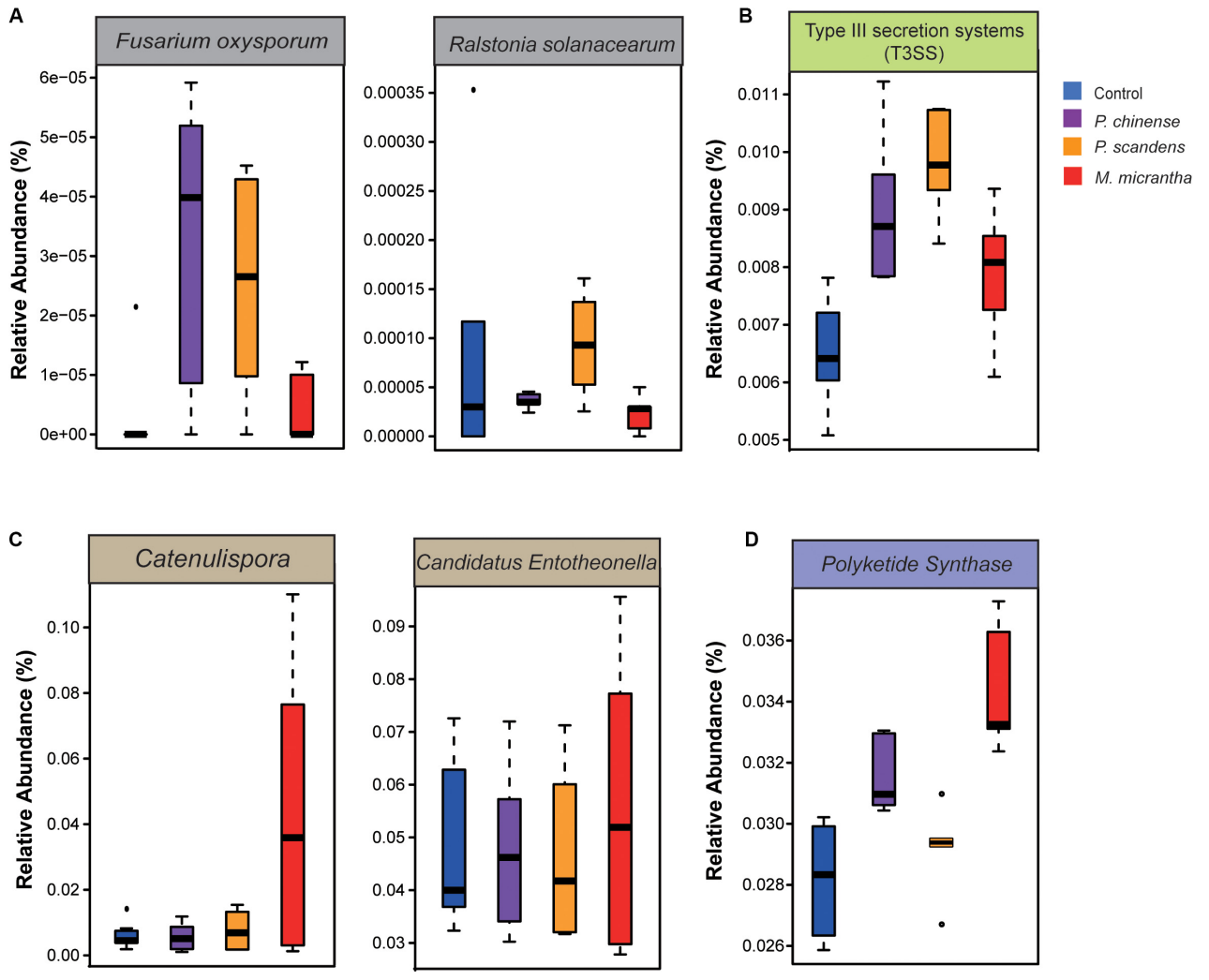

FIGURE 4 | Fewer pathogens and more biocontrol bacteria in *M. micrantha* rhizosphere. **(A)** The relative abundance of pathogens of *Fusarium oxysporum* and *Ralstonia solanacearum*. **(B)** The relative abundance of pathogenic genes of type III secretion systems (T3SS). **(C)** The relative abundance of biocontrol bacteria of *Catenulispora* and *Candidatus Entotheonella*. **(D)** The relative abundance of genes coding for type II polyketide synthase (PKS). On each boxplot, the central line indicates the median, the bottom and top edges of the box indicate the interquartile range (IQR), and the whiskers represent the maximum and minimum data points.

antimicrobials or recruiting the biocontrol microbes that have the relevant antimicrobial gene cluster (Berendsen et al., 2012). The biocontrol microbes (**Supplementary Table S5**), such as *Pseudomonas*, *Catenulispora*, and *Candidatus Entotheonella*, were more abundant in rhizosphere of *M. micrantha* than that in two native plants (**Figures 3A**, **4C**). It is known that some species belonging to *Catenulispora*, *Pseudomonas*, and *Candidatus Entotheonella* could suppress pathogen by producing antibiotics and polyketides (Zettler et al., 2014; Kurnia et al., 2017). In our results, type II PKS genes (**Supplementary Table S6**) that were involved in synthesizing aromatic polyketides that could control plant disease (Han et al., 2018) were also enriched in *M. micrantha* rhizosphere (P = 0.002) (**Figure 4D**), whereas type III PKS genes were not different among three plants. These results indicated that the biocontrol bacteria might contribute to the less pathogens by antimicrobial aromatic polyketides in *M. micrantha* rhizosphere.

DISCUSSION

The success of plant invasion depends on enemy release, enhanced nutrient acquisition, and adaptations to the physical environment (Dawkins and Esiobu, 2016). Recently, increased attention has been paid to the interactions between soil microbes and plant invasions (Dawkins and Esiobu, 2018). In this study, we investigated the role of soil microbes in plant invasions by comparing the taxonomic and functional difference of rhizosphere community between the invasive plant *M. micrantha* and two native plants (*P. chinense* and *P. scandens*) at invaded field site and pot experiment. Since the pot experiment lasted only 3 months, and each plant grew independently without competition, obvious microbial differences between pot experiment and invaded site were observed (**Figure 1C**). However, many plant-associated microbes were enriched in rhizospheres both in the pot experiment and the invaded site, and these genera were generally higher in the invaded field than those in the pot experiment, indicating their important roles in the natural environment. The interactions between an invasive plant and associated soil communities changed across the invaded range (Nunes et al., 2019). In our study, we found that there is a difference of *M. micrantha* rhizosphere between the pot experiment and the field site. As a plant killer, more field samples of the rhizosphere microbes of *M. micrantha* across latitudinal gradients in its invaded range should be analyzed to understand the interactions between its performance and soil microbes. This could provide an important basis for controlling its spread. By comparing the microbes in the rhizospheres and in the control soil, we found that the relative abundance of *Proteobacteria* and *Actinobacteria* was higher in the rhizosphere than in the control soil, whereas *Acidobacteria* was more abundant in control soil (**Figure 1C**). The distinct enrichment may be attributed to the abundant nutrients in rhizosphere, which promote the copiotrophic microorganisms (Ling et al., 2017) and the inhibited growth of oligotrophic microorganisms (Fierer et al., 2007).

The competition of invasive species with native species depends largely on the abilities of accession in resources (Seabloom et al., 2003). P is an essential macronutrient for plant growth and development (Lidbury et al., 2016), and microorganisms play an important role in soil P cycling and in regulating P availability (Dai et al., 2019). In this study, we found that *Enterobacter* and *Pseudomonas* might contribute to the increased soil available P content, and helped *M. micrantha* to outcompete native species and ultimately facilitate plant invasion (**Figure 3A**). Although the gene of *gcd* was not significantly different when the field and potted samples were analyzed together (**Figure 3C**), it was found significantly enriched in rhizosphere of *M. micrantha* (P = 0.0008) and *P. chinense* (P = 0.02) (**Supplementary Figure S1**) in the invaded site. The relative abundance of the *gcd* gene in *M. micrantha* rhizosphere was 1.2-fold of that in *P. chinense* rhizosphere and 1.5-fold of that in *P. scandens* rhizosphere in the field. Besides, even genes coding for alkaline phosphatase were at a similar level in the rhizosphere of *M. micrantha* and *P. chinense*, and the highly elongated, deep, and extensive root system of *M. micrantha* may still promote the efficient uptake of the released available P in soil.

Invasive plants may benefit from introduction to new regions where they can escape pathogens on the native ranges (Lu-Irving et al., 2017). Recently, Ramirez et al. (2019) found that the range-expanding plants harbored fewer pathogens compared to native species in the new range, through the analysis of the microbiome of European continental range-expanding plant species along a latitudinal gradient. This result was consistent with our study, which revealed that the pathogens and pathogenic genes, including the soil-borne pathogen *F. oxysporum* and *R. solanacearum*, as well as T3SS, were depleted in *M. micrantha* rhizosphere compared to the native plants (**Figures 4A,B**). Correspondingly, many biocontrol bacteria such as *Catenulispora*, *Pseudomonas*, and *Candidatus Entotheonella*, which release antibiotics and polyketides to inhibit pathogens (Kurnia et al., 2017; Mori et al., 2018), were enriched in *M. micrantha* rhizosphere. In addition, Mikania sesquiterpene lactones (STLs) have allelopathic effects on native plants and antibacterial activities (Li Y. et al., 2013), which may also contribute to the fewer pathogens in *M. micrantha* rhizosphere. In summary, the fewer pathogens and more protective microorganisms that inhabit the *M. micrantha* rhizosphere potentially benefit root growth and nutrient uptake, thus possibly enabling the successful invasion. However, there is a lack of difference in the soil microbes in *M. micrantha* between the origin and invaded one. Evidences for the resource availability and pathogen release in soil of invasive plants would require combined tests in the native and invaded ranges. Hence, in order to comprehensively understand the role of soil microorganisms in *M. micrantha* invasion, the metagenome of *M. micrantha* rhizosphere in the native range and the differences to their introduced range would need to be studied in the future. Although we showed the differences of microbial community and functional genes among the rhizosphere of three plants, the observed changes would require further experimental study.

CONCLUSION

Mikania micrantha rhizosphere has a distinct bacteria community structure that is clearly separated from the native plants and the bulk soil. Although some common microbes are observed in the rhizosphere of both *M. micrantha* and two native plants, *M. micrantha* rhizosphere specifically recruited *Cupriavidus*, *Enterobacter*, *Pseudomonas*, and *Phenylobacterium*, which played important roles in resource acquisition, plant hormone regulation, and pathogen suppression. On the other hand, the rhizosphere of native plants *P. chinense* and *P. scandens* recruited some other distinctive plant microbes. According to our analysis, the previously found higher soil available P content in *M. micrantha* rhizosphere was possibly contributed by the enrichment of P-solubilizing bacteria *Enterobacter* and *Pseudomonas*. Moreover, pathogens including *F. oxysporum* and *R. solanacearum* and pathogenic genes of T3SS were less abundant in *M. micrantha* rhizosphere compared to the two native plants. In contrast, the biocontrol bacteria such as *Catenulispora*, *Pseudomonas*, and *Candidatus Entotheonella*, as well as the PKS genes were enriched in *M. micrantha* rhizosphere to develop antibacterial activities. Taken together, these findings deepen our understanding of the microbial composition and function in *M. micrantha* rhizosphere, as well as the two native plants, and thus provide useful information that would help develop efficient technologies to control the invasion of *M. micrantha*.

DATA AVAILABILITY STATEMENT

Publicly available datasets were analyzed in this study. These data can be found in the NCBI under the accession numbers SRR8936416–SRR8936475.

AUTHOR CONTRIBUTIONS

BL, WF, and WQ conceived the study. LY, HeW, YZ, FJ, and SW collected the samples and analyzed the data. YR, CL, HL, WQ, HaW, and FW provided suggestions and helped in the checking. YZ, SW, BL, WQ, and WF helped to revise the manuscript. All authors contributed to the article and approved the submitted version.

FUNDING

This work was supported by the Shenzhen Science and Technology Program (JCYJ20170303154245825), the Associated Fund of Dapeng District (PT20170310 and PT20170309), and the research program of Urban Management Bureau of Shenzhen Municipality (No. 201914), as well as the Agricultural Science and Technology Innovation Program and The Elite Young Scientists Program of CAAS, Fundamental Research Funds for Central Non-Profit Scientific Institution (No. Y2017JC01), the Agricultural Science and Technology Innovation Program Cooperation and Innovation Mission (CAAS-XTCX2016), and Fund of Key Laboratory of Shenzhen (ZDSYS20141118170111640). Projects subsidized by special funds for science technology innovation and industrial development of Shenzhen Dapeng New District (Grant No. KJYF202001-03).

SUPPLEMENTARY MATERIAL

The Supplementary Material for this article can be found online at: https://www.frontiersin.org/articles/10.3389/fmicb.2020.01462/full#supplementary-material

REFERENCES

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

Alori, E. T., Glick, B. R., and Babalola, O. O. (2017). Microbial phosphorus solubilization and its potential for use in sustainable agriculture. *Front. Microbiol.* 8:971. doi: 10.3389/fmicb.2017.00971

Babalola, O. O., and Glick, B. R. (2012). The use of microbial inoculants in African agriculture: current practice and future prospects. *J. Food Agricult. Environ.* 10, 540–549.

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

Beth Mudgett, M. (2005). New insights to the function of phytopathogenic bacterial type III effectors in plants. *Ann. Rev. Plant Biol.* 56, 509–531. doi: 10.1146/annurev.arplant.56.032604.144218

Bruisson, S., Zufferey, M., L'haridon, F., Trutmann, E., Anand, A., Dutartre, A., et al. (2019). Endophytes and epiphytes from the grapevine leaf microbiome as potential biocontrol agents against phytopathogens. *Front. Microbiol.* 10:2726. doi: 10.3389/fmicb.2019.02726

Buchfink, B., Xie, C., and Huson, D. H. (2015). Fast and sensitive protein alignment using DIAMOND. *Nat. Methods* 12:59. doi: 10.1038/nmeth.3176

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

Buszewski, B., Railean-Plugaru, V., Pomastowski, P., Rafińska, K., Szultka-Mlynska, M., Golinska, P., et al. (2018). Antimicrobial activity of biosilver nanoparticles produced by a novel Streptacidiphilus durhamensis strain. *J. Microbiol. Immunol. Infect.* 51, 45–54. doi: 10.1016/j.jmii.2016.03.002

Callaway, R. M., Thelen, G. C., Rodriguez, A., and Holben, W. E. (2004). Soil biota and exotic plant invasion. *Nature* 427, 731–733. doi: 10.1038/nature02322

Castro-Díez, P., Godoy, O., Alonso, A., Gallardo, A., and Saldaña, A. (2014). What explains variation in the impacts of exotic plant invasions on the nitrogen cycle? A meta-analysis. *Ecol. Lett.* 17, 1–12. doi: 10.1111/ele.12197

Chaia, E. E., Wall, L. G., and Huss-Danell, K. (2010). Life in soil by the actinorhizal root nodule endophyte Frankia. A review. *Symbiosis* 51, 201–226. doi: 10.1007/s13199-010-0086-y

Chapuis-Lardy, L., Vanderhoeven, S., Dassonville, N., Koutika, L.-S., and Meerts, P. (2006). Effect of the exotic invasive plant Solidago gigantea on soil phosphorus status. *Biol. Fertil. Soils* 42, 481–489. doi: 10.1007/s00374-005-0039-4

Chen, B.-M., Peng, S.-L., and Ni, G.-Y. (2009). Effects of the invasive plant Mikania micrantha HBK on soil nitrogen availability through allelopathy in South China. *Biol. Invas.* 11, 1291–1299. doi: 10.1007/s10530-008-9336-9

Cordovez, V., Schop, S., Hordijk, K., De Boulois, H. D., Coppens, F., Hanssen, I., et al. (2018). Priming of plant growth promotion by volatiles of root-associated *Microbacterium* spp. *Appl. Environ. Microbiol.* 84, e1865–e1818.

Dai, Z., Liu, G., Chen, H., Chen, C., Wang, J., Ai, S., et al. (2019). Long-term nutrient inputs shift soil microbial functional profiles of phosphorus cycling in diverse agroecosystems. *ISME J.* 14, 1–14.

Dawkins, K., and Esiobu, N. (2016). Emerging insights on Brazilian pepper tree (*Schinus terebinthifolius*) invasion: the potential role of soil microorganisms. *Front. Plant Sci.* 7:712. doi: 10.3389/fpls.2016.00712

Dawkins, K., and Esiobu, N. (2018). The invasive Brazilian Pepper Tree (*Schinus terebinthifolius*) is colonized by a root microbiome enriched with alphaproteobacteria and unclassified spartobacteria. *Front. Microbiol.* 9:876. doi: 10.3389/fmicb.2018.00876

Dawson, W., and Schrama, M. (2016). Identifying the role of soil microbes in plant invasions. *J. Ecol.* 104, 1211–1218. doi: 10.1111/1365-2745.12619

Day, M. D., Clements, D. R., Gile, C., Senaratne, W. K. A. D., Shen, S. C., Weston, L. A., et al. (2016). Biology and Impacts of Pacific Islands Invasive Species. 13. Mikania micrantha Kunth (Asteraceae). *Pacific Sci.* 70, 257–285. doi: 10.2984/70.3.1

De La Cruz-Barrón, M., Cruz-Mendoza, A., Navarro-Noya, Y. E., Ruiz-Valdiviezo, V. M., Ortíz-Gutiérrez, D., Ramírez-Villanueva, D. A., et al. (2017). The bacterial community structure and dynamics of carbon and nitrogen when maize (*Zea mays* L.) and its neutral detergent fibre were added to soil from Zimbabwe with contrasting management practices. *Microb. Ecol.* 73, 135–152. doi: 10.1007/s00248-016-0807-8

Edwards, J., Johnson, C., Santos-Medellín, C., Lurie, E., Podishetty, N. K., Bhatnagar, S., et al. (2015). Structure, variation, and assembly of the root-associated microbiomes of rice. *Proc. Natl. Acad. Sci.* 112, E911–E920.

Fierer, N., Bradford, M. A., and Jackson, R. B. (2007). Toward an ecological classification of soil bacteria. *Ecology* 88, 1354–1364. doi: 10.1890/05-1839

Garbeva, P. V., Van Veen, J., and Van Elsas, J. (2004). Microbial diversity in soil: selection of microbial populations by plant and soil type and implications for disease suppressiveness. *Ann. Rev. Phytopathol.* 42, 243–270. doi: 10.1146/annurev.phyto.42.012604.135455

Genin, S., and Denny, T. P. (2012). Pathogenomics of the Ralstonia solanacearum species complex. *Ann. Rev. Phytopathol.* 50, 67–89.

Gerlach, W., and Stoye, J. (2011). Taxonomic classification of metagenomic shotgun sequences with CARMA3. *Nucleic Acids Res.* 39, e91–e91. doi: 10.1093/nar/gkr225

Han, J. W., Choi, G. J., and Kim, B. S. (2018). Antimicrobial aromatic polyketides: a review of their antimicrobial properties and potential use in plant disease control. *World J. Microbiol. Biotechnol.* 34, 163.

Hu, Y., and But, P. (1994). A study on life cycle and response to herbicides of Mikania micrantha. *ACTA Scientiar. Nat. Univ. SunYatSeni* 33, 88–95.

Huang, P., Zhang, Y., Xiao, K., Jiang, F., Wang, H., Tang, D., et al. (2018). The chicken gut metagenome and the modulatory effects of plant-derived benzylisoquinoline alkaloids. *Microbiome* 6, 1–17.

Kanehisa, M., Goto, S., Kawashima, S., Okuno, Y., and Hattori, M. (2004). The KEGG resource for deciphering the genome. *Nucleic Acids Res.* 32, D277–D280.

Khan, A. A., Jilani, G., Akhtar, M. S., Naqvi, S. M. S., and Rasheed, M. (2009). Phosphorus solubilizing bacteria: occurrence, mechanisms and their role in crop production. *J. Agricult. Biol. Sci.* 1, 48–58.

Khan, M. S., Zaidi, A., Ahemad, M., Oves, M., and Wani, P. A. (2010). Plant growth promotion by phosphate solubilizing fungi–current perspective. *Arch. Agron. Soil Sci.* 56, 73–98. doi: 10.1080/03650340902806469

Kouas, S., Labidi, N., Debez, A., and Abdelly, C. (2005). Effect of P on nodule formation and N fixation in bean. *Agron. Sustain. Dev.* 25, 389–393. doi: 10.1051/agro:2005034

Kourtev, P. S., Ehrenfeld, J. G., and Häggblom, M. (2002). Exotic plant species alter the microbial community structure and function in the soil. *Ecology* 83, 3152–3166. doi: 10.1890/0012-9658(2002)083[3152:epsatm]2.0.co;2

Kurnia, N. M., Uria, A. R., Kusnadi, Y., Dinawati, L., Zilda, D. S., Hadi, T. A., et al. (2017). Metagenomic survey of potential symbiotic bacteria and polyketide synthase genes in an indonesian marine sponge. *HAYATI J. Biosci.* 24, 6–15. doi: 10.1016/j.hjb.2017.04.004

Li, H., and Durbin, R. (2009). Fast and accurate short read alignment with Burrows–Wheeler transform. *Bioinformatics* 25, 1754–1760. doi: 10.1093/bioinformatics/btp324

Li, X., Shen, Y., Huang, Q., Fan, Z., and Huang, D. (2013). Regeneration capacity of small clonal fragments of the invasive Mikania micrantha HBK: effects of burial depth and stolon internode length. *PLoS One* 8:e84657. doi: 10.1371/journal.pone.0084657

Li, Y., Li, J., Li, Y., Wang, X.-X., and Cao, A.-C. (2013). Antimicrobial Constituents of the Leaves of Mikania micrantha HB K. *PLoS One* 8:e76725. doi: 10.1371/journal.pone.0076725

Lidbury, I. D., Murphy, A. R., Scanlan, D. J., Bending, G. D., Jones, A. M., Moore, J. D., et al. (2016). Comparative genomic, proteomic and exoproteomic analyses of three *Pseudomonas* strains reveals novel insights into the phosphorus scavenging capabilities of soil bacteria. *Environ. Microbiol.* 18, 3535–3549. doi: 10.1111/1462-2920.13390

Ling, N., Chen, D., Guo, H., Wei, J., Bai, Y., Shen, Q., et al. (2017). Differential responses of soil bacterial communities to long-term N and P inputs in a semi-arid steppe. *Geoderma* 292, 25–33. doi: 10.1016/j.geoderma.2017.01.013

Liu, B., Yan, J., Li, W., Yin, L., Li, P., Yu, H., et al. (2020). Mikania micrantha genome provides insights into the molecular mechanism of rapid growth. *Nat. Commun.* 11, 1–13.

Lowe, S., Browne, M., Boudjelas, S., and De Poorter, M. (2000). *100 of the World's Worst Invasive Alien Species: A Selection From the Global Invasive Species Database*. Auckland: Invasive Species Specialist Group Auckland.

Lu-Irving, P., Harenčár, J., Sounart, H., Welles, S. R., Swope, S. M., Baltrus, D. A., et al. (2017). Escape from bacterial diversity: potential enemy release in invading yellow starthistle (Centaurea solstitialis) microbiomes. *bioRxiv [Preprint]* doi: 10.1101/119917

Lu-Irving, P., Harenčár, J. G., Sounart, H., Welles, S. R., Swope, S. M., Baltrus, D. A., et al. (2019). Native and invading yellow starthistle (*Centaurea solstitialis*) microbiomes differ in composition and diversity of bacteria. *mSphere* 4, e88–e19.

Macanawai, A., Day, M., Tumaneng-Diete, T., and Adkins, S. (2012). Impact of *Mikania micrantha* on crop production systems in Viti Levu. Fiji. *Pak. J. Weed Sci. Res.* 18, 357–365.

Marler, M. J., Zabinski, C. A., and Callaway, R. M. (1999). Mycorrhizae indirectly enhance competitive effects of an invasive forb on a native bunchgrass. *Ecology* 80, 1180–1186. doi: 10.1890/0012-9658(1999)080[1180:mieceo]2.0.co;2

Meena, V. S., Meena, S. K., Verma, J. P., Kumar, A., Aeron, A., Mishra, P. K., et al. (2017). Plant beneficial rhizospheric microorganism (PBRM) strategies to improve nutrients use efficiency: a review. *Ecol. Eng.* 107, 8–32. doi: 10.1016/j.ecoleng.2017.06.058

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. doi: 10.1038/ismej.2014.17

Mitchell, C. E., and Power, A. G. (2003). Release of invasive plants from fungal and viral pathogens. *Nature* 421, 625–627. doi: 10.1038/nature01317

Mori, T., Cahn, J. K., Wilson, M. C., Meoded, R. A., Wiebach, V., Martinez, A. F. C., et al. (2018). Single-bacterial genomics validates rich and varied specialized metabolism of uncultivated Entotheonella sponge symbionts. *Proc. Natl. Acad. Sci.* 115, 1718–1723. doi: 10.1073/pnas.1715496115

Możejko-Ciesielska, J., and Serafim, L. S. (2019). Proteomic response of *Pseudomonas putida* KT2440 to dual carbon-phosphorus limitation during mcl-PHAs synthesis. *Biomolecules* 9:796. doi: 10.3390/biom9120796

Nunes, K. A., Fitzpatrick, C. R., and Kotanen, P. M. (2019). Soil biota composition and the performance of a noxious weed across its invaded range. *Ecography* 42, 1671–1681. doi: 10.1111/ecog.04562

Philippot, L., Raaijmakers, J. M., Lemanceau, P., Van, D. P., and Wim, H. (2013). Going back to the roots: the microbial ecology of the rhizosphere. *Nat. Rev. Microbiol.* 11, 789–799. doi: 10.1038/nrmicro3109

Piper, C. L., Lamb, E. G., and Siciliano, S. D. (2015). Smooth brome changes gross soil nitrogen cycling processes during invasion of a rough fescue grassland. *Plant Ecol.* 216, 235–246. doi: 10.1007/s11258-014-0431-y

Pyšek, P., and Richardson, D. M. (2008). "Traits associated with invasiveness in alien plants: where do we stand?," in *Biological Invasions. Ecological Studies (Analysis and Synthesis)*, W. Nentwig (Heidelberg: Springer), 97–125. doi: 10.1007/978-3-540-36920-2_7

Raaijmakers, J. M., and Mazzola, M. (2012). Diversity and natural functions of antibiotics produced by beneficial and plant pathogenic bacteria. *Ann. Rev. Phytopathol.* 50, 403–424. doi: 10.1146/annurev-phyto-081211-172908

Raj, R., Paul, D., and Babyson, R. S. (2014). Molecular characterization of phosphate solubilizing bacteria (PSB) and plant growth promoting rhizobacteria (PGPR) from pristine soils. *Int. J. Innovat. Sci. Eng. Technol.* 1, 317–324.

Ramirez, K. S., Snoek, L. B., Koorem, K., Geisen, S., Bloem, L. J., ten Hooven, F., et al. (2019). Range-expansion effects on the belowground plant microbiome. *Nat. Ecol. Evol.* 3, 604–611. doi: 10.1038/s41559-019-0828-z

Reinhart, K. O., Packer, A., Van Der Putten, W. H., and Clay, K. (2003). Plant–soil biota interactions and spatial distribution of black cherry in its native and invasive ranges. *Ecol. Lett.* 6, 1046–1050. doi: 10.1046/j.1461-0248.2003.00539.x

Reinhold-Hurek, B., Bünger, W., Burbano, C. S., Sabale, M., and Hurek, T. (2015). Roots shaping their microbiome: global hotspots for microbial activity. *Ann. Rev. Phytopathol.* 53, 403–424. doi: 10.1146/annurev-phyto-082712-102342

Rilling, J. I., Acuña, J. J., Sadowsky, M. J., and Jorquera, M. A. (2018). Putative nitrogen-fixing bacteria associated with the rhizosphere and root endosphere of wheat plants grown in an andisol from southern chile. *Front. Microbiol.* 9:2710. doi: 10.3389/fmicb.2018.02710

Seabloom, E. W., Harpole, W. S., Reichman, O., and Tilman, D. (2003). Invasion, competitive dominance, and resource use by exotic and native California grassland species. *Proc. Natl. Acad. Sci.* 100, 13384–13389. doi: 10.1073/pnas.1835728100

Sharma, S. B., Sayyed, R. Z., Trivedi, M. H., and Gobi, T. A. (2013). Phosphate solubilizing microbes: sustainable approach for managing phosphorus deficiency in agricultural soils. *SpringerPlus* 2:587.

Srinivas, C., Devi, D. N., Murthy, K. N., Mohan, C. D., Lakshmeesha, T., Singh, B., et al. (2019). *Fusarium oxysporum* f. sp. lycopersici causal agent of vascular wilt disease of tomato: biology to diversity–A review. *Saudi J. Biol. Sci.* 26, 1315–1324. doi: 10.1016/j.sjbs.2019.06.002

Sun, F., Ou, Q., Yu, H., Li, N., and Peng, C. (2019). The invasive plant Mikania micrantha affects the soil foodweb and plant-soil nutrient contents in orchards. *Soil Biol. Biochem.* 139:107630. doi: 10.1016/j.soilbio.2019.107630

Teng, Z., Chen, Z., Zhang, Q., Yao, Y., Song, M., and Li, M. (2019). Isolation and characterization of phosphate solubilizing bacteria from rhizosphere soils of the Yeyahu Wetland in Beijing. China. *Environ. Sci. Pollut. Res.* 26, 33976–33987. doi: 10.1007/s11356-018-2955-5

Thorpe, A. S., Archer, V., and Deluca, T. H. (2006). The invasive forb, *Centaurea maculosa*, increases phosphorus availability in Montana grasslands. *Appl. Soil Ecol.* 32, 118–122. doi: 10.1016/j.apsoil.2005.02.018

Van Der Heijden, M. G., Bardgett, R. D., and Van Straalen, N. M. (2008). The unseen majority: soil microbes as drivers of plant diversity and productivity in terrestrial ecosystems. *Ecol. Lett.* 11, 296–310. doi: 10.1111/j.1461-0248.2007.01139.x

Van Kleunen, M., Weber, E., and Fischer, M. (2010). A meta-analysis of trait differences between invasive and non-invasive plant species. *Ecol. Lett.* 13, 235–245. doi: 10.1111/j.1461-0248.2009.01418.x

Vives-Peris, V., Gómez-Cadenas, A., and Pérez-Clemente, R. M. (2018). Salt stress alleviation in citrus plants by plant growth-promoting rhizobacteria *Pseudomonas putida* and *Novosphingobium* sp. *Plant Cell Rep.* 37, 1557–1569. doi: 10.1007/s00299-018-2328-z

Weller, D. M., Raaijmakers, J. M., Gardener, B. B. M., and Thomashow, L. S. (2002). Microbial populations responsible for specific soil suppressiveness to plant pathogens. *Ann. Rev. Phytopathol.* 40, 309–348.

Wu, B., Wang, S., Wei, M., Zhou, J., Jiang, K., Du, D., et al. (2019). The invasive tree staghorn sumac affects soil N2-fixing bacterial communities in north China. *Plant Biol.* 21, 951–960. doi: 10.1111/plb.13003

Xueping, C., Zhang, X., Xi'e, Z., Zhang, H., Liang, X., Yanrui, L., et al. (2016). Exotic plant Alnus trabeculosa alters the composition and diversity of native rhizosphere bacterial communities of *Phragmites australis*. *Pedosphere* 26, 108–119. doi: 10.1016/s1002-0160(15)60027-3

Zettler, J., Xia, H., Burkard, N., Kulik, A., Grond, S., Heide, L., et al. (2014). New aminocoumarins from the rare actinomycete *Catenulispora acidiphila* DSM 44928: identification, structure elucidation, and heterologous production. *Chembiochem* 15, 612–621. doi: 10.1002/cbic.201300712

Zhang, J., Liu, Y., Zhang, N., Hu, B., Jin, T., Xu, H., et al. (2019). NRT1.1B is associated with root microbiota composition and nitrogen use in field-grown rice. *Nat. Biotechnol.* 37, 676–684. doi: 10.1038/s41587-019-0104-4

Zhao, M., Lu, X., Zhao, H., Yang, Y., Hale, L., Gao, Q., et al. (2019). Ageratina adenophora invasions are associated with microbially mediated differences in biogeochemical cycles. *Sci. Total Environ.* 677, 47–56. doi: 10.1016/j.scitotenv.2019.04.330

Zheng, M. M., Wang, C., Li, W. X., Song, W. F., and Shen, R. F. (2019). Soil nutrients drive function and composition of phoC-Harboring bacterial community in acidic soils of Southern China. *Front. Microbiol.* 10:2654. doi: 10.3389/fmicb.2019.02654

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

ORIGINAL RESEARCH
published: 08 July 2020
doi: 10.3389/fpls.2020.01023

A Diketopiperazine, *Cyclo*-(L-Pro-L-Ile), Derived From *Bacillus thuringiensis* JCK-1233 Controls Pine Wilt Disease by Elicitation of Moderate Hypersensitive Reaction

Ae Ran Park[1†], Se-In Jeong[1†], Hee Won Jeon[1], Jueun Kim[2], Namgyu Kim[3], Manh Tuan Ha[4], Mohamed Mannaa[3], Junheon Kim[5], Chul Won Lee[2], Byung Sun Min[4], Young-Su Seo[3] and Jin-Cheol Kim[1*]*

[1] Department of Agricultural Chemistry, College of Agriculture and Life Sciences, Institute of Environmentally Friendly Agriculture, Chonnam National University, Gwangju, South Korea, [2] Department of Chemistry, Chonnam National University, Gwangju, South Korea, [3] Department of Integrated Biological Science, College of Natural Science, Pusan National University, Busan, South Korea, [4] Drug Research and Development Center, College of Pharmacy, Daegu Catholic University, Gyeongbuk, South Korea, [5] Forest Insect Pests and Diseases Division, National Institute of Forest Science, Seoul, South Korea

OPEN ACCESS

Edited by:
Paulo José Pereira Lima Teixeira, University of São Paulo, Brazil

Reviewed by:
Monica Calvo-Polanco, University of Salamanca, Spain
Manuel G. M. Mota, University of Evora, Portugal
Atsushi Watanabe, Kyushu University, Japan

***Correspondence:**
Young-Su Seo
yseo0000@gmail.com
Jin-Cheol Kim
kjinc@jnu.ac.kr

[†]These authors have contributed equally to this work

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

Received: *17 April 2020*
Accepted: *22 June 2020*
Published: *08 July 2020*

Citation:
Park AR, Jeong S-I, Jeon HW, Kim J, Kim N, Ha MT, Mannaa M, Kim J, Lee CW, Min BS, Seo Y-S and Kim J-C (2020) A Diketopiperazine, Cyclo-(L-Pro-L-Ile), Derived From Bacillus thuringiensis JCK-1233 Controls Pine Wilt Disease by Elicitation of Moderate Hypersensitive Reaction. Front. Plant Sci. 11:1023. doi: 10.3389/fpls.2020.01023

Pine wilt disease (PWD) caused by the pine wood nematode (PWN) *Bursaphelenchus xylophilus* is one of the devastating diseases affecting pine forests worldwide. Although effective control measurements are still missing, induction of resistance could represent a possible eco-friendly alternative. In this study, induced resistance-based *in vitro* and *in vivo* screening tests were carried out for selection of bacteria with the ability to suppress PWD. Out of 504 isolated bacteria, *Bacillus thuringiensis* JCK-1233 was selected for its ability to boost pathogenesis-related 1 (*PR1*) gene expression, a marker of systemic acquired resistance. Moreover, treatment of pine seedlings with *B. thuringiensis* JCK-1233 resulted in increased expression of other defense-related genes, and significantly inhibited PWD development under greenhouse conditions. However, *B. thuringiensis* JCK-1233 showed no direct nematicidal activity against *B. xylophilus*. To identify the effective compound responsible for the induction of resistance in *B. thuringiensis* JCK-1233, several diketopiperazines (DPKs) including *cyclo*-(D-Pro-L-Val), *cyclo*-(L-Pro-L-Ile), *cyclo*-(L-Pro-L-Phe), and *cyclo*-(L-Leu-L-Val) were isolated and tested. Foliar treatment of pine seedlings with *Cyclo*-(L-Pro-L-Ile) resulted in suppression of PWD severity and increased the expression of defense-related genes similarly to *B. thuringiensis* JCK-1233 treatment. Interestingly, treatment with *B. thuringiensis* JCK-1233 or *cyclo*-(L-Pro-L-Ile) showed moderately enhanced expression of *PR-1*, *PR-2*, *PR-3*, *PR-4*, *PR-5*, and *PR-9* genes following inoculation with PWN compared to that in the untreated control, indicating that they mitigated the burst of hypersensitive reaction in susceptible pine seedlings. In contrast, they significantly increased the expression levels of *PR-6* and *PR-10* before

PWN inoculation. In conclusion, foliar spraying with either *B. thuringiensis* JCK-1233 culture suspension or DPKs could induce resistance in pine seedlings, thereby alleviating the serious damage by PWD. Taken together, this study supports aerial spraying with eco-friendly biotic or abiotic agents as a valuable strategy that may mark an epoch for the control of PWD in pine forests.

Keywords: pine wood nematodes, diketopiperazine, resistance-inducing bacteria, foliar application, moderate hypersensitive reaction, *cyclo*-(L-Pro-L-Ile), *Bacillus thuringiensis*

INTRODUCTION

Pine wilt disease (PWD) caused by the pine wood nematode (PWN) *Bursaphelenchus xylophilus* is one of the most destructive diseases damaging pine forests. The transmission of PWN occurs *via* pine sawyer beetles (*Monochamus* spp.), which are attracted to pine trees for feeding or oviposition (Mamiya and Enda, 1972). Since the first incidence of PWD was reported in 1905 in Nagasaki, Japan (Yano, 1913), PWD has spread quickly throughout East Asia, Europe, and even North America, threatening pine forests worldwide (Yi et al., 1989; Mota et al., 1999).

Despite the advances in the study of PWD, effective control measures have not yet been developed. As the habitat of pine trees is very wide areas and, in many cases, poorly accessible, such as cliffs and steep mountains, operations by manual labor are impractical. In addition, most pine trees infected by PWN are killed rapidly, as PWN is an endoparasite that is very difficult to control. Consequentially, PWD has caused enormous economic losses with environmental impacts worldwide and thus, is considered a serious threat to be dealt with (Tóth, 2011).

Current PWD control methods depend mainly on the removal of infection sources or other preventative measures, such as fumigation, burning, clear-cutting, breeding, aerial insecticide spraying, and trunk injection (Takai et al., 2003; Kwon et al., 2005; Nose and Shiraishi, 2008; Bonifácio et al., 2014). However, traditional chemical control using chemical compounds, including methyl bromide and phosphine, is known to cause severe environmental problems because of the high toxicity and potential to induce resistance among parasitic nematodes (Bell, 2000). Recently, with the growing public interest in eco-friendly control methods, biological control agents of plant-parasitic nematodes have received greater attention as an environmentally safe alternative for plant protection. Specifically, agricultural application of plant-associated bacteria, originated from phyllosphere or rhizosphere, exhibited the ability to reduce the incidence or severity of soil-borne diseases (Vallad and Goodman, 2004). Induced resistance is among the reported biocontrol mechanisms for management of diseases that do not exert a direct selective pressure on the pathogen population. Specifically, systemic acquired resistance (SAR) is activated throughout higher plants after being exposed to elicitors from virulent, avirulent, or nonpathogenic microbes, or chemical stimuli such as salicylic acid (SA), which then confers long-lasting protection against a broad spectrum of phytopathogenic microorganisms (Vallad and Goodman, 2004).

Studies on plant immunity have indicated that endophytic bacteria increase plant resistance to pathogens through signaling crosstalk in various plants. However, few studies have addressed the use of bacteria-mediated induction of resistance for PWD management. Previously, inoculation with avirulent *B. xylophilus* was shown to induce resistance against PWD in pine trees, suggesting that the mechanism of induced resistance in pine trees has the potential for biological control against PWD (Kosaka et al., 2001). Interestingly, foliar sprays of SAR inducers in pineapple reduced the reproduction of plant-parasitic nematodes such as *Meloidogyne javanica* and *Rotylenchulus reniformis*, which damage the pineapple root system (Rohrbach and Apt, 1986; Chinnasri et al., 2006). Some rhizobacteria also elicit systemic resistance that may be dependent on SA (Kloepper and Ryu, 2006). It was reported that even though susceptible pine trees are infected with virulent PWNs by vector beetles feeding, the ability of pine trees to activate defensive responses to the infection may reduce the nematode migration and proliferation rates within the plant tissues to some extent (Kuroda, 2008). Therefore, we predicted that the induction of resistance by foliar application with biological agents, such as endophytic bacteria, could suppress the dispersal of PWN and limit the serious damage caused by PWD.

The induced resistance in plants is divided into systemic acquired resistance (SAR) and induced systemic resistance (ISR) (Van Loon et al., 1998). Although SAR is induced by a prior pathogen infection at a local tissue, it can protect the rest of the plant from a second infection. ISR is elicited by plant growth-promoting rhizobacteria (PGPR) and confers protection of plants to a broad spectrum of attackers. SAR is associated with the SA signaling pathway, whereas ISR is mediated by the JA and ET signaling pathways. Although it has been known that SAR and ISR are clearly different, recent studies have been reported that they are interconnected by crosstalk of SA, ET, and jasmonic acid signaling from some rhizospheric *Bacillus* strains (Niu et al., 2016; Song et al., 2017). For example, PGPR *Bacillus cereus* AR156 installs ISR and enhances SAR with increased PR-1 protein expression in plants (Niu et al., 2016). Pathogenesis-related (PR) genes are widely expressed downstream the SA, jasmonic acid (JA), and ethylene (ET) pathways in plants, which play important roles in the inducible defense mechanism in plants against pathogens, facilitating plant adaptation to the environment (Hoffmann-Sommergruber, 2002). Specifically, the *PR-1* gene is used as a marker for the SA-dependent signal transduction pathway and for the study of defense gene expression in plants (Ono et al., 2004). Therefore, transgenic

Arabidopsis plants transformed with the *PR-1*-promoter fused to the *β-glucuronidase* (*GUS*) or *luciferase* have been used as a model system for high-throughput screening of bacterial activators that enhance disease-resistance mechanisms in various plants (Ogura et al., 2005; Narusaka et al., 2006). Therefore, we predicted that endophytic bacteria may stimulate the expression of the *PR-1* gene and influence their resistance-inducing activities.

In this study, endophytic bacteria isolated from several sources were screened for their possible induction of resistance against PWD, using a sequence of *Arabidopsis* plants with the *PR-1*-promoter fused to *GUS*, *in vitro* pine callus, and *in vivo* pine seedling assay systems. The objectives of this study were to select resistance-inducing bacteria capable of managing PWD by foliar application, identify the bioactive compounds responsible for the induction of resistance, and characterize the functional mechanism employed in pine trees by the selected bacteria and their bioactive compounds.

MATERIALS AND METHODS

Nematodes, Plant Materials, and Callus Culture

The pine wood nematode (PWN) *B. xylophilus* was isolated from infected pine trees and provided by the National Institute of Forest Science (NIFoS; Seoul, South Korea). Initially, PWN was cultured on the mycelia of *Botrytis cinerea* fully grown on potato dextrose agar (PDA, Difco; Becton, Dickinson and Company, MD, USA) at 25°C for propagation (Maehara and Futai, 2000). After 7 days of incubation, the propagated nematode was harvested using the funnel technique (Baermann, 1917), rinsed three times with sterilized distilled water and then prepared as an aqueous suspension of *B. xylophilus* for subsequent experiments.

Seeds of the *Arabidopsis* plant (*Arabidopsis thaliana* ecotype Columbia (Col-0)) genetically engineered with the *GUS* reporter gene fused to the *PR-1* promoter were provided by Y. C. Kim (College of Agriculture and Life Science, Chonnam National University, South Korea). The seeds were surface sterilized in 5% sodium hypochlorite followed by immersion for 3 min in 70% ethanol. After washing with sterile distilled water, they were left to imbibe in sterile water containing 0.1% agarose (Gibco; Thermo Fisher Scientific INc., MA, USA) in the dark at 4 for 3 days. Subsequently, the seeds were allowed to germinate on plates containing 1× Murashige and Skoog (MS) salt mixture and 0.5 g/L 2-(*N*-morpholino) ethanesulfonic acid (MES), pH 5.8, in 1% Duchefa agar (Duchefa Biochemie, Haarlem, The Netherlands). Seedlings were grown in a growth chamber (VS-3DM-600; Hanbaek, Bucheon, South Korea) under photoperiodic cycles of 16-hour light/8-hour dark at 22 with 70% humidity.

For the *in vivo* pathogenicity assay, three- or four-year old red pine (*Pinus densiflora*) and black pine (*P. thunbergii*) saplings with average height of 40 cm and average diameter of 0.5 cm were obtained from Daelim Farm (Okcheon, South Korea) and then transplanted to 15-cm diameter pots containing sterilized nursery soil in the greenhouse, keeping an average temperature of 25°C.

For the *in vitro* assay, *Pinus* calli were obtained from NIFoS (Seoul, South Korea). Calli were taken aseptically from embryos of *P. densiflora* and cultured in Litvay medium (LM; Thomas Scientific Inc., NJ, USA), including vitamins with 2 μg/ml 2,4-chlorophenoxyacetic acid and 1 μg/ml 6-benzyl-aminopurine solution at 24 under dark conditions.

Isolation and Incubation of Endophytic Bacteria

Endophytic strains were isolated from agronomic plants and grove trees of five regions in South Korea (Daejeon, Gwangju, Jeongeup, Busan, and Sacheon). The agronomic plants tested were tomato, pepper, and onion. The grove trees used in this study included cherry and peach trees. Individual leave, stem, and root samples were put into plastic bags, placed in a cool box for transportation, and stored at 4°C. Plant samples were surface sterilized for 10 s with 2% sodium hypochlorite and rinsed five times in sterile distilled water. Sterilized plant samples were dissected into 1-cm pieces and macerated with a sterile mortar and pestle. Each 1 g plant sample was suspended in 10 ml of sterile distilled water and shaken vigorously for 2 min. The supernatant was serially diluted in sterile distilled water (10^{-1} to 10^{-7}), and plated on tryptic soy agar medium (TSA, Difco, MD, USA). After incubation at 30°C for 1–2 days, each strain was streaked on TSA and then a single colony was isolated. Isolated bacterial strains were stored cryogenically in 20% glycerol at −70°C. For *in vitro* and *in vivo* bioassays, bacterial strains were inoculated in tryptic soy broth (TSB, Difco, MD, USA) for 3 days at 30°C with agitation (200 rpm).

Histochemical Staining for GUS Activity in Arabidopsis Leaves

Four-week-old *Arabidopsis* seedlings from the *PR-1pro::GUS* line were used to assess the resistance inducting activity of endophytic bacteria, which were isolated from several plants and their rhizospheric soils. For GUS staining of *Arabidopsis* leaves, leaf discs (5 mm diameter) were placed in 96 well plates containing the culture filtrate of bacterial strains and then the plates were incubated for 12 h at 22 with relative humidity above 70% under light conditions. After treatment, GUS activity was measured as described by Jefferson et al. (1987). Prior to the staining reactions, the treated leaves were fixed in a fixation solution (0.3% formaldehyde, 10 mM MES, pH 5.6, and 0.3 M mannitol) for 1 h on ice. The staining reaction was performed in 50 mM sodium phosphate buffer (pH 7.0) that contained 10 mg/ml 5-bromo-4-chloro-3-indolyl-β-$_D$-glucuronic acid (X-Gluc) and 0.02% (w/v) Triton X-100 for 24 h at 22 in the dark. After staining, leaf discs were decolorized in 7% (v/v) ethanol for 24 h and rinsed with water. Each experiment was run in triplicates.

In Vitro Screening of Bacterial Strains That Induce PR-1 Gene in Pinus callus

The endophytic bacteria that were selected based on GUS activity in *PR-1pro::GUS* Arabidopsis line were used for the *in vitro* assay that analyzed their *PR-1* gene expression inducing activity in *Pinus* callus. Selected endophytic bacteria were cultured up to an OD_{600} = 0.8 at 30 in TSB for the *in vitro* assay. A bacterial suspension (500 µl) was treated with *P. densiflora* callus (100 mg) and then incubated at 24 using a digital rocker at 50 rpm under dark conditions. The untreated controls for the *in vitro* experiments were performed applying the same amount of sterile TSB. Each experiment was run in triplicates.

After incubation with the bacterial suspension for 1 day, total *P. densiflora* callus RNA was extracted using CTAB extraction buffer with elimination of high viscosity and excessive polysaccharides (Azevedo et al., 2003). Then, total RNA was further purified using RNeasy mini kit (Qiagen, Valencia, CA, USA), according to the manufacturer's recommendations. cDNA libraries were prepared from total callus RNA with oligo (dT) primers and SuperScript™ IV reverse transcriptase (Invitrogen Inc., Carlsbad, CA, USA), according to the manufacturer's protocols. The PCR primers of the *PR-1* gene used in this study (**Table 1**) were synthesized by Genotech (Daejeon, Korea).

Determination of relative mRNA expression was carried out in a real-time PCR detection system (Bio-Rad CFX 96; Bio-Rad Laboratories, Hercules, CA, USA). cDNA was analyzed using iQ™ SYBR Green supermix (Bio-Rad Laboratories) in a 20 µl volume. Data were analyzed using BioRad CFX Manager Version 2.1. Relative fold changes in mRNA between treatments were determined based on the ΔΔCT method after normalizing to the housekeeping gene elongation factor 1α (EF-1α) (Livak and Schmittgen, 2001). Samples were run in triplicate and averaged.

TABLE 1 | Primers used in this study.

Gene	Sequence (5'→3')	Reference
PR-1 For	TGCCCCTTCAGGTAAATCGT	Hirao et al. (2012)
PR-1 Rev	GCGGGTCGTAGTTGCAGATAA	
PR-2 For	CGACAACATTCGCCCCTTCT	
PR-2 Rev	CTGCAGCGCGGTTTGAATAT	
PR-3 For	CCATCGAAGCCCAGGTAATTT	
PR-3 Rev	AGCCGGGAAGCAATATTATGGT	
PR-4 For	CCCCGTTACTGTCAATTGCAT	
PR-4 Rev	AAAGCGTGACGGTGCGTATT	
PR-5 For	GAACCAGTGCCCATACACAGTCT	
PR-5 Rev	CCTGCGGCAACGTTAAAAGTC	
PR-6 For	TGCTGGCGGCATCTATTTTA	
PR-6 Rev	TAACACCTGCGCAAATGCA	
PR-9 For	ACACCACCGTGCTGGACATT	
PR-9 Rev	GTGCGGGAGTCGGTGTAGAG	
PR-10 For	TGTCTCAAGTGGAGGCAAGGA	
PR-10 Rev	AAGCGACAATTTCAGGCAAAAC	
EF-1α For	GGGAAGCCACCCAAAGTTTT	
EF-1α Rev	TACATGGGAAGACGCCGAAT	
PdPR-4 For	TGTGACGAATCCTTCAACGC	Lee et al. (2019)
PdPR-4 Rev	AAAGCCGCGGTTTCAAGATC	
PdCHI For	TTCATCACAGCTGCCAATGC	
PdCHI Rev	ATGCTCCAGTTTCGTGCATC	
PdBGL2 For	AAGTCCGTGCATTCTCAACG	
PdBGL2 Rev	TCCGCCATGGAAAATTTGGG	

Efficacy of JCK-1233 in the Control of PWD by B. xylophilus on Pinus densiflora and P. thunbergii Seedlings

The disease control efficacy of the JCK-1233 bacterial strain was evaluated against PWD on three- and four-year-old *P. densiflora* (black pine) and *P. thunbergii* (red pine) seedlings with an average height of 40 cm and an average root-collar calliper of 0.5 cm. JCK-1233 were cultured in TSB at 30°C for 24 h with shaking at 150 rpm. Each culture was diluted using distilled water containing Tween 20 (250 mg/l) to a final concentration of 8×10^8 colony-forming units (cfu)/ml using a UV–VIS spectrophotometer (UV-1601; Shimadzu Co., Kyoto, Japan). Black pine and red pine seedlings pre-treated with Tween 20 (5 ml, 250 mg/l) per seedling were foliar sprayed twice with a JCK-1233 bacterial suspension (5 ml/seedling) at one-week interval. Distilled water containing Tween 20 (250 mg/l) was used as an untreated control. Emamectin benzoate (20 mg/ml) was supplied from Syngenta Korea (Seoul, South Korea) and used once as a positive control for treatment by trunk injection (100 µl/seedling). After one week from trunk injection with emamectin benzoate or the second treatment with the bacterial suspension, pine seedlings were inoculated with PWN as previously reported by Kwon et al. (2010). After making a small slit with a surface-sterilized knife in the stem of the seedlings, a small piece of absorbent cotton was inserted into the slit, and a water suspension of nematodes (2,000 nematodes/100 µl) was pipetted onto the absorbent cotton. The slits were then covered with Parafilm to prevent drying. PWD severity was evaluated according to the wilting and consequent discoloration area of the needles (Proença et al., 2010). The experiments were repeated twice in five replicates.

In Vitro Nematicidal Activity of JCK-1233 Culture Filtrates Against B. xylophilus

The nematicidal activity of JCK-1233 culture filtrates was evaluated testing their effect on the mortality of PWN *B. xylophilus*. Treatments were performed in 96 well tissue culture plates containing approximately 50 PWNs/well. To prevent solution evaporation, the plates were covered and kept in the dark at 25°C with gentle shaking. Three days after exposure, the PWNs were moved to tap water and grouped into motile and immotile categories based on observations made under a light microscope (Leica DM IL LED; Leica Microsystems CMS GmbH, Wetzlar, Germany) after pricking their bodies with a fine needle. PWNs that did not move and retained a stiff and straight body shape even after pricking with a needle were considered dead. TSB medium was used as a negative control. The experiment was repeated twice with triplicate. To analyze the nematicidal activity of JCK-1233 against PWNs, the mortality of PWNs was converted to percentage mortality and corrected using the formula of Schneider-Orelli (1947): Mortality (%) = [(mortality percentage in treatment – mortality percentage in the negative control)/(100 – mortality percentage in the negative control)] × 100. The nematicidal activities of JCK-1233 were evaluated analyzing the mortality of PWNs over a

concentration range of 0.63 to 20%. The experiments were repeated twice in triplicate.

Molecular Identification of JCK-1233

A JCK-1233 isolate showing induced resistance activity in pine seedlings was identified by *recA* nucleotide sequence analysis. The genomic DNA of the JCK-1233 isolate was prepared using a DNeasy Blood and Tissue kit (Qiagen, Hilden, Germany) following the manufacturer's recommendations. PCR amplification of the *recA* gene was performed using the universal bacterial primer pair recA-F (5′-GATCGTCA RGCAGSCYTWGAT-3′)/recA-R (5′-TTWCCRACC ATAACSCCRAC-3′) in a 20 µl reaction mixture containing genomic DNA (2 µl), primers (1 µl of each, 10 pM), sterilized distilled water (16 µl), and Accupower® PCR premix (1 µl) (Bioneer Corp., Daejeon, South Korea). The PCR conditions were 95 for 10 min, followed by 35 cycles of 95 for 30 s, 49 for 30 s, and 72 for 1 min, and then a final extension at 72 for 5 min. The result from the *recA* sequencing was used to identify JCK-1233 based on the National Center for Biotechnology Information (NCBI) blast database. Sequence alignment and phylogenetic analysis were performed using the neighbor-joining (NJ) method with MEGA 6, with the number of bootstrap trials set to 1000. The Kimura 2-parameter model was selected as the best model to construct the tree for NJ (Tamura et al., 2013).

Extraction and Isolation of Potential Resistance Inducers From B. thuringiensis JCK-1233 Strain Cultures

To find the chemicals giving inducible resistance to plants from the culture filtrates of *B. thuringiensis* JCK-1233, the strain was pre-cultured in in tryptic soy broth (TSB) medium overnight at 37 . Then, JCK-1233 was grown in TSB medium to an OD_{600} of 0.8. The cultured broth of TSB-1233 (72 L) was condensed to 10 L on a rotary evaporator *in vacuo* at 40°C. Then, the condensed broth was partitioned with CH_2Cl_2 to yield different fractions. The CH_2Cl_2 soluble fraction was subjected to silica gel column chromatography (CC) and eluted with CH_2Cl_2-MeOH (100:0 to 0:100, gradient, v/v), producing 13 fractions (C1–C13). Fraction C7 was further fractionated by silica gel CC and eluted with CH_2Cl_2-acetone (20:1, v/v) to give eight sub-fractions (C7.1–C7.8). Sub-fraction C7.5 was purified by semi-preparative RP-HPLC [Gilson Trilution System, Middleton, WI, USA; YMC Pak ODS-A column (20 × 250 mm, 5 µm particle size), YMC Co., Kyoto, Japan; UV detection at 210 nm] using MeOH and H_2O in a 0.1% TFA gradient (40:60–70:30, v/v) at a flow rate of 5 ml/min as a mobile phase. Fraction C11 was further fractionated by silica gel CC and eluted with CH_2Cl_2-acetone (10:1, v/v) to produce seven sub-fractions (C11.1–C11.7). Following a similar procedure to that used for C7.5, sub-fraction C11.4 was subjected to semi-preparative RP-HPLC using MeOH and H_2O in a 0.1% TFA gradient (50:50–70:30, v/v) at a flow rate of 5 ml/min as a mobile phase.

Characterization of Potential Resistance Inducers Isolated From B. thuringiensis JCK-1233 Strain Cultures

The optical rotations were measured using a Jasco P-1020 polarimeter (JASCO, Tokyo, Japan). The electrospray ionization (ESI) mass spectra were performed on an AGILENT 1100 LC-MSD trap spectrometer (Agilent Technologies, Palo Alto, CA, USA). High-resolution electrospray ionization mass spectra (HR-ESI-MS) were obtained from an Agilent 6530 Accurate-Mass Q-TOF LC/MS system (Agilent technology, Santa Clara, CA, USA). NMR spectra were recorded with a Bruker 500 MHz spectrometer (Bruker, Karlsruhe, Germany) using tetramethylsilane (TMS) as the internal standard. Silica gel (Merck, Darmstadt, Germany; 63 –200 µm particle size) and RP-18 (Merck, 75 µm particle size) were used for CC. TLC was performed using Merck silica gel 60 F_{254} and RP-18 F_{254} plates. Preparative reversed-phase (RP)-HPLC was performed using a Gilson Trilution System with an UV detector (UV/VIS-156) and a YMC Pak ODS-A column (20 × 250 mm, 5 µm particle size, YMC Co., Kyoto, Japan). HPLC solvents were purchased from Burdick & Jackson, USA.

Effect of Foliar Spray and Trunk Injection of DPKs Produced by B. thuringiensis JCK-1233 Against PWD

The disease control efficacy of diketopiperazines (DPKs) produced by JCK-1233 was evaluated against PWD on three- and four-year-old *P. thunbergii* (black pine) seedlings. Four DPKs isolated from JCK-1233 culture broth were diluted using distilled water containing Tween 20 (250 mg/l) to a working concentration of 1 mM and then used for trunk injection and foliar spray treatments. For trunk injection, four DPKs (1 mM) and emamectin benzoate (20 mg/ml) containing 5% MeOH were treated with 100 µl per seedling. For foliar application, four DPKs (1 mM, 5 ml per seedling) and JCK-1233 culture (OD_{600} = 0.8, 5 ml per seedling) were foliar sprayed on Tween 20 pre-treated seedlings twice at one-week interval. For untreated controls, the same amount of sterile TSB in distilled water containing Tween 20 (250 mg/l) for foliar spray and 5% MeOH for trunk injection was applied. After one week from the second foliar spray and trunk injection treatments, pine seedlings were inoculated with PWN (2,000 nematodes/100 µl). PWD severity was evaluated according to the wilting area of the seedling. The experiments were repeated twice in five replicates.

Effect of B. thuringiensis JCK-1233 and cyclo-(L-Pro-L-Ile) on the Expression of Defense Related Genes In Vivo

P. thunbergii (black pine) was used to analyze the effect on the defense related genes expression in pines. JCK-1233 was cultured to OD_{600} = 0.8 at 30 in TSB and then JCK-1233 bacterial suspension containing Tween 20 (250 mg/l, 5 ml per seedling) used for foliar spray. The selected diketopiperazine, *cyclo*-(L-Pro-L-Ile), was diluted using distilled water containing Tween 20

(250 mg/l) to a working concentration of 1 mM and then used for foliar spray (5 ml per Tween 20 pre-treated seedling twice at one-week interval). For untreated controls, the same amount of sterile TSB in distilled water containing Tween 20 (250 mg/l) was applied. After one week from the second treatment, pine seedlings were inoculated with PWN (2,000 nematodes/100 µl). Three replicates were performed for each treatment.

At 1 day after the first treatment (1 DAT), 1 day after the second treatment/8 days after the first treatment (8 DAT), and 1 day and 3 days after inoculation with PWN (1 DAI and 3 DAI), *P. thunbergii* total RNA was extracted from the pine needles using CTAB extraction buffer with elimination of high viscosity and excessive polysaccharides (Azevedo et al., 2003). Then, total RNA was further purified using IQeasy™ plus plant RNA extraction mini kit (iNtRON, Seongnam, South Korea), according to the manufacturer's recommendations. cDNA libraries were prepared from total pine needle RNA using oligo (dT) primers and SuperScript™ IV reverse transcriptase (Invitrogen Inc., Carlsbad, CA, USA), according to the manufacturer's protocols. The PCR primers used in this study (**Table 1**) were synthesized by Genotech (Daejeon, Korea).

Determination of relative mRNA expression was carried out in a real-time PCR detection system (Bio-Rad CFX 96; Bio-Rad Laboratories, Hercules, CA, USA). cDNA was analyzed using iQ™ SYBR Green supermix (Bio-Rad Laboratories) in a 20 µl volume. The data were analyzed using BioRad CFX Manager Version 2.1. Relative fold changes in mRNA between treatments were determined based on the ΔΔCT method after normalizing to the housekeeping gene elongation factor 1 alpha (Livak and Schmittgen, 2001). The samples were run in triplicate and averaged.

Statistical Analysis

The parameters measured in this study were designed to evaluate the efficacy of JCK-1233 and DPKs against PWN. The analyses were conducted separately for *in vitro* and *in vivo* experiments. All data were analyzed for homogeneity of variance using the SPSS statistical analysis software (version 21.0 for Windows; SPSS, Chicago, IL, USA). The data were expressed as means ± standard error of replicates and evaluated by one-way analysis of variance (ANOVA). Statistical differences among treatments were determined according to Duncan's multiple-range test ($p < 0.05$).

RESULTS

Primary Screening of Bacteria-Induced Resistance in Arabidopsis

Five hundred and four bacterial strains were isolated from plants of five different regions in Korea. The isolated bacteria were screened for their potential resistance-inducing abilities using transgenic *Arabidopsis* plant lines containing the β-glucuronidase (*GUS*) construct fused to the *PR-1* promoter, which are known to visualize the ability to elicit the SA signaling pathway when exposed to potential resistance inducers. After co-incubation of bacterial cultures with leaf discs from the *PR-1pro::GUS Arabidopsis* line, 24 isolates out of 504 endophytic bacteria showed increased transcriptional GUS activity compared to that of the untreated control (**Supplementary Data S1**).

Effect of the Selected Bacteria on PR-1 Transcript Expression in Pinus Calli

Among the 24 selected bacterial strains from the previous *PR-1pro::GUS Arabidopsis* assay, only 8 strains were shown in the pine callus assay to increase the expression of *PR-1* at least 1.3-fold compared to that in untreated controls (**Table 2**). Specifically, bacterial strain JCK-1233-treated calli showed the highest increase in *PR-1* gene expression (3.59-fold compared to that in the untreated control). Based on these results, JCK-1233 was selected for further experiments as a potent candidate for the induction of resistance in pine trees.

Efficacy of JCK-1233 in the Control of PWD by B. xylophilus on P. densiflora and P. thunbergii Seedlings

Treatment with a JCK-1233 culture suspension significantly reduced PWD severity in nematode-inoculated *P. densiflora* and *P. thunbergii* seedlings (**Figure 1**). Disease severity in *P. densiflora* seedlings treated by foliar spray with a JCK-1233 culture suspension was significantly reduced compared to the control (24.2% compared to 89.8% in treated and control samples, respectively). The control efficacy of JCK-1233 treatment was comparable to that of EB-treated seedlings, in which disease severity was 14.5% (**Figure 1A**). Moreover, wilting in EB and JCK-1233-treated *P. densiflora* seedlings appeared gradually, starting from 36 DAI with PWNs, while wilting in untreated controls of *P. densiflora* seedlings advanced rapidly starting from 20 DAI. Although the control efficacy of EB treatment (83.9%) was slightly higher than that of JCK-1233 treatment, JCK-1233 treatment showed a significant control efficacy of 73.1% against PWD in *P. densiflora* seedlings (**Figure 1C**).

A similar control efficacy of JCK-1233 treatment was observed in *P. thunbergii* seedlings, in which disease severity reached 42.5%, compared to 96.3% in untreated controls and

TABLE 2 | Relative transcription level of the SA marker *PR1* in *Pinus densiflora* calli inoculated with the initially selected endophytic bacterial strains.

Strain	*PR1*	Strain	*PR1*
JCK-757	2.19 ± 0.16	JCK-1229	1.08 ± 0.42
JCK-758-1	0.94 ± 0.41	JCK-1233	3.59 ± 0.88
JCK-758-2	0.58 ± 0.12	JCK-1266	0.73 ± 0.19
JCK-761	1.30 ± 0.14	JCK-1287	0.66 ± 0.09
JCK-767	0.24 ± 0.10	JCK-1288	1.49 ± 0.34
JCK-947	0.82 ± 0.13	JCK-1307	0.42 ± 0.16
JCK-1005	0.67 ± 0.09	JCK-1308	0.64 ± 0.21
JCK-1180	2.04 ± 0.49	JCK-1309	1.31 ± 0.42
JCK-1182	0.55 ± 0.12	JCK-1318	1.89 ± 0.33
JCK-1187	2.25 ± 0.68	JCK-1320	0.94 ± 0.34
JCK-1217	0.75 ± 0.11	JCK-1328	1.09 ± 0.15
JCK-1222	0.12 ± 0.02	JCK-1333	0.85 ± 0.31

Data was presented as the mean ± standard deviation of three biological replicates.

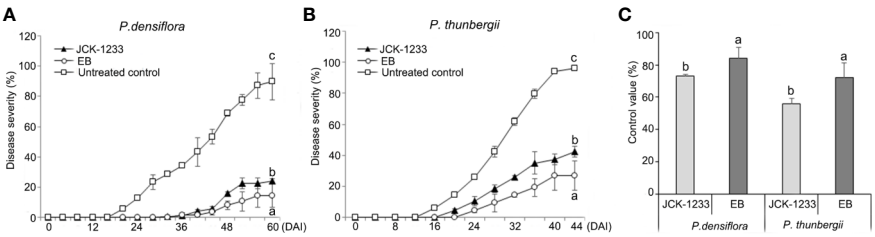

FIGURE 1 | Effect of the JCK-1233 culture broth against pine wilt disease caused by pine wood nematodes in pine seedlings. Disease severity after inoculation with pine wood nematodes in **(A)** red pine (*Pinus densiflora*) seedlings, and **(B)** black pine (*Pinus thunbergii*) seedlings. **(C)** Disease control efficacy at 28 days after inoculation (DAI) in *P. densiflora* and *P. thunbergii* seedlings. Data was represented as the mean and standard error of two runs with five replicates per run. Different lower case letters shown values that are significantly different ($p < 0.05$) level by Duncan's test.

26.7% in EB treated seedlings (**Figure 1B**). In addition, wilting in untreated *P. thunbergii* appeared earlier (16 DAI) than in EB- and JCK-1233-treated-*P. thunbergii* seedlings, in which wilting appeared gradually from 24 and 20 DAI, respectively. In agreement with the results on *P. densiflora* seedlings, JCK-1233 treatment also showed a significant control efficacy against PWD in *P. thunbergii* seedlings, although its control efficacy (55.8%) was a little lower than that of EB treatment (72.3%) (**Figure 1C**).

In Vitro Nematicidal Activity of JCK-1233 Culture Suspensions Against B. xylophilus

The effect of JCK-1233 culture suspensions on *B. xylophilus* juvenile mortality was determined at 3 days after exposure. There was no effect of JCK-1233 treatment on *B. xylophilus* juvenile mortality at the tested concentrations (0.63–20%) compared to the TSB control treatment (0.8–1.9 and 2.3%, respectively), whereas treatment with EB exhibited more than 99% mortality from a concentration of 0.33 μg/ml (**Table 3**). Therefore, JCK-1233 does not seem to have a direct nematicidal activity against *B. xylophilus*.

Molecular Identification of JCK-1233

The selected bacterial isolate JCK-1233 was identified as *B. thuringiensis* based on BLAST and phylogenetic analyses of the amplified *recA* gene sequence (**Figure 2**). The amplified genes were registered in GenBank under the accession number MT024187. *B. thuringiensis* JCK-1233 was deposited in the KCCM (Korean Culture Center of Microorganisms, Seoul, Korea) as KCCM 14085BP.

TABLE 3 | The *in vitro* nematicidal activity of JCK-1233 culture filtrates.

Sample	Concentration	Mortality (%)	STD
Emamectin	3	100.0a	0.0
Benzoate	1	99.0a	0.9
(μg/ml)	0.33	98.9a	2.0
	0.11	64.0b	2.8
	0.04	51.3c	5.3
	0.01	17.2d	1.5
JCK-1233	20	1.9e	2.0
(%)	10	1.9e	1.8
	5	0.8e	1.3
	2.5	0.7e	1.3
	1.25	2.2e	0.1
	0.63	0.8e	1.3
TSB	–	2.3e	0.8

Each value represents the mean and standard deviation of two runs with three replicates per run. Different lower case letters shown values that are significantly different ($p < 0.05$) level by Duncan's test.

Extraction and Isolation of Putative Active Compounds From B. thuringiensis JCK-1233 Strain Culture

The condensed broth of JCK-1233 (10 L) was partitioned with CH_2Cl_2, yielding CH_2Cl_2 (2.3 g) fractions. Using silica gel column chromatography (CC), the CH_2Cl_2 soluble fraction (2.3 g) was divided into 13 fractions (C1–C13). Fraction C7 (510 mg) was further fractionated by silica gel CC and eluted with CH_2Cl_2-acetone (20:1, v/v) to give eight sub-fractions (C7.1–C7.8). Sub-fraction C7.5 (253.5 mg) was purified by semi-preparative RP-HPLC using MeOH and H_2O in a 0.1% TFA gradient (40:60–70:30, v/v) at a flow rate of 5 ml/min as a mobile phase to give compounds 2 (40 mg) and 3 (55 mg). Fraction C11 (255 mg) was fractionated by silica gel CC and eluted with CH_2Cl_2-acetone (10:1, v/v) to produce seven sub-fractions (C11.1–C11.7). Following a similar procedure to that used for C7.5, sub-fraction C11.4 (140 mg) was subjected to semi-preparative RP-HPLC using MeOH and H_2O in a 0.1% TFA gradient (50:50–70:30, v/v) at a flow rate of 5 ml/min as a mobile phase to give compounds 1 (5.5 mg) and 4 (5.0 mg).

Characterization of Potential Resistance Inducers Isolated From B. thuringiensis JCK-1233 Strain Culture

The presence of the DKP ring system in compounds 1–4 was evident from the characteristic carbon chemical shifts of two amide and carbonyl groups (δ_C 166.6–172.6 ppm), and proton chemical shifts of the two methine residues (δ_H 3.63–4.45 ppm) (**Supplementary Data S2**) (Jayatilake et al., 1996; Fdhila et al., 2003). The evidence of proline as one of the components of DKP (compounds 1–3) was deduced from the presence of three broad methylene multiplets (δ_H 1.25–3.65 ppm) in these compounds. Based on the analysis of 1-dimensional NMR data, COSY correlations and literature values, valine, isoleucine, and phenylalanine were identified as the second amino acid residue

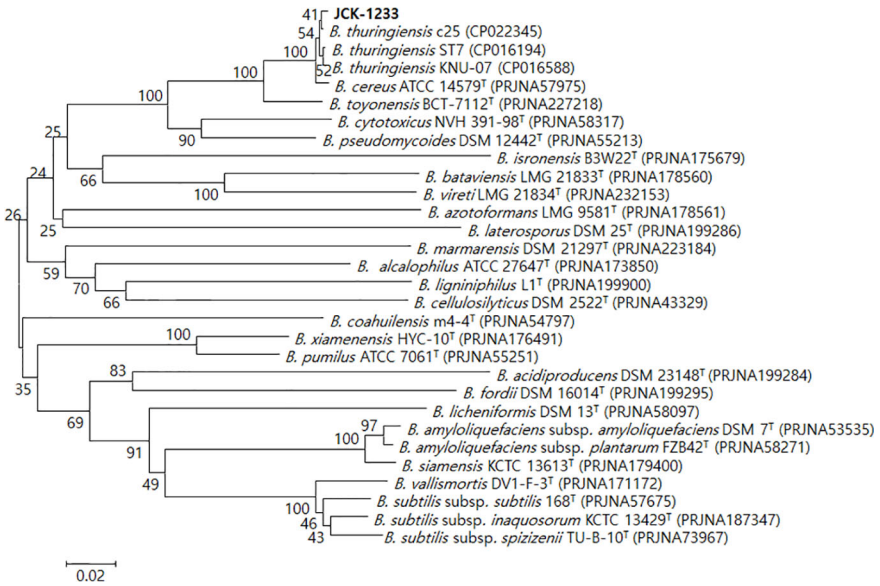

FIGURE 2 | Phylogenetic tree derived from a distance analysis of *recA* gene sequences in JCK-1233. The sequences were aligned using MEGA 6.0 software. The phylogenetic tree was constructed using the neighbor-joining (NJ) method with bootstrap analysis (1,000 trials). The selected strain was identified as *Bacillus thuringiensis*, which was separated in an exclusive cluster. Bars indicate the percentage of sequence divergence. T represents the type strains.

in compounds 1–3, respectively (Jayatilake et al., 1996; Campo et al., 2009; Li et al., 2012; Ding et al., 2013; Jiang and Yang, 2013), while in compound 4 it was the combination of leucine and valine (Ding et al., 2013). The configuration of the DKPs was determined by analysis of the NOESY spectrum and comparison with the optical rotations in the literature. In addition, in the NOESY spectrums, the NOE interactions between H-6 and H-9 observed in compounds 2 and 3, but not in compound 1, indicated that these two methine protons have the same orientation in compounds 2 and 3, and a different orientation in compound 1. Based on the above analysis and combined with the positive optical rotation values of compounds 1, and the negative optical rotation values of compounds 2 and 3, the structures of compounds 1–3 were elucidated as *cyclo*-(D-Pro-L-Val) (Jayatilake et al., 1996; Shigemori et al., 1998; Fdhila et al., 2003; Campbell et al., 2009), *cyclo*-(L-Pro-L-Ile) (Jayatilake et al., 1996; Fdhila et al., 2003), and *cyclo*-(L-Pro-L-Phe) (Jayatilake et al., 1996; Fdhila et al., 2003), respectively (**Figure 3**). By a similar analysis, the structure of compound 4 was determined as *cyclo*-(L-Leu-L-Val) (Ding et al., 2013).

cyclo-(D-Pro-L-Val) (compound 1). Yellowish oil. $[\alpha]_D^{23}$ +43.8 (*c* 0.1, MeOH). ^{1}H NMR (500 MHz, CD_3OD): δ 4.26 (1H, m, H-7), 3.63 (1H, m, H-9), 3.65, 3.51 (each 1H, m, H-3), 2.37, 1.96 (each 1H, m, H-5), 2.17 (1H, m, H-10), 2.04, 1.91 (each 1H, m, H-4), 1.05 (3H, d, *J* = 7.0, H-11), 1.02 (3H, d, *J* = 7.0, H-12). ^{13}C NMR (125 MHz, CD_3OD): δ 171.5 (C-7), 168.1 (C-1), 64.5 (C-9), 59.8 (C-6), 46.8 (C-3), 34.5 (C-10), 30.4 (C-5), 23.0 (C-4), 19.5 (C-11), 18.5 (C-12). HRESI-MS *m*/*z* 197.1283 $[M+H]^+$ (calcd for $C_{10}H_{17}N_2O_2$, 197.1290).

cyclo-(L-Pro-L-Ile) (compound 2). Yellowish oil. $[\alpha]_D^{23}$ −25.5 (*c* 0.14, MeOH). ^{1}H NMR (500 MHz, CD_3OD): δ 4.22 (1H, m, H-6), 4.10 (1H, m, H-9), 3.52-3.59 (2H, m, H-3), 2.34, 1.96 (each 1H, m, H-5), 2.20 (1H, m, H-10), 2.04, 1.95 (each 1H, m, H-4), 1.47, 1.34 (each 1H, m, H-11), 1.10 (3H, d, *J* = 7.0, H-13), 0.96 (3H, t, *J* = 7.5, H-12). ^{13}C NMR (125 MHz, CD_3OD): δ 172.6 (C-7), 167.6 (C-1), 60.0 (C-6), 61.3 (C-9), 46.2 (C-3), 37.1 (C-10), 29.6 (C-5), 25.5 (C-11), 23.3 (C-4), 15.6 (C-13), 12.7 (C-12). HRESI-MS *m*/*z* 211.1443 $[M+H]^+$ (calcd for $C_{11}H_{19}N_2O_2$, 211.1447).

cyclo-(L-Pro-L-Phe) (compound 3). Yellowish oil. $[\alpha]_D^{23}$–43.9 (*c* 0.16, MeOH). ^{1}H NMR (500 MHz, CD_3OD): δ 7.32 (2H, m, H-3', 5'), 7.30 (2H, m, H-2', 6'), 7.28 (1H, m, H-4'), 4.45 (1H, m, H-6), 4.07 (1H, m, H-9), 3.55, 3.34 (each 1H, m, H-3), 3.18 (2H, t, *J* = 4.5, H-10), 2.11, 1.27 (each 1H, m, H-5), 1.80 (2H, m, H-4). ^{13}C NMR (125 MHz, CD_3OD): δ 171.0 (C-7), 166.9 (C-1), 137.5 (C-1'), 131.1 (C-3', 5'), 129.5 (C-2', 6'), 128.1 (C-4'), 60.1 (C-9), 57.4 (C-6), 46.1 (C-3), 38.1 (C-10), 29.4 (C-5), 22.9 (C-4). HRESI-MS *m*/*z* 245.1295 $[M+H]^+$ (calcd for $C_{14}H_{17}N_2O_2$, 245.1290).

cyclo-(L-Leu-L-Val) (compound 4). White amorphous powder. $[\alpha]_D^{23}$ −38.1 (*c* 0.15, MeOH). ^{1}H NMR (500 MHz, CD_3OD): δ 3.97 (1H, m, H-3), 3.80 (1H, m, H-10), 2.24 (1H, m, H-11), 1.88 (1H, m, H-5), 1.77, 1.63 (each 1H, m, H-4), 1.08 (3H, d, *J* = 7.0, H-12), 1.00 (3H, d, *J* = 6.5, H-7), 0.98 (3H, d, *J* = 7.0, H-13), 0.97 (3H, d, *J* = 6.5, H-6). ^{13}C NMR (125 MHz, CD_3OD): δ 171.4 (C-8), 169.8 (C-1), 61.6 (C-10), 54.5 (C-3), 46.1 (C-4), 33.8 (C-11), 25.4 (C-5), 23.7 (C-7), 21.9 (C-6), 19.4 (C-12), 17.9 (C-13). HRESI-MS *m*/*z* 213.1611 $[M+H]^+$ (calcd for $C_{11}H_{21}N_2O_2$, 213.1603).

Effect of Foliar Spray and Trunk Injection of DPKs Produced by B. thuringiensis JCK-1233 Against PWD

The effect of trunk injection and foliar spray with four DPKs produced by *B. thuringiensis* JCK-1233, *cyclo*-(D-Pro-L-Val), *cyclo*-(L-Pro-L-Ile), *cyclo*-(L-Pro-L-Phe), and *cyclo*-(L-Leu-L-

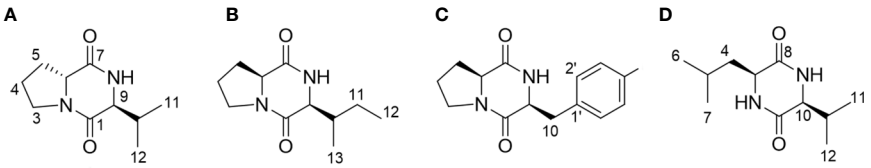

FIGURE 3 | Structural analyses of the isolated diketopiperazines from *Bacillus thuringiensis* JCK-1233. **(A)** *cyclo*-(D-Pro-L-Val), **(B)** *cyclo*-(L-Pro-L-Ile), **(C)** *cyclo*-(L-Pro-L-Phe), and **(D)** *cyclo*-(L-Leu-L-Val).

Val), on PWD control was determined in nematode-inoculated *P. thunbergii* seedlings 21 and 28 days after inoculation. Both trunk injection and foliar spray with a JCK-1233 culture filtrate or the four DPKs showed efficacy in reducing the severity of PWD in nematode-inoculated *P. thunbergii* seedlings (**Figure 4**). For trunk injection, the disease severity after treatment with compounds *cyclo*-(D-Pro-L-Val), *cyclo*-(L-Pro-L-Ile), *cyclo*-(L-Pro-L-Phe), and *cyclo*-(L-Leu-L-Val) were 30.0, 40.0, 51.7, and 25.8%, respectively, after 21 days of inoculation, and progressed to 79.2, 57.5, 81.7, and 51.7%, respectively, after 28 days (**Figure 4A**). The disease control efficacy at 28 days after inoculation was excellent in *cyclo*-(L-Leu-L-Val) and *cyclo*-(L-Pro-L-Ile) (**Figure 4B**).

In foliar spray, disease severity after treatment with *cyclo*-(D-Pro-L-Val), *cyclo*-(L-Pro-L-Ile), *cyclo*-(L-Pro-L-Phe), and *cyclo*-(L-Leu-L-Val) was 21.7, 28.3, 47.3, and 42.2%, respectively, after 21 days of inoculation, and progressed to 71.7, 45.8, 75.8, and 65.8%, respectively, after 28 days (**Figure 4C**). The disease severity and control value trends of the four DPKs upon foliar spray were similar to those observed upon trunk injection, except for *cyclo*-(L-Leu-L-Val) (**Figure 4D**). Specifically, after 28 days of inoculation, the disease control efficacy of *cyclo*-(L-Pro-L-Ile) by foliar spray (50.0%) was the highest among the four compounds, and its disease control efficacy by trunk injection (37.3%) against PWD was also as good as that of *cyclo*-(L-Leu-L-Val) (33.7%), which showed the highest efficacy when trunk injected (**Figures 4C, E**).

Effect of B. thuringiensis JCK-1233 and the Selected Bacterial Active Compound on the Expression of Defense Related Genes In Vivo

The effect of foliar spray with either *B. thuringiensis* JCK-1233 or the bioactive compound *cyclo*-(L-Pro-L-Ile) was tested on the expression of defense-related genes in pine seedlings. The relative expression level of *PR-1* gene was higher at 1 DAT with JCK-1233 and *cyclo*-(L-Pro-L-Ile) treatment (4.64-fold and 3.36-fold increase, respectively) compared to that in untreated control (**Figure 5A**). At 1 DAI with PWN, 6.84-fold and 6.58-fold increases in expression were observed in JCK-1233 and *cyclo*-(L-Pro-L-Ile) treated seedlings, respectively, compared to the untreated control at 1 DAT. However, untreated control seedlings showed a dramatic increase (16.40-fold) in *PR-1* gene expression at 1 DAI with PWN. The relative expression levels of *PR-2*, *PR-3*, *PR-4*, *PR-5*, *PR-9*, *PdBGL2*, and *PdPR-4* also showed a similar pattern to that of *PR-1* (**Figures 5A, B**).

On the contrary, the expression of *PR-10* in the untreated control at 1 DAI only increased 4.42-fold compared to that before inoculation, which was similar to the expression after JCK-1233 and *cyclo*-(L-Pro-L-Ile) treatments at the same time point. Moreover, unlike the expression of most pathogenesis-related genes, including *PR-1*, the expression of *PR-10* in JCK-1233 and *cyclo*-(L-Pro-L-Ile) treated plants after 8 days treatment was markedly enhanced (13.57-fold and 13.00-fold, respectively). The expression level of *PR-6* increased markedly at 1 DAI and did not significantly differ between the untreated control (9.63-fold), JCK-1233 treated (8.27-fold), and *cyclo*-(L-Pro-L-Ile) treated (8.49-fold) plants. In addition, after treatment and before nematode inoculation (at 1 DAT and 8 DAT), JCK-1233 and *cyclo*-(L-Pro-L-Ile) treated plants showed a significant increase in the *PR-6* expression level compared to that in untreated plants (**Figure 5A**).

These results suggest that *B. thuringiensis* JCK-1233 and *cyclo*-(L-Pro-L-Ile) enhance the expression of some pathogenesis-related genes in pine plants. However, such enhancement is lower than that produced in nematode-inoculated untreated control plants, except for *PR-6* and *PR-10* genes, indicating that the pathogenesis-related genes evaluated in this study may induce resistance against PWN in a different manner than the general hyposensitive reaction known to occur during SAR.

DISCUSSION

In this study, *B. thuringiensis* JCK-1233 was selected among 504 isolated bacteria for its ability to induce systemic resistance and suppress the severity of PWD. A DPK *cyclo*-(L-Pro-L-Ile) was identified as a bioactive compound from the selected strain and was shown to induce systemic resistance in pine calli and seedlings. Foliar treatment with the selected strain or the identified compound resulted in a significant reduction in the severity of PWD in inoculated pine seedlings. In general, foliar application using a biocontrol agent or its culture metabolite could represent a less expensive and more applicable approach compared to trunk injection with conventional chemical nematicides in largescale operations and mountainous forests with poor access.

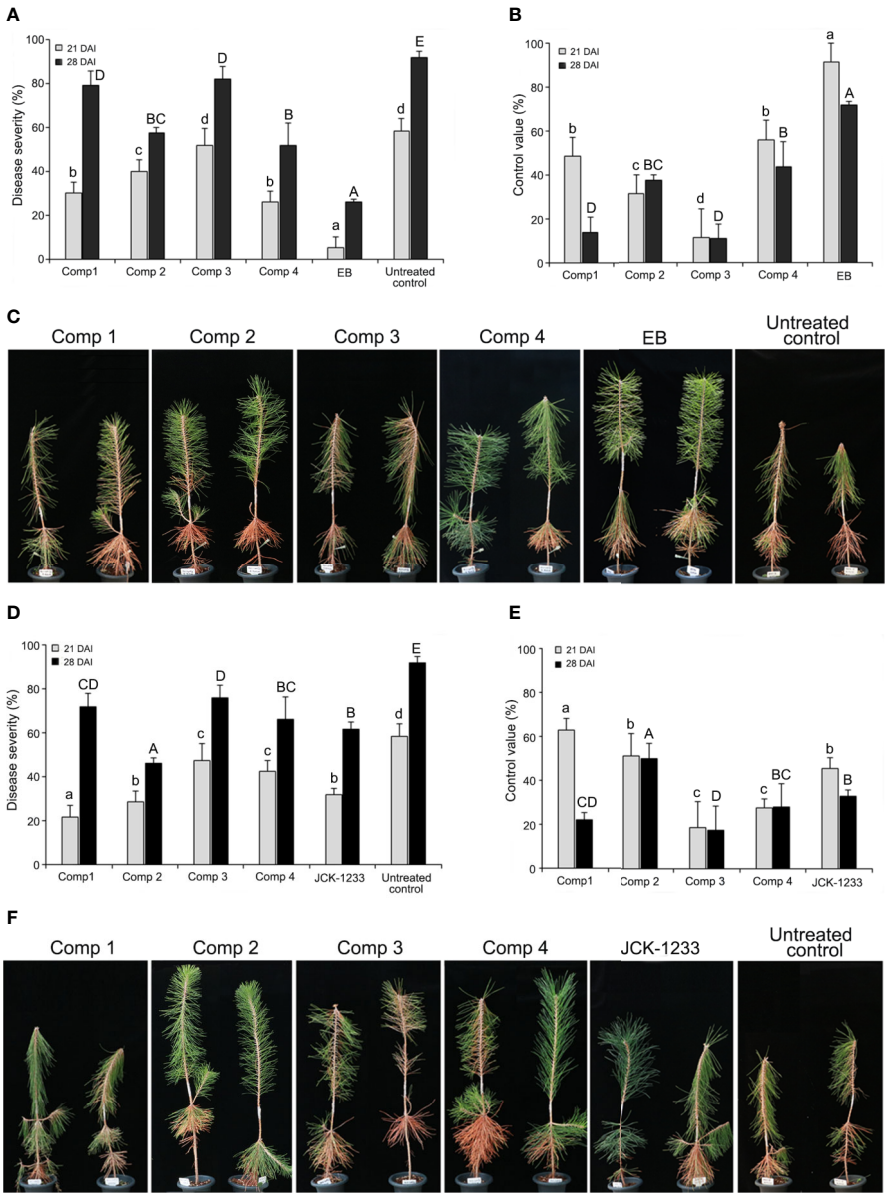

FIGURE 4 | Effect of trunk injection and foliar spray treatments with diketopiperazines on pine wilt disease (PWD). **(A)** Disease severity and **(B)** disease control efficacy of diketopiperazines against PWD at 21 and 28 days after inoculation (DAI) by trunk injection. **(C)** Photographs of the PWD wilting symptoms on pine seedlings treated with diketopiperazines by trunk injection at 28 DAI. **(D)** Disease severity and **(E)** disease control efficacy of diketopiperazines against PWD at 21 and 28 DAI by foliar spray. **(F)** Photographs of the PWD wilting symptoms on pine seedlings treated with diketopiperazines by foliar spray at 28 DAI. Comp 1, *cyclo*-(D-Pro-L-Val); Comp 2, *cyclo*-(L-Pro-L-Ile); Comp 3, *cyclo*-(L-Pro-L-Phe); Comp 4, *cyclo*-(L-Leu-L-Val); EB, emamectin benzoate as a positive control. Error bars represent standard deviation from five replicates. Data was represented as the mean and standard error of two runs with five replicates per run. Different lower and upper case letters shown values that are significantly different ($p < 0.05$) level by Duncan's test with data at 21 and 28 DAI, respectively.

Several DPKs were obtained from *B. thuringiensis* JCK-1233, including *cyclo*-(D-Pro-L-Val), *cyclo*-(L-Pro-L-Ile), *cyclo*-(L-Pro-L-Phe), and *cyclo*-(L-Leu-L-Val). DPKs are among the most common peptide derivatives found in natural products as well as in processed foods and beverages. Of the identified DPKs, foliar application of *cyclo*-(L-Pro-L-Ile) efficiently reduced the incidence of PWD and resulted in the elevated expression of defense-related genes, similar to the effect of *B. thuringiensis* JCK-1233 culture broth. Previous studies have reported the isolation of *cyclo*-(Pro-Ile) from *Aspergillus terreus* (mangrove-associated fungus), *Bacillus pumilus*, *Callyspongia* sp. (marine sponge), and *Trichoderma citrinoviride* (marine-derived fungus) (Shen et al., 2012; Brack et al., 2014; Chen et al., 2014; Zhang et al., 2014). Many studies have reported that DPKs exhibit various effects, including antibacterial, antifungal, antiviral, antitumor, and antitoxin activities (Yan et al., 2004; Noh et al.,

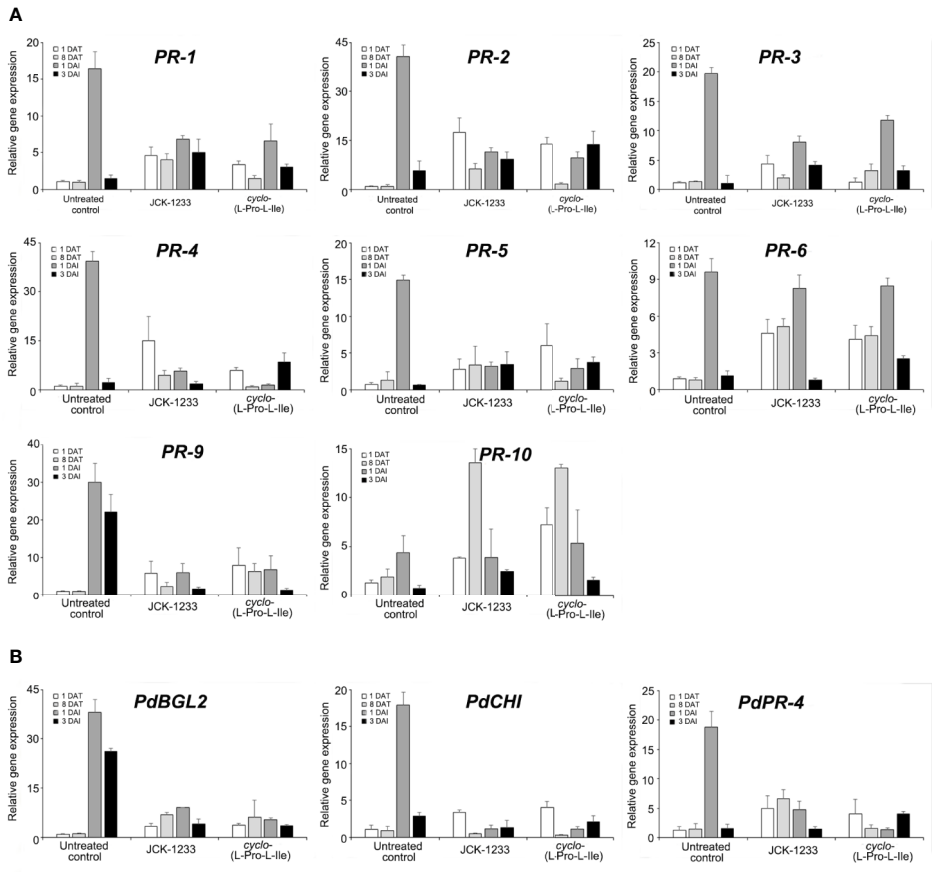

FIGURE 5 | Transcript levels of defense related genes in pine seedlings treated with *Bacillus thuringiensis* JCK-1233 and the bacterial active compound *cyclo*-(L-Pro-L-Ile). The expression of pathogenesis-related (PR) genes amplified with primers oriented from black pine trees (*Pinus thunbergii*) **(A)** and red pine trees (*Pinus densiflora*) **(B)**. Data was presented as the mean and standard error bars of three biological replicates.

2017). Plant growth promoting rhizobacteria (PGPRs) are also known to produce various DPKs that can induce resistance in plants (Noh et al., 2017). Although the plant signaling pathway mediated by DPKs is not clearly characterized yet, *cyclo*-(L-Ala-L-Ile), *cyclo*-(L-Ala-L-Leu), and *cyclo*-(L-Leu-L-Pro) isolated from *Bacillus vallismortis* BS07 elicited disease resistance in *Arabidopsis* against bacterial infection (Noh et al., 2017). However, little is known about the biological functions of *cyclo*-(L-Pro-L-Ile).

It was reported that SAR is associated with the SA signaling pathway, whereas ISR is mediated by the JA and ET signaling pathways (Van Loon et al., 1998). However, they share a lot of similarities both in the result and the mechanisms, and are interconnected by complex signaling networks and crosstalk phenomena (Pieterse et al., 2009; Niu et al., 2016). Several researches have also reported that PGPRs could trigger ISR by concurrently activating the SA- and JA-/ET-signaling pathways, and even activate SA-dependent SAR (De Meyer et al., 1999; Andreasson and Ellis, 2010; Niu et al., 2011; Niu et al., 2016). Song et al. (2017) reported that seed defense biopriming by root-associated *Bacillus gaemokensis* PB69 exhibited combined transcriptional responses with the upregulation of SA, ET, and jasmonic acid signaling. Furthermore, *B. cereus* AR156 is a PGPR that installs ISR to *Pseudomonas syringae* pv. *tomato* in *Arabidopsis* and enhances SAR with increased PR-1 protein expression in plants (Niu et al., 2016). Therefore, we predicted that endophytic bacteria may be able to stimulate the *PR-1* gene expression as well as resistance-inducing activities.

Since the molecular background of induced resistance mechanism in pine trees is not fully understood, we isolated resistance-inducing endophytic bacteria through mass screening using *Arabidopsis* seedlings of the *PR-1pro::GUS* line. Although *PR-1* is the marker gene for *Arabidopsis* SAR and SA-induced defense, it is a good indicator involved in pathogen- or microbe-associated molecular pattern (PAMPs/MAMPs) recognition. The induced resistance mechanism in different plant species was evaluated based on *PR-1* gene expression in *Pinus* calli after treatment with the endophytic bacteria that were selected based on their GUS activity in the *PR-1pro::GUS Arabidopsis* line. Here, we selected *B. thuringiensis* JCK-1233, which stimulated the expression of the *PR-1* gene in *Arabidopsis* and pine calli.

SA-mediated SAR responses are directed against biotrophic pathogens, occurring after the hypersensitive response (HR), which is a highly specific interaction between a plant resistance

protein and a pathogenic avirulent, leading to programmed cell death and pathogen growth arrest in the infected plant tissue (Glazebrook, 2005). However, this is literally the case of biotrophic pathogens, such as *Peronospora parasitica*, *Erysiphe* spp., and *Pseudomonas syringae*, not PWNs. Interestingly, the development of PWD caused by PWN *B. xylophilus* has been reported to be closely associated with the HR. HR as a part of the plant immune system is a successful strategy for the control of many potential pests and pathogens, but, in susceptible pine trees against PWNs, this same system causes pine death. Myers (1988) suggested that invasion and early migration of PWNs through tissues enforces a typical HR, such as parenchymal death, toxin production, and leakage of oleoresins and other materials into tracheids. With the rapid migration and propagation of PWNs, the HR spreads throughout the whole plant, and shortly after, susceptible pine trees die. Indeed, several studies in pines have shown a significant increase in the expression of resistance genes to virulent PWNs in susceptible pine trees (Hirao et al., 2012; Lee et al., 2019).

In *P. thunbergii*, the expression of *PR-1*, *PR-2*, *PR-3*, *PR-4*, *PR-5*, and *PR-6* was increased in susceptible trees but not in resistant trees after inoculation with PWNs (Hirao et al., 2012). In *Pinus densiflora*, inoculation of PWN also increased the expression of genes involved in the defense response, such as PR proteins (Lee et al., 2019). Consistent with these results, we observed a marked rise in the expression levels of *PR-1*, *PR-2*, *PR-3*, *PR-4*, *PR-5*, *PR-9*, *PdBGL*, *PdCHI*, and *PdPR-4* in the untreated control compared to JCK-1233 or *cyclo*-(L-Pro-L-Ile) treatment after inoculation with PWNs. In our gene expression analysis using the susceptible species *Pinus thunbergii*, we observed that the multitude of PR genes were upregulated up to 15–41-fold at 1 day after infection with PWNs compared to before infection, indicating that the HR can occur rapidly in pine seedlings infected with PWNs. Importantly, the untreated control group developed an HR much faster than the *B. thuringiensis* JCK-1233 and *cyclo*-(L-Pro-L-Ile) treated groups. Therefore, if there are treatments that can inhibit the migration of PWNs and alleviate the HR during infection, they may be helpful to control PWD. Our results suggest that disease resistance in pine trees may be caused by a moderate hypersensitive reaction.

Fitness-defense balance is important in terms of plant resistance against pathogens (Hirao et al., 2012). It is reasonable to think that plants express their inducible defense only if the protection against pathogens outweighs the costs of the resistance. However, in susceptible pine trees, plants develop an intense HR against PWNs. Although susceptible pine trees are unable to overcome infection, they develop excessive resistance systems, losing their fitness-defense balance and eventually dying. Pine seedlings treated with either *B. thuringiensis* JCK-1233 or its active compound DPK *cyclo*-(L-Pro-L-Ile) moderately increased expression of PR genes compared to that of the untreated control before and after inoculation with PWNs, suggesting that a moderate hypersensitive reaction can be a factor in their resistance against PWD. Although the exact mechanism behind the resistance induced by JCK-1233 and its active compound, DPK *cyclo*-(L-Pro-L-Ile), was not investigated, we hypothesize that it is involved in maintaining the fitness-defense balance. In addition, when PWNs infect susceptible pine trees, JCK-1233 and its active compound DPK *cyclo*-(L-Pro-L-Ile) may elicit a resistance consisting of interconnected complex signaling networks and, consequently, result in a moderate hypersensitive reaction. Therefore, we emphasize the importance of future investigations using molecular biological analyses to determine the functional mechanisms involved in the moderate HR induced by endophytic bacteria or DPKs, especially in susceptible pine trees, such as *P. thunbergii*, *P. koraiensis*, *P. densiflora*, and *P. pinaster*.

Among the tested PR genes, the expression patterns of *PR-6* and *PR-10* were different from those of other genes related to the HR, which were markedly expressed when susceptible pine trees were infected with PWNs. After inoculation with PWNs, there was no significant difference in the expression of *PR-6* and *PR-10* between the untreated and treated groups (**Figure 5**). Moreover, *B. thuringiensis* JCK-1233 and *cyclo*-(L-Pro-L-Ile) treated pine seedlings exhibited significantly higher expression levels of *PR-6* and *PR-10* than that in the untreated control before inoculation with PWN. PR-6 is known to be active in nematodes and insects, acting as a proteinase inhibitor (Devi et al., 2017). In plants, induced proteinase inhibitors often have putative proteinases targeted to the digestive tract of specific insect predators (Heitz et al., 1999). Thus, a protein fraction from soybean inhibited growth and proteolytic activity of the meal worm *Tribolium confusum in vitro* (Lipke et al., 1954). PR-10 was shown to be a ribonuclease-like protein acting against a digestive proteinase secreted by the root knot nematode *Meloidogyne incognita*, which results in a nematostatic condition *in vitro* (Andrade et al., 2010). Along the same lines, PR-10 is predicted to act as a proteinase against cellulases, β-1,3-glucanase, and pectate lyases secreted by PWNs (Kikuchi et al., 2004; Kikuchi et al., 2005; Kikuchi et al., 2006; Hirao et al., 2012). Although both PR-6 and PR-10 are not potent nematicidal proteins, they might have a role in the suppression of PWN propagation and migration during the early infection stage, representing an element in the induced resistance theory, moderate HR, proposed in this study.

In summary, *B. thuringiensis* JCK-1233 was selected among 504 isolated bacterial strains for its possible pine systemic resistance-inducing activity against PWD. Although the selected *B. thuringiensis* JCK-1233 did not have a direct nematicidal effect, foliar treatment of pine seedlings resulted in a significant reduction in PWD severity to a level comparable to that of EB trunk injection. In addition, out of the four DPKs isolated from the selected strain, the activity of *cyclo*-(L-Pro-L-Ile) was considered to be a main factor involved in the induction of pine seedling resistance by *B. thuringiensis* JCK-1233. Foliar application with *cyclo*-(L-Pro-L-Ile) showed better control efficacy compared to trunk injection, as observed at 28 DAI with PWN. Foliar application has two major advantages; it can effectively control PWD at a low cost, and it can be applied in the management of PWD in forests or areas that are inaccessible to humans. Treatment with *B. thuringiensis* JCK-1233 or the bioactive compound, the DPK *cyclo*-(L-Pro-L-Ile), moderately

enhanced the expression of various pathogenesis-related genes associated with plant immunity. As a result, a rapid and intense HR was suppressed, and a fitness-defense balance was adequately maintained. Based on our results, it may be possible to develop an eco-friendly agent for the control of PWD utilizing our proposed agent as an aerial application. This study could be the cornerstone for prospective studies on the induced resistance against PWD in susceptible pine trees worldwide.

DATA AVAILABILITY STATEMENT

The original contributions presented in the study are included in the article/**Supplementary Material**; further inquiries can be directed to the corresponding authors.

AUTHOR CONTRIBUTIONS

AP, S-IJ, Y-SS, and J-CK conceived this study. AP, S-IJ, HJ, JuK, NK, MH, MM, JunK, CL, BM, Y-SS, and J-CK performed the experiments. AP, S-IJ, CL, MM, BM, Y-SS, and J-CK analyzed the data. AP, S-IJ, MH, BM, Y-SS, and J-CK wrote the manuscript. All authors contributed to the article and approved the submitted version.

FUNDING

This research was supported by the National Institute of Forest Science, South Korea (FE0702-2016-11-2020).

SUPPLEMENTARY MATERIAL

The Supplementary Material for this article can be found online at: https://www.frontiersin.org/articles/10.3389/fpls.2020.01023/full#supplementary-material

REFERENCES

Andrade, L. B. D. S., Oliveira, A. S., Ribeiro, J. K., Kiyota, S., Vasconcelos, I. M., de Oliveira, J. T. A., et al. (2010). Effects of a novel pathogenesis-related class 10 (PR-10) protein from *Crotalaria pallida* roots with papain inhibitory activity against root-knot nematode *Meloidogyne incognita*. *J. Agr. Food Chem.* 58, 4145–4152. doi: 10.1021/jf9044556

Andreasson, E., and Ellis, B. (2010). Convergence and specificity in the Arabidopsis MAPK nexus. *Trends Plant Sci.* 15, 106–113. doi: 10.1016/j.tplants.2009.12.001

Azevedo, H., Lino-Neto, T., and Tavares, R. M. (2003). An improved method for high-quality RNA isolation from needles of adult maritime pine trees. *Plant Mol. Biol. Rep.* 21, 333–338. doi: 10.1007/BF02772582

Baermann, G. (1917). Eine einfache methode zur auffindung von Ancylostomum (Nematoden) larven in erdproben. *Geneeskd. Tijdschr. Ned. Indie.* 57, 131–137.

Bell, C. (2000). Fumigation in the 21st century. *Crop Prot.* 19, 563–569. doi: 10.1016/S0261-2194(00)00073-9

Bonifácio, L. F., Sousa, E., Naves, P., Inácio, M. L., Henriques, J., Mota, M., et al. (2014). Efficacy of sulfuryl fluoride against the pinewood nematode, *Bursaphelenchus xylophilus* (Nematoda: Aphelenchidae), in *Pinus pinaster* boards. *Pest Manage. Sci.* 70, 6–13. doi: 10.1002/ps.3507

Brack, C., Mikolasch, A., and Schauer, F. (2014). 2, 5-Diketopiperazines produced by *Bacillus pumilus* during bacteriolysis of *Arthrobacter citreus*. *Mar. Biotechnol.* 16, 385–395. doi: 10.1007/s10126-014-9559-y

Campbell, J., Lin, Q., Geske, G. D., and Blackwell, H. E. (2009). New and unexpected insights into the modulation of LuxR-type quorum sensing by cyclic dipeptides. *ACS Chem. Biol.* 4, 1051–1059. doi: 10.1021/cb900165y

Campo, V. L., Martins, M. B., da Silva, C. H., and Carvalho, I. (2009). Novel and facile solution-phase synthesis of 2, 5-diketopiperazines and O-glycosylated analogs. *Tetrahedron* 65, 5343–5349. doi: 10.1016/j.tet.2009.04.069

Chen, Y., Peng, Y., Gao, C., and Huang, R. (2014). A new diketopiperazine from South China Sea marine sponge *Callyspongia* sp. *Nat. Prod. Res.* 28, 1010–1014. doi: 10.1080/14786419.2014.903397

Chinnasri, B., Sipes, B., and Schmitt, D. (2006). Effects of inducers of systemic acquired resistance on reproduction of *Meloidogyne javanica* and *Rotylenchulus reniformis* in pineapple. *J. Nematol.* 38, 319–325.

De Meyer, G., Capieau, K., Audenaert, K., Buchala, A., Métraux, J.-P., and Höfte, M. (1999). Nanogram amounts of salicylic acid produced by the rhizobacterium *Pseudomonas aeruginosa* 7NSK2 activate the systemic acquired resistance pathway in bean. *Mol. Plant Microbe In.* 12, 450–458. doi: 10.1094/MPMI.1999.12.5.450

Devi, E. L., Kumar, S., Singh, T. B., Sharma, S. K., Beemrote, A., Devi, C. P., et al. (2017). "Adaptation strategies and defence mechanisms of plants during environmental stress," in *Medicinal Plants and Environmental Challenges* (Berlin, Germany: Springer). 359–413.

Ding, G.-Z., Liu, J., Wang, J.-M., Fang, L., and Yu, S.-S. (2013). Secondary metabolites from the endophytic fungi *Penicillium polonicum* and *Aspergillus fumigatus*. *J. Asian Nat. Prod. Res.* 15, 446–452. doi: 10.1080/10286020.2013.780349

Fdhila, F., Vázquez, V., Sánchez, J. L., and Riguera, R. (2003). DD-Diketopiperazines: antibiotics active against *Vibrio anguillarum* isolated from marine bacteria associated with cultures of *Pecten maximus*. *J. Nat. Prod.* 66, 1299–1301. doi: 10.1021/np030233e

Glazebrook, J. (2005). Contrasting mechanisms of defense against biotrophic and necrotrophic pathogens. *Annu. Rev. Phytopathol.* 43, 205–227. doi: 10.1146/annurev.phyto.43.040204.135923

Heitz, T., Geoffroy, P., Fritig, B., and Legrand, M. (1999). "The PR-6 family: proteinase inhibitors in plant-microbe and plant-insect interactions," in *Pathogenesis-related proteins in plants*. Eds. S. K. Datta and M. Subbaratnam (Boca Raton, FL: CRC Press), 131–155.

Hirao, T., Fukatsu, E., and Watanabe, A. (2012). Characterization of resistance to pine wood nematode infection in *Pinus thunbergii* using suppression subtractive hybridization. *BMC Plant Biol.* 12, 13. doi: 10.1186/1471-2229-12-13

Hoffmann-Sommergruber, K. (2002). Pathogenesis-related (PR)-proteins identified as allergens. *Biochem. Soc Trans.* 30, 930–935. doi: 10.1042/bst0300930

Jayatilake, G. S., Thornton, M. P., Leonard, A. C., Grimwade, J. E., and Baker, B. J. (1996). Metabolites from an antarctic sponge-associated bacterium, *Pseudomonas aeruginosa*. *J. Nat. Prod.* 59, 293–296. doi: 10.1021/np960095b

Jefferson, R. A., Kavanagh, T. A., and Bevan, M. W. (1987). GUS fusions: β-glucuronidase as a sensitive and versatile gene fusion marker in higher plants. *EMBO J.* 6, 3901–3907. doi: 10.1002/j.1460-2075.1987.tb02730.x

Jiang, K., and Yang, S. X. (2013). "Chemical constituents from marine Streptomycete sp. S15," in *Advanced Materials Research* (Baech, Switzerland: Trans Tech Publications). 1275–1277.

Kikuchi, T., Jones, J. T., Aikawa, T., Kosaka, H., and Ogura, N. (2004). A family of glycosyl hydrolase family 45 cellulases from the pine wood nematode *Bursaphelenchus xylophilus*. *FEBS Lett.* 572, 201–205. doi: 10.1016/j.febslet.2004.07.039

Kikuchi, T., Shibuya, H., and Jones, J. T. (2005). Molecular and biochemical characterization of an endo-β-1, 3-glucanase from the pinewood nematode

Bursaphelenchus xylophilus acquired by horizontal gene transfer from bacteria. *Biochem. J.* 389, 117–125. doi: 10.1042/BJ20042042

Kikuchi, T., Shibuya, H., Aikawa, T., and Jones, J. T. (2006). Cloning and characterization of pectate lyases expressed in the esophageal gland of the pine wood nematode *Bursaphelenchus xylophilus*. *Mol. Plant Microbe Interact.* 19, 280–287. doi: 10.1094/MPMI-19-0280

Kloepper, J. W., and Ryu, C.-M. (2006). "Bacterial endophytes as elicitors of induced systemic resistance," in *Microbial root endophytes*. Eds. B. J. E. Schulz, C. J. C. Boyle and T. N. Sieber (Berlin, Germany: Springer-Verlag), 33–52.

Kosaka, H., Aikawa, T., Ogura, N., Tabata, K., and Kiyohara, T. (2001). Pine wilt disease caused by the pine wood nematode: the induced resistance of pine trees by the avirulent isolates of nematode. *Eur. J. Plant Pathol.* 107, 667–675. doi: 10.1023/A:1011954828685

Kuroda, K. (2008). "Defense systems of Pinus densiflora cultivars selected as resistant to pine wilt disease," in *Pine Wilt Disease: A Worldwide Threat to Forest Ecosystems*. Eds. M. Mota and P. Vieiral (Berlin, Germany: Springer), 313–320.

Kwon, T.-S., Song, M.-Y., Shin, S.-C., and Park, Y.-S. (2005). Effects of aerial insecticide sprays on ant communities to control pine wilt disease in Korean pine forests. *Appl. Entomol. Zool.* 40, 563–574. doi: 10.1303/aez.2005.563

Kwon, H. R., Choi, G. J., Choi, Y. H., Jang, K. S., Sung, N. D., Kang, M. S., et al. (2010). Suppression of pine wilt disease by an antibacterial agent, oxolinic acid. *Pest Manage. Sci.* 66, 634–639. doi: 10.1002/ps.1920

Lee, I. H., Han, H., Koh, Y. H., Kim, I. S., Lee, S.-W., and Shim, D. (2019). Comparative transcriptome analysis of *Pinus densiflora* following inoculation with pathogenic (*Bursaphelenchus xylophilus*) or non-pathogenic nematodes (*B. thailandae*). *Sci. Rep.* 9, 1–11. doi: 10.1038/s41598-019-48660-w

Li, X.-J., Zhang, Q., Zhang, A.-L., and Gao, J.-M. (2012). Metabolites from *Aspergillus fumigatus*, an endophytic fungus associated with *Melia azedarach*, and their antifungal, antifeedant, and toxic activities. *J. Agric. Food Chem.* 60, 3424–3431. doi: 10.1021/jf300146n

Lipke, H., Fraenkel, G., and Liener, I. E. (1954). Growth inhibitors. Effect of soybean inhibitors on growth of *Tribolium confusum*. *J. Agr. Food Chem.* 2, 410–414. doi: 10.1021/jf60028a003

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

Maehara, N., and Futai, K. (2000). Population changes of the pinewood namatode, *Bursaphelenchus xylophilus* (Nematoda: Aphelenchoididae), on fungi growing in pine-branch segments. *Appl. Entomol. Zool.* 35, 413–417. doi: 10.1303/aez.2000.413

Mamiya, Y., and Enda, N. (1972). Transmission of *Bursaphelenchus lignicolus* (Nematoda: Aphelenchoididae) by *Monochamus alternatus* (Coleoptera: Cerambycidae). *Nematologica* 18, 159–162. doi: 10.1163/187529272X00395

Mota, M. M., Braasch, H., Bravo, M. A., Penas, A. C., Burgermeister, W., Metge, K., et al. (1999). First report of *Bursaphelenchus xylophilus* in Portugal and in Europe. *Nematology* 1, 727–734. doi: 10.1163/156854199508757.

Myers, R. F. (1988). Pathogenesis in pine wilt caused by pinewood nematode, Bursaphelenchus xylophilus. *J. Nematol.* 20, 236–244.

Narusaka, M., Abe, H., Kobayashi, M., Kubo, Y., Kawai, K., Izawa, N., et al. (2006). A model system to screen for candidate plant activators using an immune-induction system in *Arabidopsis*. *Plant Biotech.* 23, 321–327. doi: 10.5511/plantbiotechnology.23.321

Niu, D.-D., Liu, H.-X., Jiang, C.-H., Wang, Y.-P., Wang, Q.-Y., Jin, H.-L., et al. (2011). The plant growth–promoting rhizobacterium *Bacillus cereus* AR156 induces systemic resistance in *Arabidopsis thaliana* by simultaneously activating salicylate-and jasmonate/ethylene-dependent signaling pathways. *Mol. Plant Microbe In.* 24, 533–542. doi: 10.1094/MPMI-09-10-0213

Niu, D., Wang, X., Wang, Y., Song, X., Wang, J., Guo, J., et al. (2016). *Bacillus cereus* AR156 activates PAMP-triggered immunity and induces a systemic acquired resistance through a *NPR1*-and SA-dependent signaling pathway. *Biochem. Bioph. Res. Co.* 469, 120–125. doi: 10.1016/j.bbrc.2015.11.081

Noh, S. W., Seo, R., Park, J.-K., Manir, M. M., Park, K., Sang, M. K., et al. (2017). Cyclic dipeptides from *Bacillus vallismortis* BS07 require key components of plant immunity to induce disease resistance in *Arabidopsis* against *Pseudomonas* Infection. *Plant Pathol. J.* 33, 402–409. doi: 10.5423/PPJ.OA.11.2016.0255

Nose, M., and Shiraishi, S. (2008). "Breeding for resistance to pine wilt disease," in *Pine wilt disease* (Tokyo, Japan: Springer). 334–350.

Ogura, R., Matsuo, N., Wako, N., Tanaka, T., Ono, S., and Hiratsuka, K. (2005). Multi-color luciferases as reporters for monitoring transient gene expression in higher plants. *Plant Biotech.* 22, 151–155. doi: 10.5511/plantbiotechnology.22.151

Ono, S., Tanaka, T., Watakabe, Y., and Hiratsuka, K. (2004). Transient assay system for the analysis of *PR-1a* gene promoter in tobacco BY-2 cells. *Biosci. Biotechnol. Biochem.* 68, 803–807. doi: 10.1271/bbb.68.803

Pieterse, C. M., Leon-Reyes, A., Van der Ent, S., and Van Wees, S. C. (2009). Networking by small-molecule hormones in plant immunity. *Nat. Chem. Biol.* 5, 308–316. doi: 10.1038/nchembio.164

Proença, D. N., Francisco, R., Santos, C. V., Lopes, A., Fonseca, L., Abrantes, I. M., et al. (2010). Diversity of bacteria associated with *Bursaphelenchus xylophilus* and other nematodes isolated from *Pinus pinaster* trees with pine wilt disease. *PloS One* 5, e15191–e15151. doi: 10.1371/journal.pone.0015191

Rohrbach, K. G., and Apt, W. J. (1986). Nematode and disease problems of pineapple. *Plant Dis.* 70, 81–87. doi: 10.1094/PD-70-81

Schneider-Orelli, O. (1947). *Entomologisches praktikum: Einführung in die land-und forstwirtschaftliche Insektenkunde* (Verlag Sauerländer Switzerland: Aarau).

Shen, Y., Zou, J., Xie, D., Ge, H., Cao, X., and Dai, J. (2012). Butyrolactone and cycloheptanetrione from mangrove-associated fungus *Aspergillus terreus*. *Chem. Pharm. Bull.* 60, 1437–1441. doi: 10.1248/cpb.c12-00616

Shigemori, H., Tenma, M., Shimazaki, K., and Kobayashi, J. I. (1998). Three new metabolites from the marine yeast *Aureobasidium pullulans*. *J. Nat. Prod.* 61, 696–698. doi: 10.1021/np980011u

Song, G. C., Choi, H. K., Kim, Y. S., Choi, J. S., and Ryu, C.-M. (2017). Seed defense biopriming with bacterial cyclodipeptides triggers immunity in cucumber and pepper. *Sci. Rep.* 7, 1–15. doi: 10.1038/s41598-017-14155-9

Tóth, Á. (2011). *Bursaphelenchus xylophilus*, the pinewood nematode: its significance and a historical review. *Acta Biol. Szeged.* 55, 213–217.

Takai, K., Suzuki, T., and Kawazu, K. (2003). Development and preventative effect against pine wilt disease of a novel liquid formulation of emamectin benzoate. *Pest Manage. Sci.* 59, 365–370. doi: 10.1002/ps.651

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

Vallad, G. E., and Goodman, R. M. (2004). Systemic acquired resistance and induced systemic resistance in conventional agriculture. *Crop Sci.* 44, 1920–1934. doi: 10.2135/cropsci2004.1920

Van Loon, L., Bakker, P., and Pieterse, C. (1998). Systemic resistance induced by rhizosphere bacteria. *Annu. Rev. Phytopathol.* 36, 453–483. doi: 10.1146/annurev.phyto.36.1.453

Yan, P.-S., Song, Y., Sakuno, E., Nakajima, H., Nakagawa, H., and Yabe, K. (2004). Cyclo (L-leucyl-L-prolyl) produced by *Achromobacter xylosoxidans* inhibits aflatoxin production by *Aspergillus parasiticus*. *Appl. Environ. Microbiol.* 70, 7466–7473. doi: 10.1128/AEM.70.12.7466-7473.2004

Yano, S. (1913). Investigation on pine death in Nagasaki prefecture. *Sanrin-Kouhou* 4, 1–14.

Yi, C. K., Byun, B. H., Park, J. D., Yang, S., and Chang, K. H. (1989). First finding of the pine wood nematode, *Bursaphelenchus xylophilus* (Steiner et Buhrer) Nickle and its insect vector in Korea. *Res. Rep. For. Res. Ins. Seoul* 38, 141–149.

Zhang, Q.-Q., Chen, L., Hu, X., Gong, M.-W., Zhang, W.-W., Zheng, Q.-H., et al. (2014). Novel cytotoxic metabolites from the marine-derived fungus *Trichoderma citrinoviride*. *Heterocycles* 89, 189–196. doi: 10.3987/COM-13-12874

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 Microbiology

REVIEW
published: 14 July 2020
doi: 10.3389/fmicb.2020.01619

Modes of Action of Microbial Biocontrol in the Phyllosphere

*Marie Legein[1], Wenke Smets[1,2], Dieter Vandenheuvel[1], Tom Eilers[1], Babette Muyshondt[1], Els Prinsen[3], Roeland Samson[1] and Sarah Lebeer[1]**

[1] *Environmental Ecology and Applied Microbiology, Department of Bioscience Engineering, University of Antwerp, Antwerp, Belgium,* [2] *Department of Plant and Microbial Biology, University of California, Berkeley, Berkeley, CA, United States,* [3] *Laboratory for Integrated Molecular Plant Physiology Research, Department of Biology, University of Antwerp, Antwerp, Belgium*

A fast-growing field of research focuses on microbial biocontrol in the phyllosphere. Phyllosphere microorganisms possess a wide range of adaptation and biocontrol factors, which allow them to adapt to the phyllosphere environment and inhibit the growth of microbial pathogens, thus sustaining plant health. These biocontrol factors can be categorized in direct, microbe–microbe, and indirect, host–microbe, interactions. This review gives an overview of the modes of action of microbial adaptation and biocontrol in the phyllosphere, the genetic basis of the mechanisms, and examples of experiments that can detect these mechanisms in laboratory and field experiments. Detailed insights in such mechanisms are key for the rational design of novel microbial biocontrol strategies and increase crop protection and production. Such novel biocontrol strategies are much needed, as ensuring sufficient and consistent food production for a growing world population, while protecting our environment, is one of the biggest challenges of our time.

Keywords: biocontrol, phyllosphere, plant immune system, induced systemic resistance, antipathogenic mechanisms, plant pathogens, beneficial microbes, probiotics

OPEN ACCESS

Edited by:
Ioannis Stringlis,
Utrecht University, Netherlands

Reviewed by:
Matthew Agler,
Friedrich-Schiller University of Jena, Germany
Massimiliano Morelli,
Institute for Sustainable Plant Protection (CNR), Italy

****Correspondence:***
Sarah Lebeer
sarah.lebeer@uantwerpen.be

Specialty section:
This article was submitted to Microbe and Virus Interactions with Plants, a section of the journal Frontiers in Microbiology

Received: *19 March 2020*
Accepted: *22 June 2020*
Published: *14 July 2020*

Citation:
Legein M, Smets W, Vandenheuvel D, Eilers T, Muyshondt B, Prinsen E, Samson R and Lebeer S (2020) Modes of Action of Microbial Biocontrol in the Phyllosphere. Front. Microbiol. 11:1619. doi: 10.3389/fmicb.2020.01619

INTRODUCTION

Pathogens and pests cause between 20% and 30% of global crop yield losses (Savary et al., 2019). To ensure a sufficient and consistent yield we depend on chemical crop protection and growth-promoting products such as pesticides, herbicides, and fertilizers. Many of these chemical products pose a threat to human health and the environment, which fuels a demand for safer products (Nishimoto, 2019). A promising alternative is the use of microbial based products that can protect crops against diseases. Such microbial products are classified under biological control agents, defined as "a natural enemy, antagonist, or other organism, used for pest control" (ISPM 05, International Standards for Phytosanitary Measures). Although biocontrol is a broad term, including eukaryotic biocontrol agents such as yeasts, fungi, beneficial insects, and other non-microbial pests, in this review we focus on bacterial biocontrol agents. We will use the term biocontrol agent defined similarly as probiotics, "live microorganisms which when administered in adequate amounts confer a health benefit on the host" (Hill et al., 2014). We use this interpretation of a biocontrol agent because it does not only focus on antagonizing the pathogen, but also on improving plant health. Moreover, this definition allows to draw parallels between probiotic and biocontrol research. We will focus on the mechanisms of bacterial biocontrol agents targeting microbial pathogens.

The phyllosphere, the above-ground surface of plants, is a complex ecosystem where microorganisms and the host plant interact extensively to create specific, yet dynamic, communities. Microbial communities inhabit both the external surfaces (epiphytes) as the internal spaces (endophytes) and these communities play an important role in protecting the plant against diseases. Pathogens often have an epiphytic phase before entering the plant cell or the apoplast (intercellular space) (Pfeilmeier et al., 2016). In this review, we focus on external leaf applications of biocontrol agents, unless otherwise specified.

In analogy to a successful probiotic micro-organism, a successful biocontrol agent needs both specific adaptations that allow survival in the phyllosphere habitat (adaptation factors), as well as factors that contribute to the health of the host plant, by inhibiting the pathogen (probiotic or biocontrol factors) (Lebeer et al., 2008). To exert their beneficial properties, biocontrol agents need to be adapted to abiotic environmental factors as well as biotic host-specific factors. A general overview of environmental adaptation factors for the phyllosphere can be found in a review by Vorholt (2012). Adaptation factors are often overlooked in biocontrol research. However, low efficacy of biocontrol agents in field studies is often due to a lack of adaptation rather than a lack of biocontrol factors (Zeriouh et al., 2014; Salvatierra-Martinez et al., 2018). Moreover, a successful biocontrol agent needs a variety of adaptation and biocontrol factors to inhibit a pathogen and improve plant health (Köhl et al., 2019). Biocontrol factors can be related to direct or indirect microbial interactions (**Figure 1**). Direct interactions occur between the pathogen and the biocontrol agent. Indirect interactions are the interactions between the biocontrol agent and the host plant which improvesthe plant's fitness, like its resistance to the disease. In this review, we will give an overview of direct and indirect biocontrol and adaptation mechanisms relevant for biocontrol in the phyllosphere. Furthermore, we will describe these mechanisms and the genetic basis in detail, and indicate whether these mechanisms have been validated in the field, *in vitro* or in greenhouse experiments. An overview of biocontrol and adaptation factors discussed in the text is given in **Table 1**.

THE PHYLLOSPHERE MICROBIAL HABITAT

The phyllosphere is inhabited by a complex and dynamic community. The composition of this community depends on which microbes reach the phyllosphere in addition to abiotic factors such as climate, season and surrounding land use, and biotic factors such as leaf characteristics and host plant species (Maignien et al., 2014; Agler et al., 2016; Laforest-Lapointe et al., 2016; Smets et al., 2016). Microbes arrive on the phyllosphere rather stochastically via the air, soil, rain or insects. However, only selected taxa successfully colonize the phyllosphere (Maignien et al., 2014). Frequently occurring genera in phyllosphere communities are *Methylobacterium*, *Sphingomonas*, and *Pseudomonas* (Delmotte et al., 2009; Vorholt, 2012). These common phyllosphere bacteria possess specific adaptation factors to the phyllosphere. For example, *Methylobacterium* spp. have adapted to the low-nutrient environment by metabolizing single-carbon compounds such as methanol (Kutschera, 2007). *Sphingomonas* spp. cope with the scarcity of nutrients by being able to metabolize a wide range of carbon sources (Delmotte et al., 2009). *Pseudomonas* spp. use flagellar motility to reach more favorable sites (Haefele and Lindow, 1987), synthesize the biosurfactant syringafactin to increase the water availability on leaf surfaces (Hernandez and Lindow, 2019), and use effectors to leak water from the cells into the apoplast (Xin et al., 2016).

DIRECT INTERACTIONS

Antibiotic Metabolites and Binary Inhibitory Interactions

A key first step in the identification of novel biocontrol agents is the screening of antagonistic activities. Such screenings are increasingly applied at a larger scale. For example, Helfrich et al. (2018) recently screened more than 200 leaf isolates from *Arabidopsis thaliana* for binary inhibitory interactions, novel antagonistic strains and interesting metabolites. Most of these strains (88%) engaged in such inhibitory interactions. The orders *Bacillales* and *Pseudomonadales* were especially strong inhibitors, making up only 8% of the tested isolates but engaging in over 60% of the observed inhibitions. Most of the inhibitions also took place between distinct phylogenetic groups rather than within the same family or genus. Genome analysis using the antiSMASH tool (Blin et al., 2019) revealed that many of the inhibitory strains contained more biosynthetic gene clusters than average. These clusters can encode for metabolites with inhibitory effects. The top inhibitor of the collection, *Brevibacillus* sp. Leaf182, was shown to produce several non-ribosomal peptides with antimicrobial activity, such as marthiapeptide A (an anti-infective and cytotoxic polythiazole cyclopeptide previously isolated from deep-sea *Marinactinospora thermotolerans*), streptocidin D (a cyclic decapeptide antibiotic from the tyrocidine family, named after tyrothricin, the first commercially available antibiotic containing tyrocidine and gramicidin), and an unusual lysophospholipid (a bioactive molecule that possesses a large polar or charged head and a single hydrophobic carbon chain), which was active against Gram-negative bacteria. Previously, biocontrol activity by a *Brevibacillus brevis* strain against *Botrytis cinerea* had been observed in the phyllosphere of Chinese cabbage (Edwards and Seddon, 2001). This strain produces the antibiotic gramicidin S, another cyclic antibiotic non-ribosomal decapeptide and major constituent of tyrothricin. Comparison of biocontrol activity with an antibiotic-negative mutant and pure gramicidin S showed that gramicidin S was the mechanism behind the observed biocontrol.

The *Pseudomonas* genus is frequently found in the phyllosphere in relatively high abundances (Delmotte et al., 2009; Maignien et al., 2014). The *Pseudomonas* genus includes several commercialized biocontrol strains, such as *Pseudomonas chlororaphis* MA342 and *Pseudomonas* sp. DSMZ 13134. However, also several plant pathogens belong to this genus, such as the model phyllosphere pathogen *Pseudomonas syringae*

TABLE 1 | Overview of several known mechanisms by which phyllosphere microbes can inhibit pathogen growth.

In vitro screening	*In silico* screening	Compound	Mechanism/specific activity	Identified in	References	BC	A
1.1 Antibiotic metabolites							
Binary inhibitory interactions, purification and identification of compounds in supernatant	Screening for biosynthetic gene clusters using the antiSMASH tool	Antimicrobial secondary metabolites	Various	Various species	Helfrich et al., 2018	X	
	*srf*AC, *srf*AD	Lipopeptide, surfactin	Triggers biofilm formation	*Bacillus* spp.	Chen et al., 2007; Ongena and Jacques, 2008; Dunlap et al., 2013; Kim et al., 2015		X
	*fen*F, *myc*ABC	Lipopeptide, iturin	Interferes with lipid layers	*Bacillus* spp.	Chen et al., 2007; Ongena and Jacques, 2008; Dunlap et al., 2013; Kim et al., 2015	X	
	*pps*ABCDE	Lipopeptide, fengycin	Interferes with lipid layers	*Bacillus* spp.	Chen et al., 2007; Ongena and Jacques, 2008; Dunlap et al., 2013; Kim et al., 2015	X	
	phz gene cluster, *ehp* gene cluster	Phenazine	Interferes with histone acetylation and biofilm formation	*Pseudomonas* spp., *Pantoea* spp.	Giddens et al., 2002; Chin-A-Woeng et al., 2003	X	X
	*dda*A-I	Herbicolin I		*Pantoea vagans C9-1*	Kamber et al., 2012	X	
1.2 Hydrolytic enzymes							
Zymogram, or specific colorimetric assays	*chi*A, *chi*B, *chi*C or other genes encoding for glycosyl hydrolases from family 18 or 19 in the CAZy database	Hydrolytic enzymes: e.g., chitinase	Hydrolyses fungal cell wall	*Bacillus subtilis*	Essghaier et al., 2012	X	
	msp1 (p75)	Bifunctional peptidoglycan hydrolase/chitinase	Inhibits hyphae formation	*Lactobacillus casei* group species	Allonsius et al., 2019	X	
1.3 Quorum quenching and sensing							
	nis gene cluster (nisin), *spa* gene cluster (subtilin), *lux*I and *lux*R (AHLs)	Signalling molecules. Some gr- bacteria use bacteriocins (nisin and subtilin) that also have a signalling function	Quorum sensing	Nisin in *lactococcus lactis* and subtilin in *Bacillus subtilis*	Kleerebezem, 2004		X
Screening of bacteria interfering with the transcription of a reporter gene, induced by the signalling molecule of interest	*carAB* (degradation signaling molecule of *Xylella fastidiosa*), *aii*A (AHL lactonase)	Enzymes involved in degradation signalling factors	Quorum quenching	*Bacillus, Paenibacillus, Microbacterium, Staphylococcus*, and *Pseudomonas*	Newman et al., 2008; Morohoshi et al., 2009; Alymanesh et al., 2016	X	
1.4 Competition for nutrients and space							
Carbon source profiling and calculation of NOI	Genes related in carbohydrate metabolism (e.g., glycosyl hydrolases), or transport (e.g., Tonb receptors), using the CAZy database	Enzymes ensuring flexible carbohydrate metabolism, e.g., high diversity of TonB receptors	Increased competitiveness in a carbon limited environment	*Sphingomonas* spp.	Delmotte et al., 2009	X	X

(Continued)

TABLE 1 | Continued

In vitro screening	*In silico* screening	Compound	Mechanism/specific activity	Identified in	References	BC	A
Selective media with methanol as sole carbon source	*mxa*F	Conserved enzyme responsible for methanol dehydrogenase	Methylotrophy, increased adaptability in a carbon limited environment	*Methylobacterium* spp.	Mcdonald and Murrell, 1997		X
1.5 Siderophores							
Plate assay with indicator for detection of siderophores (Chrome azurol S assay)	Screening for siderophore gene clusters, using antiSMASH	Siderophores	Primary function is iron chelation. Siderophores can also have antibacterial properties trough the production of ROS and play a role in motility on the phyllosphere	*Pseudomonas protegens* CS1	Burbank et al., 2015; Santos Kron et al., 2020	X	X
2.1 Modulation plant hormone levels							
Colorimetric assays	*iac* gene cluster	Enzymes responsible for the degradation of indole-3-acetic acid (IAA)	IAA is used as an energy source and modulation of IAA levels induces physiological changes in the plant	*Pseudomonas putida* 1290	Leveau and Gerards, 2008	X?	X
HPLC analysis of extracts of the supernatant	*ipdC/ald*H *or dcc/ald*H *or iaa*M*/iaa*H *or nth*A	Enzymes involved in the production of IAA, several pathways possible, described in text	Modulation of IAA levels can enhance plant growth, enhanced protection against pathogens has not been demonstrated so far	*P. agglomerans*	Brandl et al., 2001; Duca et al., 2014; de Souza et al., 2019	X?	X
Cultivation with 1-aminocyclopropane-1 carboxylate as nitrogen source and by measuring production α-ketobutyrate (end-product) spectrophotometrically	*acd*S or *acc*D	Enzymes responsible for lowering ethylene levels	1-aminocyclopropane-1-carboxylate deaminase, modulation of ethylene levels induces physiological changes in the plant. Enhanced protection against pathogens has not been demonstrated so far	*Methylobacterium* spp., *R. fascians*	Chinnadurai et al., 2009; Francis et al., 2016	X?	X
HPLC analysis of extracts of the supernatant	*fas*4 or *IPT*	Enzymes responsible for production cytokinins	Isopentenyl transferase, modulation of cytokinins levels induces physiological changes in the plant. Enhanced protection against pathogens has not been demonstrated so far	*Methylobacterium* spp., *R. fascians*	Madhaiyan et al., 2006; Francis et al., 2016; Jorge et al., 2019	X?	X
2.2 Induced systemic response							
Transcriptomics of the host plant	Creation of a MAMP database, compare between beneficial and pathogenic microbes	MAMPs that trigger an immune response, that increases protection against pathogens	Detection results in immune response	*Sphingomonas melonis* fr1	Ryffel et al., 2016; Vogel et al., 2016	X	X
Transcriptomics of the host plant	Creation of an effector database, screening for type III secretion system gene clusters	effectors that trigger an immune response, that increases protection against pathogens	Detection results in immune response	*Pseudomonas* spp., *Parabulkholderia* sp.	Stringlis et al., 2019; Herpell et al., 2020	X	X

The table includes (i) information on in vitro assays to test for the presence of these mechanisms, (ii) known genes involved in these mechanisms (in silico screening), (iii) the compound and (iv) the mechanism resulting in antipathogenic activity, (v) microbes in which the mechanism has been identified, (vi) references and the last two columns indicate whether the mechanism is (vii) a biocontrol factor (BC) and/or (viii) an adaptation factor (A). The screening methods, strains and references are not exhaustive but rather examples, which are also discussed in the text. The table follows the same structure as the review.

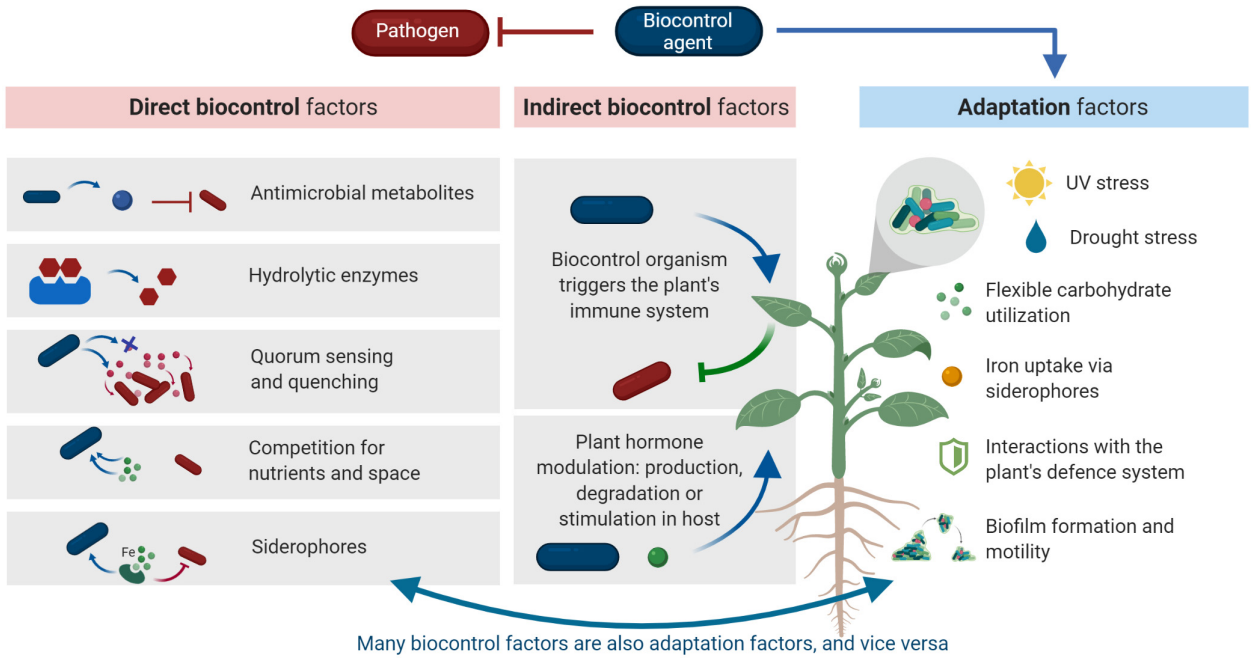

FIGURE 1 | Overview of mechanisms described in this review. A successful biocontrol agent possesses biocontrol factors as well as adaptation factors. Biocontrol factors can be divided in direct and indirect interactions. Direct interactions are interactions directly between biocontrol agent (blue rod) and pathogen (red rod). Indirect interactions are interactions between biocontrol agent and host plant that lead to an enhanced immunity of the host plant against pathogen infection. Adaptation factors are factors that are required to adapt to the specific phyllosphere conditions, such as high levels of UV stress, low availability of water and nutrients and responses from the host immune system. Finally, adaptation factors are often biocontrol factors and vice versa, this is further explained in the text. Created with BioRender.com.

pv. *tomato* DC3000 (Innerebner et al., 2011). Biocontrol *Pseudomonas* strains have been observed to directly inhibit the growth of a wide variety of pathogens (such as *P. syringae* and *B. cinerea*) in lab and in field experiments (Völksch and May, 2001; Romero et al., 2016; Simionato et al., 2017). Biocontrol activity of *Pseudomonas* spp. is often attributed to the production of phenazines, a group of heterocyclic nitrogen-containing secondary metabolites (Chin-A-Woeng et al., 2003). Biosynthesis in *Pseudomonas* spp. is encoded by the *phz* gene cluster. Phenazines inhibit the growth of a variety of fungal pathogens, such as *B. cinerea* and *Fusarium oxysporum* [a more detailed overview is given in Chin-A-Woeng et al. (2003)]. The antifungal mode of action of phenazines is multifaceted. For example, Chen et al. (2018) demonstrated that phenazines inhibit mycelial growth of *Fusarium graminearum* by interference with fungal histone acetylation, and are involved in the formation of a bacterial biofilm on the hyphae, further decreasing pathogenicity. Biofilm formation on fungal hyphae is a widespread trait in soil bacteria (Guennoc et al., 2018). More studies are needed to determine how frequent this occurs in the phyllosphere. Next to phenazines, many other potential biocontrol metabolites have been identified in *Pseudomonas* spp. such as siderophores (see section "Siderophores"), 4-hydroxy-2-alkylquinolines (Yasmin et al., 2017), volatile compounds such as cyanide and other volatile organic compounds (Bailly and Weisskopf, 2017), and cyclic lipopeptides (non-ribosomal peptides) and rhamnolipids (glycolipids synthesized in a three-step biosynthetic pathway including *rhl*ABC enzymes) (Nielsen et al., 2006; Strano et al., 2017; Yasmin et al., 2017). Rhamnolipids are effective against zoosporic root-infecting pathogens such as *Phytium* and *Phytotophtera* spp. Furthermore, spraying purified rhamnolipids on leaves of Arabidopsis triggers an immune response in the host characterized by the accumulation of signaling molecules and defense genes (Sanchez et al., 2012) (this is an indirect biocontrol mechanism and is further discussed in the section "Plant Hormones"). Interestingly, cyclic lipopeptides and rhamnolipids are also biosurfactants. Biosurfactants generally improve surface motility, biofilm formation and colonization of plant surfaces. Therefore, these adaptation factors could play an important role in the effectiveness of *Pseudomonas* biocontrol agents. Although, to our knowledge, the importance of rhamnolipids in adaptation, has not yet been investigated in the phyllosphere. Recently, Santos Kron et al. (2020) investigated the role of three antibiotic compounds in the antagonism by *Pseudomonas orientalis* F9 via experiments with mutants deficient in the production of the siderophore pyoverdine (also see section "Siderophores"), safracin (a tetrahydroisoquinoline alkaloid) and phenazine. *In vitro* double-layer assays showed antagonism against *Erwinia amylovora* and three *P. syringae* pathovars by the parental strain *P. orientalis* F9 and surprisingly also by the pyoverdine and phenazine deficient mutants. Only the mutant deficient in safracin production did not inhibit the pathogens. This

indicates that safracin rather than pyoverdine and phenazine was causing the *in vitro* antagonism. In contrast, all mutants were able to inhibit the pathogen *Pythium ultimum, in vivo* in a soil microcosm and *E. amylovora, in vitro* in a detached blossom assay. These unexpected results indicate that the biocontrol mechanism of *P. orientalis* is versatile and that other mechanisms could play a role in the observed biocontrol. Recently, Bernal et al. (2017) described the use of a type VI secretion system for the secretion of the Rhs effector Tke2 in *Pseudomonas putida*. The secretion of this effector was shown to be responsible for inhibiting *P. syringae*, *Xanthomonas campestris*, *Pectobacterium carotovorum*, and *Agrobacterium tumefaciens in vitro,* as well as reducing colonization of *X. campestris* and reducing disease symptoms on *Nicotiana benthamiana* leaves. Furthermore, Chen et al. (2016) described the secretion of the antimicrobial siderophore pyoverdine by a type VI secretion system, which inhibits *Xanthomonas oryzae* pv. *oryzae* (see also section "Siderophores"). Many *Pseudomonas* spp., both pathogenic as non-pathogenic, as well as other Gram-negative phyllosphere bacteria, harbor type VI secretion systems, suggesting that these are an adaptation factor (Bernal et al., 2018).

Less frequent inhabitants of the phyllosphere, but often used in commercial biocontrol products, are *Bacillus* spp. (Ongena and Jacques, 2008). Bacilli isolated from the phyllosphere often engage in inhibitory interactions with other microbial competitors (Helfrich et al., 2018) and their ability to form resistant endospores facilitates their formulation and shelf life (Ongena and Jacques, 2008). *Bacillus subtilis* and *Bacillus amyloliquefaciens* are the two most described biocontrol agents in this genus thus far. *B. subtilis* strains inhibit a wide range of pathogens, both fungal and bacterial, such as *F. graminearum* (Wang et al., 2007), *B. cinerea* (On et al., 2015), *Alternaria* spp. (Ali et al., 2016), *X. campestris,* and *P. carotovorum* (Zeriouh et al., 2011). The antipathogenic activity of bacilli has mainly been attributed to the synthesis of non-ribosomal peptides and polyketides (Ongena and Jacques, 2008; Chen et al., 2009). The three classes of non-ribosomal lipopeptides, surfactin, iturin, and fengycin, often act in a synergistic manner. Interestingly, surfactins produced by *B. subtilis* do not appear to play a role in the antipathogenic activity *in vitro*, whereas they are necessary for biocontrol *in planta* (Zeriouh et al., 2014). Surfactins trigger biofilm formation, allowing *B. subtilis* to successfully colonize the phyllosphere in sufficient numbers and to manage the release of antimicrobial compounds. Therefore, surfactins are rather adaptation factors than biocontrol factors. Wei et al. (2016) confirmed that *B. subtilis* QST 713, which is used in commercial products, was able to colonize the leaf surface in sufficient numbers. However, despite successful colonization of the phyllosphere, difficulty to colonize new leaves (i.e., dispersal), limited the biocontrol potential of this product. Fengycins and iturins are mostly active against filamentous fungi, by interfering with the lipid layers and altering cell membrane structures (Ongena et al., 2007), but also against the Gram-negative pathogens *X. campestris* pv. *cucurbitae* and *P. carotovorum* subsp. *carotovorum* (Zeriouh et al., 2011). *B. amyloliquefaciens* strains have been proven to be successful biocontrol agents in the field for a wide range of pathogens, such as *Sclerotinia sclerotiorum* causing canola stem rot (Fernando et al., 2007), and fusarium head blight on durum wheat (Schisler et al., 2002). Salvatierra-Martinez et al. (2018) described biocontrol activity of two *B. amyloliquefaciens* strains against *B. cinerea* on tomato plants. These two trains had similar antagonistic effect *in vitro,* while strain BBC047 showed better results *in planta*. BBC047 was also able to produce a robust biofilm and maintain higher population density over time on the plants. Therefore, it is assumed that adaptation factors explain why strain BBC047 is a more effective biocontrol agent. The genomes of biocontrol *B. amyloliquefaciens* strains contain several gene clusters encoding for the three lipopeptides surfactin, iturin and fengycin, and polyketide compounds, such as bacillaene, macrolactin and difficidin (Chen et al., 2007; Dunlap et al., 2013; Kim et al., 2015). A clear overview of the secondary metabolite synthetase gene clusters in the genome of *B. amyloliquefaciens* AS 43.3 is given in Dunlap et al. (2013). Chen et al. (2009) demonstrated that in the mix of these antimicrobial metabolites, the polyketide difficidin and the dipeptide bacilysin, are most important for biocontrol against *E. amylovora* on apple blossoms. This was proven *in planta* by applying three mutants of the commercial strain FZB42 on detached apple blossoms, one mutant deficient in production of difficidin, the second unable to synthesize non-ribosomal lipopeptides and polyketides, and a third double mutant deficient in polyketide and bacilysin synthesis. Similarly, Wu et al. (2015) also show the role of difficidin and bacilysin from strain FZB42 in the antagonistic mechanism against *X. oryzae* pv. *oryzae* and *X. oryzae* pv. *oryzicola*. Moreover, microscopic techniques revealed that difficidin and bacilysin cause changes in the cell wall of *Xanthomonas* spp.

The genus *Pantoea* contains several plant pathogens, as well as biocontrol agents effective against a range of pathogens such as *B. cinerea*, *X. campestris,* and, the most extensively studied, *E. amylovora* [as reviewed by Walterson and Stavrinides (2015)]. Several antibiotics, such as pantocins (Smits et al., 2019), herbicolins (Kamber et al., 2012), and phenazines (Giddens et al., 2003), have been identified to play a role in the inhibition of *E. amylovora*. Stockwell et al. (2002) compared biological control of *E. amylovora* in field conditions by *Pantoea agglomerans* (syn. *Erwinia herbicola*) Eh252, known to produce only one antibiotic, and by its near-isogenic derivative, strain 10:12. Strain 10:12 is deficient in the production of mccEh252, involved in the synthesis of microcin C7. Strain Eh252 reduced the incidence of fire blight more effectively then 10:12. However, the mutant strain still protected the plants more effectively than a mock treatment, indicating that other mechanisms also contribute to biocontrol. The antibiotic herbicolin I was identified and characterized in *Pantoea vagans* C9-1 via the construction of a mutant library (Kamber et al., 2012). The herbicolin I biosynthetic gene cluster responsible *dda*A-I is located on the plasmid pPag2. Remarkably, this cluster was not detected in many other biocontrol strains. Using a similar approach, another antibiotic gene cluster, PNP-1 was identified in *Pantoea ananatis* BFT175, also effective against *E. amylovora* (Walterson et al., 2014). The PNP-1 cluster shows similarities to a gene cluster encoding for phenazine in *Pseudomonas chloraphis*. Previously, the *ehp* gene cluster encoding for phenazine synthesis had been identified in the

genome of *P. agglomerans* Eh1087 (Giddens et al., 2002). However, the PNP-1 gene cluster was not found in other *Pantoea* spp., indicating again the diversity of antibiotics in this genus.

Formulating antimicrobial metabolites into a plant protection product, without the living microorganism could result in a more convenient and cheaper product. Furthermore, the problem of limited biocontrol activity due to a low survival rate of the biocontrol agent would be solved. The formulation of a product with live bacteria is challenging, the drying process needs to be optimized to ensure a long shelf life and to minimize the loss of biocontrol activity (Broeckx et al., 2016). However, the use of live microorganisms does have advantages too. Firstly, the persistence of the metabolite in the environment. Antimicrobial metabolites can degrade rapidly in field conditions and would require frequent applications, while applying a living organism might need fewer. Secondly, antagonists are likely to acquire resistance toward a frequently applied metabolite. Live microorganisms and even consortia of live microorganisms have the advantage of producing various active molecules and thus reducing the chance of resistance. Finally, live microorganism can improve the health of plants not only via antimicrobial metabolites but via other direct and indirect mechanisms, as described in the next paragraphs.

Hydrolytic Enzymes

Production of chitinases, as well as other cell wall degrading enzymes, such as β-1,3-glucanase, is a common defense mechanism of plants (Boller, 1993). Microbes can also produce chitinases, which are an important biocontrol mechanism in the rhizosphere (reviewed by Veliz et al., 2017). Their importance in the rhizosphere indicates the potential of further studying the microbial chitinase activity on the phyllosphere. It has been demonstrated that *B. subtilis* J9 strain protects strawberry plants against *B. cinerea* in field conditions and that this strain produces extracellular chitinase and protease (Essghaier et al., 2012). Recently, we observed that certain lactobacilli can inhibit hyphae formation of fungi *in vitro* by producing bifunctional enzymes with chitinase/peptidoglycan hydrolase activity (Allonsius et al., 2019). Lactobacilli are not typical phyllosphere inhabitants, and often have a low survival rate (Miller et al., 2019). However, they have been shown to dominate the endosphere of *Origanum vulgare* plants (Pontonio et al., 2018) and have been correlated negatively with disease symptoms of leaf spot on cucumber plants, presumably caused by *P. syringae* pv. *lachrymans* (Luo et al., 2019). Next to the production of hydrolytic enzymes by the biocontrol agents themselves, microbes can induce the production of chitinases in the host plant, a common defense reaction in plants. Inhibition of a pathogen by triggering a defense reaction in the host is further discussed in section "Indirect Interactions."

Quorum Sensing and Quenching

Quorum sensing systems are systems by which bacteria change their behavior once a certain concentration threshold of signaling molecules is passed. In the phyllosphere, signaling molecules mediate behavior that enables bacteria to survive on the leaf surface, such as biofilm development, adhesion, motility, and production of cell-wall-degrading enzymes. Pathogenic bacteria use quorum sensing to measure their population size and regulate the moment to enter the apoplast or plant cell (Pfeilmeier et al., 2016; Leach et al., 2017). Gram-negative bacteria often use N-acyl-homoserine lactones (AHLs) as signaling molecules, which are synthesized by AHL synthase (*luxI*) and detected by a transcriptional regulator (*luxR*). Interestingly, AHL molecules can also trigger a response in the host plant (Delalande et al., 2005; Sieper et al., 2014), which is further discussed in the section "Indirect Interactions." Gram-positive bacteria do not make use of AHL systems, but typically use small post-translationally processed peptides as signal molecules or diffusible signal factors (see further in this section). A wide variety of small communication peptides exist, and these peptides sometimes have other functions as well. For example, *Lactococcus lactis* and *B. subtilis* produce the antibiotic lantipeptides nisin and subtilin, respectively, which are also involved in quorum sensing (Kleerebezem, 2004). Both *B. subtilis* (Wei et al., 2016) and *L. lactis* (Trias et al., 2008) can survive in the phyllosphere and even have biocontrol characteristics. However, involvement of the bifunctional peptides nisin and subtilin in the biocontrol activity on the phyllosphere has not been described. Therefore, it would be interesting to investigate their role in the biocontrol mechanism of these bacteria.

Interestingly, non-pathogenic bacteria use the same signaling molecules as pathogens and can thereby contribute to disease development or inhibition, depending on the way of interfering. A shared quorum sensing system using AHL-signal molecules was observed between the symbiotic bacteria *P. agglomerans*, *Erwinia toletana* and *Pseudomonas savastanoi* pv. *savastanoi,* the causative agent for knot disease in olive trees (Hosni et al., 2011). The symbionts, or in this case opportunistic pathogens, benefit from the niche created by disease development by the pathogen and thus participate in its communication system. By cooperating with the pathogen, *E. toletana* and *P. agglomerans* aggravated the infection in the olive trees in this study (Hosni et al., 2011). In contrast, other *P. agglomerans* strains showed biocontrol activity against the pathogen *P. syringae* pv. *tomato* in tomato plants (Morella et al., 2019), but it is at present not known whether quorum sensing could be involved. It remains to be determined whether actual biocontrol agents can have this effect on target and non-target pathogens.

Next to cross-communication by producing the same signaling molecules, bacteria can degrade each other's signals, also known as quorum quenching. Strains belonging to the genera *Bacillus, Paenibacillus, Microbacterium, Staphylococcus,* and *Pseudomonas* are able to rapidly degrade the diffusible signal factor, cis-11-methyl-2-dodecenoic acid. This signal is involved in the regulation of virulence of *Xanthomonas* spp. and *Xylella fastidiosa* in a quorum-sensing AHL-independent way (Newman et al., 2008). In the quorum-quenching strains, the genes *car*AB, involved in synthesis of carbamoylphosphate, a precursor for pyrimidines and arginine, were identified to be required for the rapid degradation of this diffusible signal factor. Bacteria containing the *car*AB genes could reduce disease incidence and severity of *X. campestris* pv. *campestris* in a detached leaf assay with mustard, cabbage and turnip plants,

and of *X. fastidiosa* when co-inoculated into the xylem of grape stems. Furthermore, Wu et al. (2015) showed that difficidin and bacilysin produced by *B. amyloliquefaciens* FZB42 (see section "Antimicrobial Metabolites") can downregulate the expression of several virulence genes in *X. oryzae*, including *rpf*F, involved in the production of a diffusible signal factor.

Morohoshi et al. (2009) screened 109 isolates from the potato phyllosphere for the ability to degrade several short-chain and long-chain AHLs, as Gram-negative pathogens use AHLs as a signaling molecule to regulate their virulence. They screened the isolates *in vitro* by using AHL biosensors, i.e., bacteria that respond to the presence of AHLs by producing a reporter protein. One of the enzymes involved in AHL degradation is AHL-lactonase, encoded by the *aii*A gene, initially identified in *Bacillus* spp. *Microbacterium testaceum* strains StLB018 and StLB037 tested positive for AHL degradation and decreased disease symptoms in potato tissue caused by *P. carotovorum* subsp. *carotovorum*. In contrast, *M. testaceum* ATCC 15829, lacking AHL-degrading activity, did not decrease disease symptoms, indicating that quorum quenching was the mode of action of biocontrol. Alymanesh et al. (2016) used a similar method to screen isolates from the phyllosphere and rhizosphere from saffron, fig, and pomegranate, for the degradation of the AHL 3-oxo-C6-HSL. They concluded that quorum quenching is a common trait among the isolates tested and is most often observed in *Pseudomonas* spp. These *Pseudomonas* isolates with strong quorum quenching activity also showed biocontrol activity against *P. carotovorum* subsp. *carotovorum in vitro* and on potato tubers.

Competition for Nutrients and Space

Phyllosphere bacterial community sizes are limited by low carbon availability on the leaf surface (Mercier and Lindow, 2000). Therefore, carbon competition will likely play an important role in the community structure. Microcosm experiments show that "invaders," such as introduced biocontrol agents, with a similar metabolism as the resident species are strong competitors in environments with a low resource availability, whereas fast-growing species have an advantage when resource availability is high (Yang et al., 2017).

The dominant carbohydrates available on the leaf surface are sucrose, fructose and glucose. These sugars are specifically altered after epiphytic leaf colonization by *Sphingomonas melonis* or the pathogen *P. syringae* pv. *tomato*, but only to a minor extent by *Methylobacteria* (Ryffel et al., 2016). Phyllosphere bacteria have developed different strategies to utilize all possible carbon sources available. Methylotrophs, such as *Methylobacteria,* have specialized in the utilization of single carbon compounds, such as methane and methanol. Therefore, they do not rely as much on the available sugars on the phyllosphere (Kutschera, 2007). Methylobacteria even modulate the release of methanol, which is released as plant cells expand, by encouraging plant growth via the production of plant hormones (see further, section "Plant Hormones") (Kutschera, 2007). The *mxa*F gene, which contains the active site of a methanol oxidation complex, was found to be highly conserved among methylotrophs and is an appropriate probe to screen for methylotrophy (Mcdonald and Murrell, 1997). Methylotrophy is thus an important adaptation factor for some phyllosphere bacteria. However, methylotrophs are not likely to inhibit pathogens by competing for nutrients. Nevertheless, Methylobacteria can possess other biocontrol mechanisms such as antimicrobial metabolites (Kwak et al., 2014) or indirect mechanisms by triggering plant immunity (see further in section "Plant Immunity") (Madhaiyan et al., 2006).

Another adaptation strategy is the ability to scavenge for a wide variety of carbon sources. The presence of a high variety of TonB receptors in the phyllosphere proteome has been suggested as an indication that the residing species can metabolize a wide variety of carbon compounds (Delmotte et al., 2009). Indeed, TonB receptors are involved in the transport of carbohydrates, siderophores, and vitamin B_{12}, in Gram-negative bacteria (Schauer and Kutschera, 2013). Blanvillain et al. (2007) noted that bacteria expressing a high variety of TonB receptors, but belonging to various taxonomical lineages, share the ability to metabolize a wide variety of carbohydrates. The overrepresentation of TonB receptors in *Xanthomonas* spp. appears to facilitate their survival in the phyllosphere by making them competitive nutrient scavengers (Blanvillain et al., 2007). Additionally, community proteogenomics of the phyllosphere of *Arabidopsis*, clover, and soybean assigned a high proportion and great variety of TonB receptors to *Sphingomonas* species. This high abundance of TonB receptors is thought to allow *Sphingomonas* spp. to be more successful than other Gram-negative bacteria to withstand the carbon-stressed environment and account for their success on the phyllosphere in terms of their relative abundance (Delmotte et al., 2009). Innerebner et al. (2011) tested 17 *Sphingomonas* strains on the phyllosphere of *A. thaliana* for their ability to suppress disease symptoms of the pathogen *P. syringae* pv. *tomato* DC3000. All seven phyllosphere isolates, and four out of five rhizosphere isolates, protected the plant against developing disease symptoms. On the other hand, four out of five *Sphingomonas* non-plant isolates (isolated from air, dust, or water), did not protect the host plant against *P. syringae* infection. Carbon-source profiling of two protective and two non-protective strains suggested that substrate competition plays a role in the observed antagonistic effect. It would be interesting to verify whether the difference in carbon-source utilization is a result of a higher TonB diversity and whether plant-associated *Sphingomonas* spp. typically have a higher TonB diversity in comparison to other *Sphingomonas* spp.

The niche-overlap index (NOI) is a measure that can be used to quantify the similarity in carbon source profile of two microbes (Wilson and Lindow, 1994). Wilson and Lindow (1994) calculated the NOI as the number of carbon sources that both strains utilize as a proportion of the total number of carbon sources utilized by one strain. They demonstrated that the NOI of the epiphytic bacteria *Pseudomonas fluorescens, P. agglomerans, Stenotrophomonas maltophilia*, and *Methylobacterium organophilum* correlated inversely with their ability to coexist with the pathogen *P. syringae* on the phyllosphere of beans (*Phaseolus vulgaris*). In another study, the NOI of 36 non-pathogenic phyllosphere bacteria were correlated with the ability to suppress disease caused by *P. syringae* pv. *tomato* (Ji and Wilson, 2002). These studies

confirm the hypothesis made by Lindow (1987) that "antagonism due to competition of one strain with another would increase proportionally to the overlap of their ecological niche." This hypothesis was formulated based on a field study where ice nucleation-deficient *P. syringae* mutants successfully antagonized the *P. syringae* wild-type strain in field conditions when the mutant was applied to the plants two days before the wild-type strain (Lindow, 1987). Under such conditions, the mutants could successfully outcompete the wild-type strain and a reduction of the frost injury to the plants was noted. However, the mutants had the advantage of being able to occupy the ecological niche first. Priority effects do play an important role in competition between microbes and in the assembly of phyllosphere communities (Maignien et al., 2014). Therefore, some biocontrol agents are more effective as a preventive measure and less so as a treatment.

Berg and Koskella (2018) tested the antipathogenic properties of both a natural phyllosphere community and a simplified synthetic phyllosphere community (comprising of 12 bacterial strains), against *P. syringae* pv. *tomato*. Both the natural as the synthetic community protected the plant against the pathogen. The authors observed that addition of fertilizer to the soil canceled the observed pathogen protection of the synthetic community, but not of the natural community. Microbial loads on the leaves did not increase significantly due to fertilization. The authors hypothesize that fertilization resulted in an increase in phyllosphere nutrient availability. The synthetic communities were all cultured on KB medium before application on the plants. This is a medium on which *P. syringae* also grows well. This might have caused selection for metabolically similar strains, which would increase antagonism due to nutrient competition (cfr. Lindow, 1987). Nutrient competition might therefore play a more prominent role in the synthetic communities than in the more diverse natural communities, where other modes of action could possibly dominate. This hypothesis on nutrient-dependent effects provokes two novel research questions. Firstly, does soil fertilization increase nutrient availability in the phyllosphere and secondly, how does this have an impact on biocontrol in the phyllosphere in field conditions?

Siderophores

Apart from carbon sources, iron is often a limiting element in phyllosphere microbial communities. Siderophores are secreted by microorganisms to bind and transport iron into the cell. Siderophore production is essential for the epiphytic fitness of *P. syringae* pv. *syringae* 22d/93, a strain with biocontrol activity against the pathogen *P. syringae* pv. *glycinea* (Wensing et al., 2010). Interestingly, when inoculated in wounded leaves, siderophore production by the commensal had no effect on its own population size nor on the population size of the pathogen. This indicates that iron was not a limiting element in wounded plant cells. Siderophore production is thus not a biocontrol mechanism of importance for *P. syringae* pv. *glycinea*, when the pathogen rapidly penetrates living tissue. However, siderophore production is an important adaptation factor for biocontrol agent *P. syringae* pv. *syringae* 22d/93, as 10 days post inoculation, the population size of a siderophore-negative mutant was 2 orders of magnitude lower than that of the wild-type. Furthermore, a role for siderophores in the induced systemic resistance (ISR) (see section "Induced Systemic Responses") has been reported in several systems (Bakker et al., 2007). It is not excluded that the wounding in the experiment by Wensing et al. (2010) triggered ISR, via host jasmonic acid (JA) and ethylene mediated pathways (see section "Plant Hormones"). The wounding switched off the necessity for an additional siderophore triggered ISR and the strain did not exert any biocontrol activity in the wounded plants.

Siderophores can have alternative functions in addition to iron scavenging, such as non-iron metal transport, sequestration of toxic metals, signaling, protection from oxidative stress, and antibiotic activity. The latter occurs by attaching a bactericidal 'warhead' on a siderophore which is then taken up by the antagonized bacterium (Kramer et al., 2019). The siderophore enantio-pyochelin, produced by *Pseudomonas protegens* CS1, isolated from the lemon tree phyllosphere, showed antagonistic activity *in vitro* and in the phyllosphere of lemon plants against the pathogen *Xanthomonas citri* subsp. *citri* (Michavila et al., 2017). Additions of iron and ascorbic acid indicated that not competition for iron but oxidative stress, induced by the formation of reactive oxygen species (ROS) from pyochelin, was the mechanism of action for the observed antimicrobial activity. Indeed, ascorbic acid was able to counteract the antimicrobial activity of ROS while addition of iron had almost no effect. In contrast, experiments with *P. orientalis* F9 and a mutant deficient in the production of siderophore pyoverdine (also see section "Antibiotic Metabolites") showed that the mutant was still able to antagonize *E. amylovora* and three *P. syringae* pathovars *in vitro*, as well as *E. amylovora* on a detached flower assay and *P. ultimum* in a soil microcosm assay (Santos Kron et al., 2020). This indicates that pyoverdine did not play a role in the biocontrol mechanism by *P. orientalis* F9. Another function of siderophores on the phyllosphere was demonstrated by Ruiz et al. (2015). The siderophores pyoverdine and enantio-pyochelin, synthetized by *P. protegens*, were responsible for its resistance against the mycotoxin fusaric acid. Fusaric acid is produced by pathogenic fungi of the *Fusarium* genus and is toxic to plants and bacteria through mechanisms that are not yet fully understood. Finally, Burbank et al. (2015) showed that mutations in the *iuc*A and *iut*A genes, responsible for siderophore and receptor biosynthesis respectively, results in a loss of surface motility of the xylem-dwelling pathogen *Pantoea stewartia*, and reduced virulence in sweet corn. This indicates that siderophores also play a role in adaptation by mediating motility. However, this mechanism has not been described yet as an adaptation strategy for phyllosphere biocontrol agents.

INDIRECT INTERACTIONS

Next to direct interactions, biocontrol agents can inhibit pathogens indirectly, by modulating the plant's immune system or hormone levels (**Figure 1**). Microbe-plant interactions that protect the plant against pathogen infection are discussed here as indirect interactions.

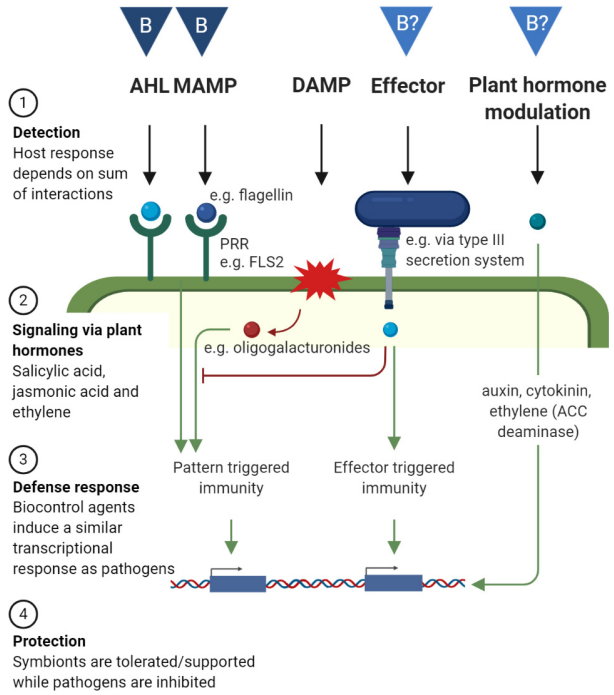

FIGURE 2 | The plant's defense system and how biocontrol agents can interact with it. The plant's immune system can be divided in four steps: (1) Detection: A micro-organism can be detected via N-acyl homoserine lactone (AHL), microbial associated molecular patterns (MAMPs) via pattern-recognition receptors (PRRs), damage associated molecular patterns (DAMPs), effectors or other ligands which are detected intracellularly. (2) Signalization via the plant hormones salicylic acid, jasmonic acid and ethylene. (3) A defense response via changes in gene expression, and (4) Protection against the pathogen, while symbionts are tolerated or supported. Micro-organisms can also modulate plant hormone levels by producing or degrading them, which induces physiological changes in the host plant. "B" in dark blue triangles indicate known biocontrol mechanisms by which biocontrol organisms interact with the host plant (AHL and MAMP), inducing protection against pathogens. "B?" in light blue triangles indicate mechanisms for which their role in biocontrol on the phyllosphere requires confirmation (effector and plant hormone modulation). Created with BioRender.com.

Plants have evolved a complex immune system to prevent infection by recognizing potential intruders and responding with an appropriate defense response. Reversely, pathogens evolve continuously to evade recognition or to interfere with the defense response. This action and counteraction are described by Jones and Dangl (2006) in the "zigzag model." A schematic representation of the host immune system as well as mechanisms by which biocontrol agents can interact with it is given in **Figure 2**. The host plant recognizes microbe-associated molecular patterns (MAMPs) by specific pattern-recognition receptors (PRRs), which leads to pattern-triggered immunity. One of the best studied MAMPs is flagellin, more specifically the epitope flg22, which is recognized by the PRR FLS2. Other MAMPs are lipopolysaccharides from Gram-negative bacteria and N-acetylglucosamine-containing glycans, such as bacterial peptidoglycan, generally more prominently in Gram-positive bacteria, fungal chitin, or rhizobacterial nodulation factors. Also volatile compounds emitted by beneficial bacteria such as *Bacillus* and *Pseudomonas* spp. can trigger the plant's immune system, however the receptors remain to be identified (Tyagi et al., 2018). An overview of PRRs, the specific MAMPs that are recognized, and the molecular basis of the following pattern triggered immunity has been reviewed by Saijo et al. (2018). Of importance here, both pathogens and mutualistic microbes are detected through MAMP–PRR interactions and detection generally leads to relatively weak immune responses. Hacquard et al. (2017) argues that the pattern-triggered immunity does not discriminate between a beneficial or pathogenic attack, but mainly functions by restricting the microbial load. The immune response can become stronger when additional virulence factors are present, such as tissue damage or plant hormones modulation (discussed further in this section) (Jones and Dangl, 2006; Hacquard et al., 2017).

Microbes can overcome this first line of defense by modifying MAMPs or by secreting effectors into the cytoplasm of host cells that interfere with the triggered immune signaling. Consequently, plants have evolved additional mechanisms to detect these microbial effectors: effector-triggered immunity (Jones and Dangl, 2006; **Figure 2**). Gram-negative bacteria use type III secretion systems to deliver effector molecules into the cytoplasm to suppress the immune system. When such a secretion system is inactivated (through mutations in *hrp* genes, which are required for a functional type III secretion system and elicitation of a hypersensitive response in plants) in pathogens, disease symptoms are reduced (Hanemian et al., 2013). Such mutants are then unable to overcome pattern-triggered immunity and are unable to infect host tissue. These mutants often reside in the apoplast without causing harm and can even protect the host against invasion of the wild-type pathogen (Hanemian et al., 2013). Mutants and wild-type pathogens colonize separate cells/niches when co-inoculated. In some studies, co-inoculation led to protection (Faize et al., 2006), while in others, it was necessary to inoculate the non-virulent mutant prior to the pathogen (Feng et al., 2012). Therefore, it is debatable whether competition for nutrients and space is an important mode of action. However, multiple studies show that inoculation with *hrp* mutants induces changes in gene expression which lead to a defense response and increased resistance against the pathogen (Faize et al., 2006; Feng et al., 2012; Hanemian et al., 2013).

Type III secretion systems and effectors have mostly been described in pathogens. However, some commensals also interact more actively with the plant's immune response via effectors. For example, nodulating rhizobacteria secrete effectors using type III and type VI secretion systems to activate nodulation in the host plant (Deakin and Broughton, 2009). Recently, Stringlis et al. (2019) identified type III secretion system gene clusters in beneficial rhizosphere *Pseudomonas* spp. These gene clusters were highly similar to type III secretion systems in other beneficial bacteria, but distinct compared to phytopathogenic *P. syringae*. Also on the phyllosphere, type III secretion systems have been identified in the genome of a non-pathogenic *Paraburkholderia isolate* (Herpell et al.,

2020). However, more research is needed to determine the presence of type III secretion systems in other beneficial phyllosphere bacteria, to identify effectors associated with pathogens versus biocontrol effectors, and to determine the role of these type III secretion systems in beneficial host–microbe interactions.

A group of molecules often forgotten that have an effect on the host immune system are N-acyl-homoserine lactones (AHLs) (Schenk and Schikora, 2015). AHLs regulate the behavior of Gram-negative pathogens (see section "Quorum Sensing and Quenching"). The host plant could benefit from the ability to respond to or interfere with this quorum sensing signal. Indeed, exposing roots to AHLs or AHL-producing bacteria has been shown to trigger the upregulation of defense genes in the plant and inducing systemic resistance via salicylic acid (SA) signaling (described further) (Schenk and Schikora, 2015). One of the AHL-induced defense reactions of *Arabidopsis* plants was stomatal closure, a common first defense reaction to limit the entry of pathogens in the apoplast. Furthermore, plants react by degrading the AHLs (Delalande et al., 2005) or transporting the AHLs into the vascular system to remove them from the bacteria that produced them (Sieper et al., 2014). Both actions could be described as a plant's equivalent to quorum quenching. Detection of AHLs in *Arabidopsis* plants is mediated through the G-protein coupled receptor encoded by *AtGPA1*. However, more research is needed to identify AHL receptors in other plants.

Finally, the plant's immune system can be triggered by the detection of host-derived danger-associated molecular patterns (DAMPs), such as oligogalacturonides which are released from the plant cell wall during pathogen invasion. Detection of DAMPs in combination with pattern or effector triggered immunity, will result in a more severe immune response against the invader (Hacquard et al., 2017). It is unlikely biocontrol agents trigger the host immune system through the release of DAMPs.

As described here, both biocontrol as pathogenic microorganisms interact with the host immune system. Similarly to pathogen and commensal host interactions in humans, the final host response depends on the sum of the interactions with host receptors. Commensal bacteria do not trigger a strong defense reaction in the host as they lack additional virulence factors, such as triggering the formation of DAMPs, modulating plant hormone levels or secreting effectors into the host cells (Lebeer et al., 2010).

Plant Hormones

The recognition of beneficial or pathogenic microbial attacks, as described above, leads to the activation of signaling hormones in the plant, as counterparts of immune modulating cytokines in human and animal cells. Relevant plant hormones include SA, JA, and ethylene, where SA and JA are considered to act antagonistic (Koornneef and Pieterse, 2008; Pieterse et al., 2014). JA and ethylene are usually involved in the defense response against necrotrophic pathogens (feeding on killed host cells), or after wounding, while SA is involved in the defense reaction against biotrophic or hemibiotrophic pathogens (feeding on living tissue) (Glazebrook, 2005). Experiments spraying bacterial produced rhamnolipids on leaves of Arabidopsis (see section "Direct Interactions") showed that SA plays a central role in rhamnolipid-mediated disease resistance (Sanchez et al., 2012).

A first example on how phyllosphere microbes can directly modulate plant hormone levels, is through the enzyme 1-aminocyclopropane-1-carboxylate (ACC) deaminase that degrades the ethylene precursor ACC. It has been detected in plant-growth promoting rhizosphere bacteria such as *Azospirillum*, *Rhizobium*, and *Pseudomonas* spp. (Gamalero and Glick, 2015; Nascimento et al., 2019), as well as in phyllosphere bacteria, such as several *Methylobacterium* spp. (Kwak et al., 2014) and *Rhodococcus fascians* (Chinnadurai et al., 2009; Francis et al., 2016). 1-Aminocyclopropane-1-carboxylate deaminase activity lowers ethylene levels, reducing the plant's defense responses and thereby facilitating symbiotic microorganisms. ACC deaminase also results in the promotion of plant growth, since plants become more resilient against environmental stress such as drought, flooding, salt stress or pathogen pressure (Gamalero and Glick, 2015; Nascimento et al., 2018; Saghafi et al., 2020). Direct evidence for a role of ethylene in modulating the community composition of the phyllosphere is given by Bodenhausen et al. (2014), as ethylene-insensitive plant mutants harbored a different phyllosphere community. Moreover, evidence is rising for a direct role of ACC in regulating plant development (Van de Poel and Van Der Straeten, 2014; Vanderstraeten and van Der Straeten, 2017) and defense responses (Tsang et al., 2011).

Levels of phytohormones that are primarily involved in plant growth, such as cytokinins and auxins, can also be modulated by microbes (Leach et al., 2017). Both production and degradation of the auxin indole-3-acetic acid (IAA) have been observed in both plant growth-promoting and pathogenic bacteria (Duca et al., 2014; Nascimento et al., 2019). Degradation of IAA can be advantageous for phyllosphere microbes in two ways. On the one hand, IAA is a good source of carbon and nitrogen (Leveau and Lindow, 2005; Nascimento et al., 2019). On the other hand, manipulation of IAA levels induces physiological changes in the plant, such as cell wall-loosening and the release of nutrients that benefit the survival or colonization of the microbe (Vanderhoef and Dute, 1981). *P. putida* 1290 is able to grow on IAA as a sole source of carbon, nitrogen, and energy (Leveau and Lindow, 2005). This ability of *P. putida* 1290 is encoded by the *iac* gene cluster. Homologs of the *iac* gene cluster have been identified in strains from various genera, such as *P. putida* GB-1, *Marinomonas* sp. MWYL1, *Burkholderia* sp. 383, *Sphingomonas wittichii* RW1, *Rhodococcus* sp. RHA, *Acinetobacter baumannii* ATCC 19606, and *Lelliottia* sp. (Leveau and Gerards, 2008; Lin et al., 2012). On the other hand, high levels of IAA, produced by plant or bacterium, can play an important role in disease development [e.g., by gall forming pathogens *Rhodococcus fascians* (Stes et al., 2012) (see further in text), knot development by *P. savastanoi* (Surico et al., 1985) or suppression of the host defense system by *P. syringae* pv. *syringae* DC3000 (McClerklin et al., 2018)]. Bacterial degradation of IAA has so far not

been directly linked with antipathogenic effects. However, IAA degradation is an important adaptation mechanism of bacteria on the phyllosphere.

Besides degradation, IAA can also be produced by plant-associated bacteria. Production of IAA can occur via several pathways, as reviewed by Duca et al. (2014). The presence of these pathways can be detected by the presence of the following essential genes: *ipd*C and *ald*H for the indole-3-pyruvate pathway (encoding the enzymes necessary for the decarboxylation of indole-3-pyruvate and subsequent oxidation, respectively), *dcc* and *ald*H for the tryptamine pathway (encoding the enzymes necessary for the decarboxylation of tryptophan and subsequent oxidation, respectively), *iaa*M and *iaa*H for the indole-3-acetamide pathway (encoding for tryptophan-2-monooxygenase and indole-3-acetamide hydrolase, respectively), and *nth*A for the indole-3-acetonitrile pathway (encoding for nitrile hydratase α) (de Souza et al., 2019). It is important to note that although the indole-3-acetamide pathway was considered as being exclusive for the excessive IAA production by gall forming bacteria like *P. savastanoi*, *Erwinia* spp., and *Agrobacterium* transformed plant tissue (Jameson, 2000), these genes are also present in methylotrophic rhizosphere microorganisms (Li et al., 2019). The *ipd*C gene is of special interest since it was demonstrated that the specific growth conditions in the phyllosphere trigger the expression of the *ipd*C gene in symbiotic *P. agglomerans* (syn. *E. herbicola*) (Brandl et al., 2001). Furthermore, the phyllosphere consists of microenvironments that induce differential expression of the *ipd*C gene. Expression of the *ipd*C gene and production of IAA is induced in the rhizosphere symbiont *Azospirillum brasilense* Sp245 when carbon source availability is limited in batch and fed-batch cultures (Ona et al., 2005). These observations indicate that IAA production, encoded on the *ipd*C gene, is used by beneficial plant-associated bacteria to adapt to the phyllosphere by improving their growth conditions and availability of carbon sources. However, as with IAA degradation, IAA production is an important adaptation factor and has not yet been linked to biocontrol activity.

Members of the genus *Methylobacterium* enhance plant growth by producing auxins and cytokinins (Koenig et al., 2002; Kwak et al., 2014; Jorge et al., 2019; Li et al., 2019). Interaction with the host is beneficial for the symbiont's growth since they metabolize the methanol released as the plant grows (Kutschera, 2007). *Methylobacterium* derived cytokinins were attributed to drought/saline stress resistance in the host (Jorge et al., 2019). Both the type of cytokinins present and the presence of a *mia*A gene indicate that methylobacterial cytokinin production is merely via tRNA (Koenig et al., 2002; Kwak et al., 2014; Jorge et al., 2019). Moreover, biocontrol activity has been observed, for example, seed inoculation of groundnut plants with *Methylobacterium* spp. increased protection against pathogens *Aspergillus niger* and *Sclerotium rolfsii* (Madhaiyan et al., 2006). The treatment with *Methylobacterium* spp. induced an increased activity of enzymes in the host plant that are typically associated with Induced Systemic Resistance (ISR) a state in which the plant's immune system is triggered in order to become resistant against pathogen infection (see further for the paragraph on Induced Systemic Responses). This indicates that the applied *Methylobacterium* spp. interacted with the host plant's defense system resulting in protection against *A. niger* and *S. rolfsii.* However, the specific role of microbial production of cytokinins and auxins in the plant's defense response has not been elucidated in this study.

Bacteria that are known to modulate plant hormone levels are *Rhodococcus fascians.* Both pathogenic as non-pathogenic *R. fascians* have the ability to both produce IAA and cytokinins, and decrease ethylene levels (Francis et al., 2016). In pathogenic bacteria, the genes encoding auxin and cytokinin production are plasmid-borne (Jameson, 2000). IAA production is higher in presence of exogenous tryptophan, a precursor of IAA. Interestingly, upon inoculation of the plant with pathogenic or non-pathogenic *R. fascians*, the metabolism of the host plant changes and more tryptophan is accumulated, possibly stimulating bacterial production of IAA (Francis et al., 2016). On the other hand, the production of cytokinins by *R. fascians* induce increased auxin production in the plant. The increased auxin levels play an important role in the development of disease symptoms (Stes et al., 2012). The non-pathogenic derivative of this strain lacks the plasmid with virulence genes. The main pathogenicity factor on the plasmid is the production of modified methylated cytokinins, which are not degraded by cytokinin oxidase activity, mimic plant cytokinins, induces increased auxin production in plants and results in the development of disease symptoms (Radhika et al., 2015). Cytokinin and auxin production in pathogenic *R. fascians* is thus detrimental and contributes to the disease development. Reversely, cytokinin and auxin production in non-pathogenic *R. fascians*, as well as in other symbionts (e.g., *Methylobacteria*, described above), is being described as a beneficial trait since it promotes plant growth (Schauer and Kutschera, 2011; Francis et al., 2016; Romero et al., 2016).

In conclusion we can postulate that, through the ability to control the auxin steady state by producing additional auxins on the one hand, and by auxin degradation when excessive auxin production occurs in case an additional pathogen is invading the host on the other hand, a benign symbiont is possibly able to optimize its ecological niche both by improving the host growth and excluding other invaders. The cross talk between auxins and ethylene, as well as the bacterial ACC-degradation might prevent ethylene induced excessive immune and senescence responses to occur. Moreover, it is of general knowledge that cytokinins act as a sink for sugar and other metabolites (Roitsch and Ehneß, 2000), therefore we postulate that it is plausible that the microbial cytokinin production works as a sink for metabolites to the benefit of the symbiont (carbon source) as well as the host by reducing senescence and as a consequence prolonging photosynthetic activities.

Induced Systemic Responses

Once a microbe is detected by the plant and its presence is signaled via plant hormones, as described above, a specific immune response is triggered in the plant (Fig 2). Beneficial microbes are able to trigger the plant's defense system at the

point of recognition and induce immunity against pathogens in the whole plant body (Pieterse et al., 2014). This phenomenon is called induced systemic resistance (ISR). Also pathogens can induce a systemic response, which then protects other parts of the plants, this is called systemic acquired resistance (SAR). The term ISR is usually used when it is triggered by rhizosphere microbes (Pieterse et al., 2014). However, in the next paragraphs, we will describe several examples of ISR by beneficial microorganisms in the phyllosphere. Also endophytic bacteria can trigger ISR (Kloepper and Ryu, 2007).

Vogel et al. (2016) studied the differences in gene expression in *Arabidopsis* plants upon inoculation with two model commensal phyllosphere bacteria; *S. melonis* Fr1 and *Methylobacterium extorquens* PA1. Colonization by *M. extorquens* PA1 resulted in very little transcriptional response from the plant whereas *S. melonis* Fr1 changed the expression of several hundreds of genes. This corresponds with the findings of Innerebner et al. (2011) where *S. melonis* Fr1 decreased disease development on *A. thaliana* while *M. extorquens* PA1 did not. The transcriptional response induced by *S. melonis* Fr1 was similar to the response induced by an encounter with the pathogenic *P. syringae* DC3000 (Vogel et al., 2016). The authors hypothesize that plants detect the presence of *S. melonis* Fr1 in a similar way as *P. syringae* and respond with an expression of defense-related genes that are involved in plant protection. However, the plant's response is less severe, probably because *S. melonis Fr1* lacks additional virulence factors which are present in *P. syringae* DC3000. The exact mechanisms still need to be unraveled, since plant mutants defective in several known defense signaling pathways, such as SA and JA signaling, and lacking the FLS2 PRR, showed that these pathways and receptors where not involved. More recently, Ryffel et al. (2016) demonstrated that *S. melonis* Fr1 induced camalexin production in the host plant. The pathogen *P. syringae* pv. *tomato* also induced production of camalexin, yet in higher concentrations. Production of the tryptophan-derived indole alkaloid camalexin, is a typical defense response of *Arabidopsis* and other plants from the Crucifereae family. Due to its lipophilic nature, camalexin is effective against a wide range of bacteria and fungi by interfering with the integrity of membranes (e.g., by binding to phospholipids). Production of camalexin by *Arabidopsis*, triggered by *S. melonis* Fr1 is thus postulated to be the mechanism behind the observed plant protection by this commensal.

The host's immune system does not only target bacterial or fungal pathogens, but can also protect against viral diseases (Lee and Ryu, 2016). Three-year field trials of foliar applications of *Bacillus amyloliquefasciens* 5B6 showed consistently reduced cucumber mosaic virus accumulation as measured by qPCR (Lee and Ryu, 2016). Observed changes in gene expression in the host plant suggested that activation of SA and ethylene signaling pathways played a key role in the acquired resistance. Also here, the genes upregulated by the biocontrol agent were the same as the genes upregulated in the defense response caused by several viruses, including cucumber mosaic virus (Park et al., 2004). *B. amyloliquefasciens* 5B6 colonized the phyllosphere successfully as their population size remained stable during seven days after administration of 10^8 CFU/ml until run-off. This contrasted with the sharp decline in population size of strain FZB42, isolated from the soil, showing that strain-specific adaptation traits are important for survival and successful biocontrol in the phyllosphere.

Another intriguing observation was made by Hong et al. (2016) on the known plant-growth promoting rhizosphere bacterium *Paenibacillus polymyxa* AC-1. This strain inhibited the growth of phyllosphere pathogens *P. syringae* pv. *tomato* DC3000 and *P. syringae* pv. *tabaci* in an *in vitro* setting. Cell-free supernatant of *P. polymyxa* AC-1 also suppressed these pathogens, suggesting that antimicrobial metabolites excreted by the antagonist play a direct antagonistic role (see section "Antimicrobial Metabolites"). Inoculation of the root tips of axenic *Arabidopsis* seedlings with bacterial suspensions of *P. polymyxa* AC-1 resulted in a SA and JA-dependent defense reaction. Interestingly, this inoculation of the roots of axenic plants resulted in colonization of the *Arabidopsis* leaf endosphere with *P. polymyxa* AC-1. Colonization of the leaf endosphere was 10-fold higher in *Arabidopsis* mutants with reduced sensitivity to JA and 10-fold lower in mutants deficient in the isoprenoid plant hormone abscisic acid, compared to wild-type plants. The colonization of the leaf endosphere by *P. polymyxa* AC-1 in JA-deficient plants even caused disease symptoms in the phyllopshere. This indicates that JA negatively impacts the detrimental endophytic growth of AC-1. This illustrates that the plant's defense system is important in regulating the total microbial load and preventing symbiotic bacteria to become invasive.

The mechanisms by which microbes are detected by the host and subsequently trigger the host's immune response, are similar in both non-pathogenic and pathogenic strains (Fig 2). However, non-pathogenic strains lack additional virulence factors, resulting in a milder defense response from the host. Biocontrol agents have the ability to trigger the immune system, and thereby inducing resistance to phylogenetically distinct pathogens. Sometimes, biocontrol agents are very closely related to pathogenic strains, or can even be opportunistic pathogens themselves (e.g., *Pseudomonas*, *R. fascians, P. polymyxa*), in this case the difference between ISR and SAR becomes less clear.

CONCLUSION AND FUTURE RESEARCH PERSPECTIVES

The phyllosphere harbors a diverse set of microbes. These microbes interact closely with each other and with the host plant. Amongst them are pathogens, causing disease in the host plant and reducing yields in agriculture, but also beneficial microbes which can be the key to environmentally friendly solutions to protect crops from diseases. These beneficial microbes can inhibit pathogen growth directly, by competing for nutrients and space, by interfering with their communication, by excreting antimicrobial metabolites or enzymes, or by parasitizing on the pathogen. On the other hand, beneficial microbes can trigger the plant's immune response and modulate plant hormone levels, and hereby indirectly inhibiting pathogen growth.

Knowledge on these mechanisms is often gained through *in vitro* experiments using gnotobiotic or sterile plants, while the efficacy of a biocontrol agent needs to be validated in field trials. In this review we linked these two types of studies and gave an overview of biocontrol mechanisms and adaptation mechanisms that play a role in the phyllosphere. Several mechanisms still need further validation, for example, the characterization of novel antimicrobial peptides, the role of type III secretion systems, the biocontrol potential of small peptides involved in quorum sensing and the impact of bacterially produced plant hormones on the host immune system. Next, we need to understand which mechanisms are at play in field conditions. This question has also been addressed by Köhl et al. (2019). Firstly, which biocontrol mechanisms are active on the field, e.g., some antimicrobial metabolites play a role in biocontrol *in vitro* but not *in vivo*, and reversely (Köhl et al., 2019; Santos Kron et al., 2020)? Secondly, is the biocontrol agent adapted to the phyllosphere in field conditions, and can it disperse in the growing plant (e.g., Wei et al., 2016)? Finally, the biocontrol agent needs to integrate in the resident microbial community. This resident community can positively or negatively influence the biocontrol activity and the success of colonization of the biocontrol agent (Massart et al., 2015).

Techniques that can help us understand the mechanisms playing a role in complex phyllosphere communities are metagenome, metatranscriptome, metaproteome, and metabolome analyses. Eventually, this understanding may allow us to go beyond the application of single strains, and toward designing communities, an idea that is often repeated in biocontrol research (Massart et al., 2015; Singh and Trivedi, 2017). Biocontrol communities possess a variety of complementary adaptation and biocontrol factors, that co-operate in suppressing the disease and surviving on the phyllosphere. Biocontrol agents and communities can be an effective and sustainable alternative to conventional pesticides, which is needed to safeguard our crop production.

AUTHOR CONTRIBUTIONS

ML and SL conceived and designed the manuscript. ML wrote the manuscript and prepared the figures. All authors critically reviewed the manuscript and contributed to figure improvement, with special attention toward their specific expertise ranging from phyllosphere bacterial communities, bacterial antimicrobial compounds, probiotics, bacterial genome analysis and functional prediction, plant hormones, plant immune system toward ecology in general and approved the final version of the manuscript.

FUNDING

ML and TE are currently funded by the Industrial Research Fund (IOF) of the University of Antwerp, in the context of the PhylloBac project. ML was previously funded by the University Research Fund (BOF) of the University of Antwerp to start her Ph.D. on biocontrol in the phyllosphere (01/2019–01/2020). BM currently holds a personal FWO-SB grant (Research Foundation of Flanders) to explore bioremediation potential of phyllosphere bacterial communities. SL currently holds an ERC grant (Lacto-Be, 85600) in which the phyllosphere is one of the habitats explored for lactobacilli. DV has received a small BOF-KP grant (BOF/KP2017 34486) to explore bacteria in the phyllosphere.

REFERENCES

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

Ali, G. S., El-Sayed, A. S. A., Patel, J. S., Green, K. B., Ali, M., Brennan, M., et al. (2016). Ex Vivo application of secreted metabolites produced by soil-inhabiting *Bacillus* spp. Efficiently controls foliar diseases caused by *Alternaria* spp. *Appl. Environ. Microbiol.* 82, 478–490. doi: 10.1128/aem.02662-15

Allonsius, C. N., Vandenheuvel, D., Oerlemans, E. F. M., Petrova, M. I., Donders, G. G. G., Cos, P., et al. (2019). Inhibition of *Candida albicans* morphogenesis by chitinase from Lactobacillus rhamnosus GG. *Sci. Rep.* 9:2900. doi: 10.1038/s41598-019-39625-0

Alymanesh, M. R., Taheri, P., and Tarighi, S. (2016). *Pseudomonas* as a frequent and important quorum quenching bacterium with biocontrol capability against many phytopathogens. *Biocontrol Sci. Technol.* 26, 1719–1735. doi: 10.1080/09583157.2016.1239065

Bailly, A., and Weisskopf, L. (2017). Mining the volatilomes of plant-associated microbiota for new biocontrol solutions. *Front. Microbiol.* 8:1638. doi: 10.3389/fmicb.2017.01638

Bakker, P. A. H. M., Pieterse, C. M. J., and Van Loon, L. C. (2007). Induced Systemic Resistance by fluorescent *Pseudomonas* spp. *Phytopathology* 97, 239–243. doi: 10.1094/PHYTO-97-2-0239

Berg, M., and Koskella, B. (2018). Nutrient- and Dose-Dependent Microbiome-Mediated Protection against a Plant Pathogen. *Curr. Biol.* 28:2487-2492.e3. doi: 10.1016/j.cub.2018.05.085

Bernal, P., Allsopp, L. P., Filloux, A., and Llamas, M. A. (2017). The *Pseudomonas putida* T6SS is a plant warden against phytopathogens. *ISME J.* 11, 972–987. doi: 10.1038/ismej.2016.169

Bernal, P., Llamas, M. A., and Filloux, A. (2018). Type VI secretion systems in plant-associated bacteria. *Environ. Microbiol.* 20, 1–15. doi: 10.1111/1462-2920.13956

Blanvillain, S., Meyer, D., Boulanger, A., Lautier, M., Guynet, C., Denancé, N., et al. (2007). Plant carbohydrate scavenging through TonB-dependent receptors: a feature shared by phytopathogenic and aquatic bacteria. *PLoS One* 2:e224. doi: 10.1371/journal.pone.0000224

Blin, K., Shaw, S., Steinke, K., Villebro, R., Ziemert, N., Lee, S. Y., et al. (2019). antiSMASH 5.0: updates to the secondary metabolite genome mining pipeline. *Nucleic Acids Res.* 47, W81–W87. doi: 10.1093/nar/gkz310

Bodenhausen, N., Bortfeld-Miller, M., Ackermann, M., and Vorholt, J. A. (2014). A synthetic community approach reveals plant genotypes affecting the phyllosphere microbiota. *PLoS Genet.* 10:e1004283. doi: 10.1371/journal.pgen.1004283

Boller, T. (1993). "Antimicrobial Functions of the Plant Hydrolases, Chitinase and ß-1,3-Glucanase," in *Mechanisms of Plant Defense Responses. Developments in Plant Pathology*, Vol 2, B. Fritig, and M. Legrand (Dordrecht: Springer), 391–400. doi: 10.1007/978-94-011-1737-1_124

Brandl, M. T., Quiñones, B., and Lindow, S. E. (2001). Heterogeneous transcription of an indoleacetic acid biosynthetic gene in Erwinia herbicola on plant surfaces. *Proc. Natl. Acad. Sci. U.S.A.* 98, 3454–3459. doi: 10.1073/pnas.061014498

Broeckx, G., Vandenheuvel, D., Claes, I. J. J., Lebeer, S., and Kiekens, F. (2016). Drying techniques of probiotic bacteria as an important step towards the

development of novel pharmabiotics. *Int. J. Pharm.* 505, 303–318. doi: 10.1016/j.ijpharm.2016.04.002

Burbank, L., Mohammadi, M., and Roper, M. C. (2015). Siderophore-mediated iron acquisition influences motility and is required for full virulence of the xylem-dwelling bacterial phytopathogen *Pantoea stewartii* subsp. stewartii. *Appl. Environ. Microbiol.* 81, 139–148. doi: 10.1128/AEM.02503-14

Chen, W. J., Kuo, T. Y., Hsieh, F. C., Chen, P. Y., Wang, C. S., Shih, Y. L., et al. (2016). Involvement of type VI secretion system in secretion of iron chelator pyoverdine in *Pseudomonas taiwanensis*. *Sci. Rep.* 6, 1–14. doi: 10.1038/srep32950

Chen, X. H., Koumoutsi, A., Scholz, R., Eisenreich, A., Schneider, K., Heinemeyer, I., et al. (2007). Comparative analysis of the complete genome sequence of the plant growth-promoting bacterium *Bacillus amyloliquefaciens* FZB42. *Nat. Biotechnol.* 25, 1007–1014. doi: 10.1038/nbt1325

Chen, X. H., Scholz, R., Borriss, M., Junge, H., Mögel, G., Kunz, S., et al. (2009). Difficidin and bacilysin produced by plant-associated *Bacillus amyloliquefaciens* are efficient in controlling fire blight disease. *J. Biotechnol.* 140, 38–44. doi: 10.1016/j.jbiotec.2008.10.015

Chen, Y., Wang, J., Yang, N., Wen, Z., Sun, X., Chai, Y., et al. (2018). Wheat microbiome bacteria can reduce virulence of a plant pathogenic fungus by altering histone acetylation. *Nat. Commun.* 9:3429. doi: 10.1038/s41467-018-05683-7

Chin-A-Woeng, T. F. C., Bloemberg, G. V., and Lugtenberg, B. J. J. (2003). Phenazines and their role in biocontrol by *Pseudomonas* bacteria. *New Phytol.* 157, 503–523. doi: 10.1046/j.1469-8137.2003.00686.x

Chinnadurai, C., Balachandar, D., and Sundaram, S. P. (2009). Characterization of 1-aminocyclopropane-1-carboxylate deaminase producing methylobacteria from phyllosphere of rice and their role in ethylene regulation. *World J. Microbiol. Biotechnol.* 25, 1403–1411. doi: 10.1007/s11274-009-0027-1

Van de Poel, B., and Van Der Straeten, D. (2014). 1-aminocyclopropane-1-carboxylic acid (ACC) in plants: more than just the precursor of ethylene!. *Front. Plant Sci.* 5:640. doi: 10.3389/fpls.2014.00640

de Souza, R. S. C., Armanhi, J. S. L., Damasceno, N., de, B., Imperial, J., and Arruda, P. (2019). Genome sequences of a plant beneficial synthetic bacterial community reveal genetic features for successful plant colonization. *Front. Microbiol.* 10:1779. doi: 10.3389/fmicb.2019.01779

Deakin, W. J., and Broughton, W. J. (2009). Symbiotic use of pathogenic strategies: rhizobial protein secretion systems. *Nat. Rev. Microbiol.* 7, 312–320. doi: 10.1038/nrmicro2091

Delalande, L., Faure, D., Raffoux, A., Uroz, S., D'Angelo-Picard, C., Elasri, M., et al. (2005). N-hexanoyl-L-homoserine lactone, a mediator of bacterial quorum-sensing regulation, exhibits plant-dependent stability and may be inactivated by germinating Lotus corniculatus seedlings. *FEMS Microbiol. Ecol.* 52, 13–20. doi: 10.1016/j.femsec.2004.10.005

Delmotte, N., Claudia, K., Samuel, C., Gerd, I., Bernd, R., Ralph, S., 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

Duca, D., Lorv, J., Patten, C. L., Rose, D., and Glick, B. R. (2014). Indole-3-acetic acid in plant-microbe interactions. *Antonie van Leeuwenhoek* 106, 85–125. doi: 10.1007/s10482-013-0095-y

Dunlap, C. A., Bowman, M. J., and Schisler, D. A. (2013). Genomic analysis and secondary metabolite production in *Bacillus amyloliquefaciens* AS 43.3: a biocontrol antagonist of Fusarium head blight. *Biol. Control* 64, 166–175. doi: 10.1016/j.biocontrol.2012.11.002

Edwards, S. G., and Seddon, B. (2001). Mode of antagonism of Brevibacillus brevis against Botrytis cinerea in vitro. *J. Appl. Microbiol.* 91, 652–659. doi: 10.1046/j.1365-2672.2001.01430.x

Essghaier, B., Hedi, A., Halaoui, M. R., Boudabous, A., and Sadfi-Zouaoui, N. (2012). In vivo and in vitro evaluation of antifungal activities from a halotolerant Bacillus subtilis strain J9. *Afr. J. Microbiol. Res.* 6, 4073–4083. doi: 10.5897/ajmr11.403

Faize, M., Brisset, M. N., Perino, C., Vian, B., Barny, M. A., Paulin, J. P., et al. (2006). Protection of apple against fire blight induced by an hrpL mutant of *Erwinia amylovora*. *Biol. Plant.* 50, 667–674. doi: 10.1007/s10535-006-0104-3

Feng, D. X., Tasset, C., Hanemian, M., Barlet, X., Hu, J., Trémousaygue, D., et al. (2012). Biological control of bacterial wilt in Arabidopsis thaliana involves abscissic acid signalling. *New Phytol.* 194, 1035–1045. doi: 10.1111/j.1469-8137.2012.04113.x

Fernando, W. G. D., Nakkeeran, S., Zhang, Y., and Savchuk, S. (2007). Biological control of *Sclerotinia sclerotiorum* (Lib.) de Bary by *Pseudomonas* and *Bacillus* species on canola petals. *Crop Prot.* 26, 100–107. doi: 10.1016/j.cropro.2006.04.007

Francis, I. M., Stes, E., Zhang, Y., Rangel, D., Audenaert, K., and Vereecke, D. (2016). Mining the genome of *Rhodococcus fascians*, a plant growth-promoting bacterium gone astray. *N. Biotechnol.* 33, 706–717. doi: 10.1016/j.nbt.2016.01.009

Gamalero, E., and Glick, B. R. (2015). Bacterial modulation of plant ethylene levels. *Plant Physiol.* 169, 13–22. doi: 10.1104/pp.15.00284

Giddens, S. R., Feng, Y., and Mahanty, H. K. (2002). Characterization of a novel phenazine antibiotic gene cluster in *Erwinia herbicola* Eh1087. *Mol. Microbiol.* 45, 769–783. doi: 10.1046/j.1365-2958.2002.03048.x

Giddens, S. R., Houliston, G. J., and Mahanty, H. K. (2003). The influence of antibiotic production and pre-emptive colonization on the population dynamics of Pantoea agglomerans (Erwinia herbicola) Eh1087 and *Erwinia amylovora* in planta. *Environ. Microbiol.* 5, 1016–1021. doi: 10.1046/j.1462-2920.2003.00506.x

Glazebrook, J. (2005). Contrasting mechanisms of defense against biotrophic and necrotrophic pathogens. *Annu. Rev. Phytopathol.* 43, 205–227. doi: 10.1146/annurev.phyto.43.040204.135923

Guennoc, C. M., Rose, C., Labbé, J., and Deveau, A. (2018). Bacterial biofilm formation on the hyphae of ectomycorrhizal fungi: a widespread ability under controls? *FEMS Microbiol. Ecol.* 94:fiy093. doi: 10.1093/femsec/fiy093

Hacquard, S., Spaepen, S., Garrido-Oter, R., and Schulze-Lefert, P. (2017). Interplay between innate immunity and the plant microbiota. *Annu. Rev. Phytopathol.* 55, 565–589. doi: 10.1146/annurev-phyto-080516-35623

Haefele, D. M., and Lindow, S. E. (1987). Flagellar Motility Confers Epiphytic Fitness Advantages upon *Pseudomonas syringae*. *Appl. Environ. Microbiol.* 53, 2528–2533. doi: 10.1128/aem.53.10.2528-2533.1987

Hanemian, M., Zhou, B., Deslandes, L., Marco, Y., and Trémousaygue, D. (2013). Hrp mutant bacteria as biocontrol agents: toward a sustainable approach in the fight against plant pathogenic bacteria. *Plant Signal. Behav.* 8:e25678. doi: 10.4161/psb.25678

Helfrich, E. J. N. N., Vogel, C. M., Ueoka, R., Schäfer, M., Ryffel, F., Müller, D. B., et al. (2018). Bipartite interactions, antibiotic production and biosynthetic potential of the Arabidopsis leaf microbiome. *Nat. Microbiol.* 3, 909–919. doi: 10.1038/s41564-018-0200-0

Hernandez, M. N., and Lindow, S. E. (2019). *Pseudomonas syringae* increases water availability in leaf microenvironments via production of hygroscopic syringafactin. *Appl. Environ. Microbiol.* 85, e1014–e1019. doi: 10.1128/AEM.01014-19

Herpell, J. B., Schindler, F., Bejtoviæ, M., Fragner, L., Diallo, B., Bellaire, A., et al. (2020). The Potato Yam Phyllosphere Ectosymbiont *Paraburkholderia* sp. Msb3 is a potent growth promotor in tomato. *Front. Microbiol.* 11:581. doi: 10.3389/fmicb.2020.00581

Hill, C., Guarner, F., Reid, G., Gibson, G. R., Merenstein, D. J., Pot, B., et al. (2014). Expert consensus document: the international scientific association for probiotics and prebiotics consensus statement on the scope and appropriate use of the term probiotic. *Nat. Rev. Gastroenterol. Hepatol.* 11, 506–514. doi: 10.1038/nrgastro.2014.66

Hong, C. E., Kwon, S. Y., and Park, J. M. (2016). Biocontrol activity of Paenibacillus polymyxa AC-1 against *Pseudomonas syringae* and its interaction with *Arabidopsis thaliana*. *Microbiol. Res.* 185, 13–21. doi: 10.1016/j.micres.2016.01.004

Hosni, T., Moretti, C., Devescovi, G., Suarez-Moreno, Z. R., Fatmi, M. B., Guarnaccia, C., et al. (2011). Sharing of quorum-sensing signals and role of interspecies communities in a bacterial plant disease. *ISME J.* 5, 1857–1870. doi: 10.1038/ismej.2011.65

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

Jameson, P. E. (2000). "Cytokinins and auxins in plant-pathogen interactions - An overview," in *Plant Growth Regulation*, Ed. Z.-H. Chen (Berlin: Springer), 369–380. doi: 10.1023/A:1010733617543

Ji, P., and Wilson, M. (2002). Assessment of the importance of similarity in carbon source utilization profiles between the biological control agent and the pathogen in biological control of bacterial speck of tomato. *Appl. Environ. Microbiol.* 68, 4383–4389. doi: 10.1128/AEM.68.9.4383-4389.2002

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

Jorge, G. L., Kisiala, A., Morrison, E., Aoki, M., Nogueira, A. P. O., and Emery, R. J. N. (2019). Endosymbiotic *Methylobacterium oryzae* mitigates the impact of limited water availability in lentil (*Lens culinaris* Medik.) by increasing plant cytokinin levels. *Environ. Exp. Bot.* 162, 525–540. doi: 10.1016/j.envexpbot.2019.03.028

Kamber, T., Lansdell, T. A., Stockwell, V. O., Ishimaru, C. A., Smits, T. H. M., and Duffy, B. (2012). Characterization of the biosynthetic operon for the antibacterial peptide herbicolin in *Pantoea vagans* biocontrol strain C9-1 and incidence in *Pantoea* species. *Appl. Environ. Microbiol.* 78, 4412–4419. doi: 10.1128/AEM.07351-11

Kim, B.-Y., Lee, S.-Y., Ahn, J.-H., Song, J., Kim, W.-G., and Weon, H.-Y. (2015). Complete Genome Sequence of *Bacillus amyloliquefaciens* subsp. plantarum CC178, a Phyllosphere Bacterium Antagonistic to Plant Pathogenic Fungi. *Genome Announc.* 3:e01368-14. doi: 10.1128/genomeA.01368-14

Kleerebezem, M. (2004). Quorum sensing control of lantibiotic production; nisin and subtilin autoregulate their own biosynthesis. *Peptides* 25, 1405–1414. doi: 10.1016/j.peptides.2003.10.021

Kloepper, J. W., and Ryu, C.-M. (2007). "Bacterial endophytes as elicitors of induced systemic resistance," in *Microbial Root Endophytes*, eds C. J.C. Boyle, T. N. Sieber, and B. J. E. Schulz (Berlin: Springer Berlin Heidelberg), 33–52. doi: 10.1007/3-540-33526-9_3

Koenig, R. L., Morris, R. O., and Polacco, J. C. (2002). tRNA is the source of low-level trans-zeatin production in *Methylobacterium* spp. *J. Bacteriol.* 184, 1832–1842. doi: 10.1128/JB.184.7.1832-1842.2002

Köhl, J., Kolnaar, R., and Ravensberg, W. J. (2019). Mode of action of microbial biological control agents against plant diseases: relevance beyond efficacy. *Front. Plant Sci.* 10:845. doi: 10.3389/fpls.2019.00845

Koornneef, A., and Pieterse, C. M. J. (2008). Cross talk in defense signaling. *Plant Physiol.* 146, 839–844. doi: 10.1104/pp.107.112029

Kramer, J., Özkaya, Ö, and Kümmerli, R. (2019). Bacterial siderophores in community and host interactions. *Nat. Rev. Microbiol.* 18, 152–163. doi: 10.1038/s41579-019-0284-4

Kutschera, U. (2007). Plant-associated methylobacteria as co-evolved phytosymbionts: a hypothesis. *Plant Signal. Behav.* 2, 74–78. doi: 10.4161/psb.2.2.4073

Kwak, M. J., Jeong, H., Madhaiyan, M., Lee, Y., Sa, T. M., Oh, T. K., et al. (2014). Genome information of *Methylobacterium oryzae*, a plant-probiotic methylotroph in the phyllosphere. *PLoS One* 9:e106704. doi: 10.1371/journal.pone.0106704

Laforest-Lapointe, I., Messier, C., and Kembel, S. W. (2016). Host species identity, site and time drive temperate tree phyllosphere bacterial community structure. *Microbiome* 4:27. doi: 10.1186/s40168-016-0174-1

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

Lebeer, S., Vanderleyden, J., and De Keersmaecker, S. C. J. (2008). Genes and molecules of *Lactobacilli* supporting probiotic action. *Microbiol. Mol. Biol. Rev.* 72, 728–764. doi: 10.1128/mmbr.00017-08

Lebeer, S., Vanderleyden, J., and De Keersmaecker, S. C. J. (2010). Host interactions of probiotic bacterial surface molecules: comparison with commensals and pathogens. *Nat. Rev. Microbiol.* 8, 171–184. doi: 10.1038/nrmicro2297

Lee, G. H., and Ryu, C.-M. (2016). Spraying of leaf-colonizing *Bacillus amyloliquefaciens* protects pepper from Cucumber mosaic virus. *Plant Dis.* 100, 2099–2105. doi: 10.1094/PDIS-03-16-0314-RE

Leveau, J. H. J., and Gerards, S. (2008). Discovery of a bacterial gene cluster for catabolism of the plant hormone indole 3-acetic acid. *FEMS Microbiol. Ecol.* 65, 238–250. doi: 10.1111/j.1574-6941.2008.00436.x

Leveau, J. H. J., and Lindow, S. E. (2005). Utilization of the plant hormone indole-3-acetic acid for growth by *Pseudomonas putida* strain 1290. *Appl. Environ. Microbiol.* 71, 2365–2371. doi: 10.1128/AEM.71.5.2365-2371.2005

Li, Z., Yao, Q., Guo, X., Crits-Christoph, A., Mayes, M. A., Iv, W. J. H., et al. (2019). Genome-resolved proteomic stable isotope probing of soil microbial communities using 13CO2 and 13C-Methanol. *Front. Microbiol.* 10:2706. doi: 10.3389/fmicb.2019.02706

Lin, G. H., Chen, H. P., Huang, J. H., Liu, T. T., Lin, T. K., Wang, S. J., et al. (2012). Identification and characterization of an indigo-producing oxygenase involved in indole 3-acetic acid utilization by *Acinetobacter baumannii*. *Antonie van Leeuwenhoek* 101, 881–890. doi: 10.1007/s10482-012-9704-4

Lindow, S. E. (1987). Competitive exclusion of epiphytic bacteria by Ice-*Pseudomonas syringae* mutants. *Appl. Environ. Microbiol.* 53, 2520–2527. doi: 10.1128/aem.53.10.2520-2527.1987

Luo, L., Zhang, Z., Wang, P., Han, Y., Jin, D., Su, P., et al. (2019). Variations in phyllosphere microbial community along with the development of angular leaf-spot of cucumber. *AMB Express* 9:76. doi: 10.1186/s13568-019-0800-y

Madhaiyan, M., Suresh Reddy, B. V., Anandham, R., Senthilkumar, M., Poonguzhali, S., Sundaram, S. P., et al. (2006). Plant growth-promoting Methylobacterium induces defense responses in groundnut (*Arachis hypogaea* L.) compared with rot pathogens. *Curr. Microbiol.* 53, 270–276. doi: 10.1007/s00284-005-0452-9

Maignien, L., DeForce, E. A., Chafee, M. E., Murat Eren, A., and Simmons, S. L. (2014). Ecological succession and stochastic variation in the assembly of Arabidopsis thaliana phyllosphere communities. *mBio* 5:e00682-13. doi: 10.1128/mBio.00682-13

Massart, S., Margarita, M. M., and Jijakli, M. H. (2015). Biological control in the microbiome era: challenges and opportunities. *Biol. Control* 89, 98–108. doi: 10.1016/j.biocontrol.2015.06.003

McClerklin, S. A., Lee, S. G., Harper, C. P., Nwumeh, R., Jez, J. M., and Kunkel, B. N. (2018). Indole-3-acetaldehyde dehydrogenase-dependent auxin synthesis contributes to virulence of *Pseudomonas syringae* strain DC3000. *PLoS Pathog.* 14:e1006811. doi: 10.1371/journal.ppat.1006811

Mcdonald, I. R., and Murrell, J. C. (1997). The methanol dehydrogenase structural gene mxaf and its use as a functional gene probe for methanotrophs and methylotrophs. *Appl. Environ. Microbiol.* 63, 3218–3224. doi: 10.1128/aem.63.8.3218-3224.1997

Mercier, J., and Lindow, S. E. (2000). Role of leaf surface sugars in colonization of plants by bacterial epiphytes. *Appl. Environ. Microbiol.* 66, 369–374. doi: 10.1128/AEM.66.1.369-374.2000

Michavila, G., Adler, C., De Gregorio, P. R., Lami, M. J., Caram Di Santo, M. C., Zenoff, A. M., et al. (2017). *Pseudomonas* protegens CS1 from the lemon phyllosphere as a candidate for citrus canker biocontrol agent. *Plant Biol.* 19, 608–617. doi: 10.1111/plb.12556

Miller, E. R., Kearns, P. J., Niccum, B. A., Schwartz, J. O., Ornstein, A., and Wolfe, B. E. (2019). Establishment limitation constrains the abundance of lactic acid bacteria in the Napa cabbage phyllosphere. *Appl. Environ. Microbiol.* 85:AEM.00269-19. doi: 10.1128/AEM.00269-19

Morella, N. M., Zhang, X., and Koskella, B. (2019). Tomato seed-associated bacteria confer protection of seedlings against foliar disease caused by *Pseudomonas syringae*. *Phytobiomes J.* 3, 177–190. doi: 10.1094/PBIOMES-01-19-0007-R

Morohoshi, T., Someya, N., and Ikeda, T. (2009). Novel n-acylhomoserine lactone-degrading bacteria isolated from the leaf surface of solarium tuberosum and their quorum-quenching properties. *Biosci. Biotechnol. Biochem.* 73, 2124–2127. doi: 10.1271/bbb.90283

Nascimento, F. X., Glick, B. R., and Rossi, M. J. (2019). Isolation and characterization of novel soil-and plant-associated bacteria with multiple phytohormone-degrading activities using a targeted methodology. *Access Microbiol.* 1:e000053, doi: 10.1099/acmi.0.000053

Nascimento, F. X., Rossi, M. J., and Glick, B. R. (2018). Ethylene and 1-aminocyclopropane-1-carboxylate (ACC) in plant–bacterial interactions. *Front. Plant Sci.* 9:114. doi: 10.3389/fpls.2018.00114

Newman, K. L., Chatterjee, S., Ho, K. A., and Lindow, S. E. (2008). Virulence of plant pathogenic bacteria attenuated by degradation of fatty acid cell-to-cell signaling factors. *Mol. Plant-Microbe Interact.* 21, 326–334. doi: 10.1094/MPMI-21-3-0326

Nielsen, C. J., Ferrin, D. M., and Stanghellini, M. E. (2006). Efficacy of biosurfactants in the management of *Phytophthora capsici* on pepper in recirculating hydroponic systems. *Can. J. Plant Pathol.* 28, 450–460. doi: 10.1080/07060660609507319

Nishimoto, R. (2019). Global trends in the crop protection industry. *J. Pestic. Sci.* 44, 141–147. doi: 10.1584/jpestics.D19-101

On, A., Wong, F., Ko, Q., Tweddell, R. J., Antoun, H., and Avis, T. J. (2015). Antifungal effects of compost tea microorganisms on tomato pathogens. *Biol. Control* 80, 63–69. doi: 10.1016/J.BIOCONTROL.2014.09.017

Ona, O., Van Impe, J., Prinsen, E., and Vanderleyden, J. (2005). Growth and indole-3-acetic acid biosynthesis of *Azospirillum brasilense* Sp245 is environmentally controlled. *FEMS Microbiol. Lett.* 246, 125–132. doi: 10.1016/j.femsle.2005.03.048

Ongena, M., and Jacques, P. (2008). Bacillus lipopeptides: versatile weapons for plant disease biocontrol. *Trends Microbiol.* 16, 115–125. doi: 10.1016/j.tim.2007.12.009

Ongena, M., Jourdan, E., Adam, A., Paquot, M., Brans, A., Joris, B., et al. (2007). Surfactin and fengycin lipopeptides of *Bacillus subtilis* as elicitors of induced systemic resistance in plants. *Environ. Microbiol.* 9, 1084–1090. doi: 10.1111/j.1462-2920.2006.01202.x

Park, C.-J., Kim, K.-J., Shin, R., Park, J. M., Shin, Y.-C., and Paek, K.-H. (2004). Pathogenesis-related protein 10 isolated from hot pepper functions as a ribonuclease in an antiviral pathway. *Plant J.* 37, 186–198. doi: 10.1046/j.1365-313X.2003.01951

Pfeilmeier, S., Caly, D. L., and Malone, J. G. (2016). Bacterial pathogenesis of plants: future challenges from a microbial perspective: challenges in bacterial molecular plant pathology. *Mol. Plant Pathol.* 17, 1298–1313. doi: 10.1111/mpp.12427

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

Pontonio, E., Di Cagno, R., Tarraf, W., Filannino, P., De Mastro, G., and Gobbetti, M. (2018). Dynamic and assembly of epiphyte and endophyte lactic acid bacteria during the life cycle of *Origanum vulgare* L. *Front. Microbiol.* 9:1372. doi: 10.3389/fmicb.2018.01372

Radhika, V., Ueda, N., Tsuboi, Y., Kojima, M., Kikuchi, J., Kudo, T., et al. (2015). Methylated Cytokinins from the Phytopathogen *Rhodococcus fascians* Mimic Plant Hormone Activity 1[OPEN]. *Plant Physiol.* 169, 1118–1126. doi: 10.1104/pp.15.00787

Roitsch, T., and Ehneß, R. (2000). Regulation of source/sink relations by cytokinins. *Plant Growth Regulat.* 32, 359–367.

Romero, F. M., Marina, M., and Pieckenstain, F. L. (2016). Novel components of leaf bacterial communities of field-grown tomato plants and their potential for plant growth promotion and biocontrol of tomato diseases. *Res. Microbiol.* 167, 222–233. doi: 10.1016/j.resmic.2015.11.001

Ruiz, J. A., Bernar, E. M., and Jung, K. (2015). Production of siderophores increases resistance to fusaric acid in *Pseudomonas* protegens Pf-5. *PLoS One* 10:e0117040. doi: 10.1371/journal.pone.0117040

Ryffel, F., Helfrich, E. J. N., Kiefer, P., Peyriga, L., Portais, J. C., Piel, J., et al. (2016). Metabolic footprint of epiphytic bacteria on Arabidopsis thaliana leaves. *ISME J.* 10, 632–643. doi: 10.1038/ismej.2015.141

Saghafi, D., Asgari Lajayer, B., and Ghorbanpour, M. (2020). "Engineering bacterial ACC deaminase for improving plant productivity under stressful conditions," in *Molecular Aspects of Plant Beneficial Microbes in Agriculture*, eds V. Sharma, R. Salwan, and L. K. Tawfeeq Al-ani (Amsterdam: Elsevier), 259–277. doi: 10.1016/b978-0-12-818469-1.00022-5

Saijo, Y., Loo, E. P., and Yasuda, S. (2018). Pattern recognition receptors and signaling in plant–microbe interactions. *Plant J.* 93, 592–613. doi: 10.1111/tpj.13808

Salvatierra-Martinez, R., Arancibia, W., Araya, M., Aguilera, S., Olalde, V., Bravo, J., et al. (2018). Colonization ability as an indicator of enhanced biocontrol capacity—An example using two *Bacillus amyloliquefaciens* strains and *Botrytis cinerea* infection of tomatoes. *J. Phytopathol.* 166, 601–612. doi: 10.1111/jph.12718

Sanchez, L., Courteaux, B., Hubert, J., Kauffmann, S., Renault, J. H., Clément, C., et al. (2012). Rhamnolipids elicit defense responses and induce disease resistance against biotrophic, hemibiotrophic, and necrotrophic pathogens that require different signaling pathways in Arabidopsis and highlight a central role for salicylic acid. *Plant Physiol.* 160, 1630–1641. doi: 10.1104/pp.112.201913

Santos Kron, A., Zengerer, V., Bieri, M., Dreyfuss, V., Sostizzo, T., Schmid, M., et al. (2020). *Pseudomonas orientalis* F9 Pyoverdine, Safracin, and Phenazine Mutants Remain Effective Antagonists against *Erwinia amylovora* in apple flowers. *Appl. Environ. Microbiol.* 86:e02620-19. doi: 10.1128/AEM.02620-19

Savary, S., Willocquet, L., Pethybridge, S. J., Esker, P., McRoberts, N., and Nelson, A. (2019). The global burden of pathogens and pests on major food crops. *Nat. Ecol. Evol.* 3, 430–439. doi: 10.1038/s41559-018-0793-y

Schauer, S., and Kutschera, U. (2011). A novel growth-promoting microbe, *Methylobacterium funariae* sp. nov., isolated from the leaf surface of a common moss. *Plant Signal. Behav.* 6, 510–515. doi: 10.4161/psb.6.4.14335

Schauer, S., and Kutschera, U. (2013). Methylobacteria isolated from bryophytes and the 2-fold description of the same microbial species. *Plant Signal. Behav.* 8:e23091. doi: 10.4161/psb.23091

Schenk, S. T., and Schikora, A. (2015). AHL-Priming functions via oxylipin and salicylic acid. *Front. Plant Sci.* 5:784. doi: 10.3389/fpls.2014.00784

Schisler, D. A., Khan, N. I., Boehm, M. J., and Slininger, P. J. (2002). Greenhouse and field evaluation of biological control of Fusarium head blight on durum wheat. *Plant Dis.* 86, 1350–1356. doi: 10.1094/PDIS.2002.86.12.1350

Sieper, T., Forczek, S., Matucha, M., Krämer, P., Hartmann, A., and Schröder, P. (2014). N-acyl-homoserine lactone uptake and systemic transport in barley rest upon active parts of the plant. *New Phytol.* 201, 545–555. doi: 10.1111/nph.12519

Simionato, A. S., Navarro, M. O. P., de Jesus, M. L. A., Barazetti, A. R., da Silva, C. S., Simões, G. C., et al. (2017). The effect of phenazine-1-carboxylic acid on mycelial growth of Botrytis cinerea produced by *Pseudomonas aeruginosa* LV strain. *Front. Microbiol.* 8:1102. doi: 10.3389/fmicb.2017.01102

Singh, B. K., and Trivedi, P. (2017). Microbiome and the future for food and nutrient security. *Microb. Biotechnol.* 10, 50–53. doi: 10.1111/1751-7915.12592

Smets, W., Wuyts, K., Oerlemans, E., Wuyts, S., Denys, S., Samson, R., et al. (2016). Impact of urban land use on the bacterial phyllosphere of ivy (*Hedera* sp.). *Atmos. Environ.* 147, 376–383. doi: 10.1016/j.atmosenv.2016.10.017

Smits, T. H. M., Duffy, B., Blom, J., Ishimaru, C. A., and Stockwell, V. O. (2019). Pantocin A, a peptide-derived antibiotic involved in biological control by plant-associated *Pantoea* species. *Arch. Microbiol.* 201, 713–722. doi: 10.1007/s00203-019-01647-7

Stes, E., Prinsen, E., Holsters, M., and Vereecke, D. (2012). Plant-derived auxin plays an accessory role in symptom development upon *Rhodococcus fascians* infection. *Plant J.* 70, 513–527. doi: 10.1111/j.1365-313X.2011.04890.x

Stockwell, V. O., Johnson, K. B., Sugar, D., and Loper, J. E. (2002). Antibiosis contributes to biological control of fire blight by *Pantoea agglomerans* strain Eh252 in orchards. *Phytopathology* 92, 1202–1209. doi: 10.1094/PHYTO.2002.92.11.1202

Strano, C. P., Bella, P., Licciardello, G., Caruso, A., and Catara, V. (2017). Role of secondary metabolites in the biocontrol activity of *Pseudomonas corrugata* and *Pseudomonas mediterranea*. *Eur. J. Plant Pathol.* 149, 103–115. doi: 10.1007/s10658-017-1169-x

Stringlis, I. A., Zamioudis, C., Berendsen, R. L., Bakker, P. A. H. M., and Pieterse, C. M. J. (2019). Type III secretion system of beneficial rhizobacteria *Pseudomonas simiae* WCS417 and *Pseudomonas defensor* WCS374. *Front. Microbiol.* 10:1631. doi: 10.3389/fmicb.2019.01631

Surico, G., Iacobellis, N. S., and Sisto, A. (1985). Studies on the role of indole-3-acetic acid and cytokinins in the formation of knots on olive and oleander plants by *Pseudomonas syringae* pv. savastanoi. *Physiol. Plant Pathol.* 26, 309–320. doi: 10.1016/0048-4059(85)90006-2

Trias, R., Bañeras, L., Badosa, E., and Montesinos, E. (2008). Bioprotection of Golden Delicious apples and Iceberg lettuce against foodborne bacterial pathogens by lactic acid bacteria. *Int. J. Food Microbiol.* 123, 50–60. doi: 10.1016/j.ijfoodmicro.2007.11.065

Tsang, D. L., Edmond, C., Harrington, J. L., and Nühse, T. S. (2011). Cell wall integrity controls root elongation via a general 1-aminocyclopropane-1-carboxylic acid-dependent, ethylene-independent pathway. *Plant Physiol.* 156, 596–604. doi: 10.1104/pp.111.175372

Tyagi, S., Mulla, S. I., Lee, K. J., Chae, J. C., and Shukla, P. (2018). VOCs-mediated hormonal signaling and crosstalk with plant growth promoting microbes. *Crit. Rev. Biotechnol.* 38, 1277–1296. doi: 10.1080/07388551.2018.1472551

Vanderhoef, L. N., and Dute, R. R. (1981). Auxin-regulated wall loosening and sustained growth in elongation. *Plant Physiol.* 67, 146–149. doi: 10.1104/pp.67.1.146

Vanderstraeten, L., and van Der Straeten, D. (2017). Accumulation and transport of 1-aminocyclopropane-1-carboxylic acid (ACC) in plants: current status, considerations for future research and agronomic applications. *Front. Plant Sci.* 8:38. doi: 10.3389/fpls.2017.00038

Veliz, E. A., Martínez-Hidalgo, P., and Hirsch, A. M. (2017). Chitinase-producing bacteria and their role in biocontrol. *AIMS Microbiol.* 3, 689–705. doi: 10.3934/microbiol.2017.3.689

Vogel, C., Bodenhausen, N., Gruissem, W., and Vorholt, J. A. (2016). The Arabidopsis leaf transcriptome reveals distinct but also overlapping responses to colonization by phyllosphere commensals and pathogen infection with impact on plant health. *New Phytol.* 212, 192–207. doi: 10.1111/nph.14036

Völksch, B., and May, R. (2001). Biological control of *Pseudomonas syringae* pv. glycinea by epiphytic bacteria under field conditions. *Microb. Ecol.* 41, 132–139. doi: 10.1007/s002480000078

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

Walterson, A. M., Smith, D. D. N., and Stavrinides, J. (2014). Identification of a Pantoea biosynthetic cluster that directs the synthesis of an antimicrobial natural product. *PLoS One* 9:e96208. doi: 10.1371/journal.pone.0096208

Walterson, A. M., and Stavrinides, J. (2015). Pantoea: insights into a highly versatile and diverse genus within the *Enterobacteriaceae*. *FEMS Microbiol. Rev.* 39, 968–984. doi: 10.1093/femsre/fuv027

Wang, J., Liu, J., Chen, H., and Yao, J. (2007). Characterization of Fusarium graminearum inhibitory lipopeptide from *Bacillus subtilis* IB. *Appl. Microbiol. Biotechnol.* 76, 889–894. doi: 10.1007/s00253-007-1054-1

Wei, F., Hu, X., and Xu, X. (2016). Dispersal of *Bacillus subtilis* and its effect on strawberry phyllosphere microbiota under open field and protection conditions. *Sci. Rep.* 6:22611. doi: 10.1038/srep22611

Wensing, A., Braun, S. D., Büttner, P., Expert, D., Völksch, B., Ullrich, M. S., et al. (2010). Impact of siderophore production by *Pseudomonas syringae* pv. syringae 22d/93 on epiphytic fitness and biocontrol Activity against *Pseudomonas syringae* pv. glycinea 1a/96. *Appl. Environ. Microbiol.* 76, 2704–2711. doi: 10.1128/AEM.02979-09

Wilson, M., and Lindow, S. E. (1994). Coexistence among epiphytic bacterial populations mediated through nutritional resource partitioning. *Appl. Environ. Microbiol.* 60, 4468–4477. doi: 10.1128/aem.60.12.4468-4477.1994

Wu, L., Wu, H., Chen, L., Yu, X., Borriss, R., and Gao, X. (2015). Difficidin and bacilysin from *Bacillus amyloliquefaciens* FZB42 have antibacterial activity against Xanthomonas oryzae rice pathogens. *Sci. Rep.* 5, 1–9. doi: 10.1038/srep12975

Xin, X. F., Nomura, K., Aung, K., Velásquez, A. C., Yao, J., Boutrot, F., et al. (2016). Bacteria establish an aqueous living space in plants crucial for virulence. *Nature* 539, 524–529. doi: 10.1038/nature20166

Yang, T., Wei, Z., Friman, V. P., Xu, Y., Shen, Q., Kowalchuk, G. A., et al. (2017). Resource availability modulates biodiversity-invasion relationships by altering competitive interactions. *Environ. Microbiol.* 19, 2984–2991. doi: 10.1111/1462-2920.13708

Yasmin, S., Hafeez, F. Y., Mirza, M. S., Rasul, M., Arshad, H. M. I., Zubair, M., et al. (2017). Biocontrol of Bacterial Leaf Blight of rice and profiling of secondary metabolites produced by rhizospheric *Pseudomonas aeruginosa* BRp3. *Front. Microbiol.* 8:1895. doi: 10.3389/fmicb.2017.01895

Zeriouh, H., de Vicente, A., Pérez-García, A., and Romero, D. (2014). Surfactin triggers biofilm formation of *Bacillus subtilis* in melon phylloplane and contributes to the biocontrol activity. *Environ. Microbiol.* 16, 2196–2211. doi: 10.1111/1462-2920.12271

Zeriouh, H., Romero, D., García-Gutiérrez, L., Cazorla, F. M., De Vicente, A., and Pérez-García, A. (2011). The Iturin-like lipopeptides are essential components in the biological control arsenal of bacillus subtilis against bacterial diseases of cucurbits. *Mol. Plant-Microbe Interact.* 24, 1540–1552. doi: 10.1094/MPMI-06-11-0162

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 Microbiology

ORIGINAL RESEARCH
published: 08 September 2020
doi: 10.3389/fmicb.2020.559728

Dynamic Changes in the Microbiome of Rice During Shoot and Root Growth Derived From Seeds

*Mengying Wang[1], Alexander W. Eyre[1], Michael R. Thon[2], Yeonyee Oh[1] and Ralph A. Dean[1]**

[1] Fungal Genomics Laboratory, Department of Entomology and Plant Pathology, Center for Integrated Fungal Research, North Carolina State University, Raleigh, NC, United States, [2] Spanish-Portuguese Institute for Agricultural Research (CIALE), University of Salamanca, Villamayor, Spain

Microbes form close associations with host plants including rice as both surface (epiphytes) and internal (endophytes) inhabitants. Yet despite rice being one of the most important cereal crops agriculturally and economically, knowledge of its microbiome, particularly core inhabitants and any functional properties bestowed is limited. In this study, the microbiome in rice seedlings derived directly from seeds was identified, characterized and compared to the microbiome of the seed. Rice seeds were sourced from two different locations in Arkansas, USA of two different rice genotypes (Katy, M202) from two different harvest years (2013, 2014). Seeds were planted in sterile media and bacterial as well as fungal communities were identified through 16S and ITS sequencing, respectively, for four seedling compartments (root surface, root endosphere, shoot surface, shoot endosphere). Overall, 966 bacterial and 280 fungal ASVs were found in seedlings. Greater abundance and diversity were detected for the microbiome associated with roots compared to shoots and with more epiphytes than endophytes. The seedling compartments were the driving factor for microbial community composition rather than other factors such as rice genotype, location and harvest year. Comparison with datasets from seeds revealed that 91 (out of 296) bacterial and 11 (out of 341) fungal ASVs were shared with seedlings with the majority being retained within root tissues. Core bacterial and fungal microbiome shared across seedling samples were identified. Core bacteria genera identified in this study such as *Rhizobium*, *Pantoea*, *Sphingomonas,* and *Paenibacillus* have been reported as plant growth promoting bacteria while core fungi such as Pleosporales, *Alternaria* and *Occultifur* have potential as biocontrol agents.

Keywords: rice, seed and seedling, microbiome, diversity, driving factors

OPEN ACCESS

Edited by:
Christos Zamioudis, Democritus University of Thrace, Greece

Reviewed by:
Rumakanta Sapkota, Aarhus University, Denmark
Hui Li, Jinan University, China

***Correspondence:**
Ralph A. Dean
radean2@ncsu.edu

Specialty section:
This article was submitted to Microbe and Virus Interactions with Plants, a section of the journal Frontiers in Microbiology

Received: *07 May 2020*
Accepted: *17 August 2020*
Published: *08 September 2020*

Citation:
Wang M, Eyre AW, Thon MR, Oh Y and Dean RA (2020) Dynamic Changes in the Microbiome of Rice During Shoot and Root Growth Derived From Seeds. Front. Microbiol. 11:559728. doi: 10.3389/fmicb.2020.559728

INTRODUCTION

Macro-organisms such as plants form close interactions with microbes, which together can be considered as meta-organisms or holobionts (Berg et al., 2014). Fungi, bacteria, viruses, archaea and protista that are closely associated with plants are often referred to as the "second genome" (Berendsen et al., 2012). Different plant compartments such as roots, leaves, stems, flowers, fruits as

well as seeds can all be colonized, potentially with different microbes (Berg et al., 2014). Microbes accumulate not only on the outer surfaces of plants as epiphytes but also inside plant tissues as endophytes (Turner et al., 2013). With the advent of new sequencing technologies over the past few years, the composition and possible function of these microbes, which collectively form the microbiome, associated with plants has drawn much interest (Müller et al., 2016).

Attention to microbes associated with plants has risen because they can establish beneficial, neutral or detrimental interactions of varying intimacy with their host plants (Berg et al., 2014). Beneficial microbes may promote plant growth, suppress biotic as well as abiotic stress and improve product quality. For example, various rhizobia and mycorrhizal fungi have been demonstrated to improve the acquisition of nutrients by plants (Hawkins et al., 2000; Zehr et al., 2003; Richardson et al., 2009; Miransari, 2011). Fungal endophytes such as *Neotyphodium lolii* can influence host plant CO_2 fixation (Spiering et al., 2006). Bacteria including *Bacillus* and *Paenibacillus* are able to promote plant growth in desert agroecosystems, whereas fungi such as *Lewia* sp. can be used for rhizoremediation of hydrocarbons (Köberl et al., 2011; Cruz-Hernández et al., 2013). Unlike pathogenic microbes that cause disease on plants, microbes from *Proteobacteria, Firmicutes,* and *Actinobacteria* are known to suppress plant disease (Mendes et al., 2011).

Rice (*Oryza sativa*) is the most important cereal crop agriculturally and economically feeding over half of the world's population. In addition, because of its relatively small genome size and molecular tractability, it has been established as a model plant for both basic and applied research (Izawa and Shimamoto, 1996; Shimamoto and Kyozuka, 2002; Rensink and Buell, 2004; Kawahara et al., 2013). Current strategies used to increase rice yield include breeding and application of chemical fertilizers and pesticides, which can be time consuming, expensive and environmental unfriendly (Khush, 2000; Peng et al., 2006; Zhang, 2007; Mano and Morisaki, 2008; Huang et al., 2017). Other environmentally conscious alternatives are in high demand such as the identification and application of beneficial microbes. Though limited research has been done, knowledge of the microbiome associated with rice is beginning to accumulate. For example, three different root niches [rhizosphere, rhizoplane (the root surface) and root endosphere] of rice were shown to carry different microbial communities including eubacteria and methanogenic archaea (Edwards et al., 2015). Rapid and selective acquisition of root-associated microbes from the soil was demonstrated (Edwards et al., 2015). In addition, *Methylobacterium* in rice shoots, *Azospirillum* and *Herbaspirillum* in rice stems and roots, and *Burkholderia* and *Rhizobium* in roots were detected (Mano and Morisaki, 2008). Similar bacteria were also found to be associated with other plants facilitating nitrogen fixation, and stress tolerance such as high osmotic pressure, dryness and gamma-ray radiation (Mano et al., 2006; Mano and Morisaki, 2008). It was also found that microbes from *Alphaproteobacteria*, *Actinobacteria*, *Pantoea*, *Exiguobacterium,* and *Bacillus* were common in the rice phyllosphere. Such microbes may have significant effects on global carbon, nitrogen and other nutrient cycles at the ecosystem level (Venkatachalam et al., 2016).

Given the abundant evidence that various microbes influence plant growth and development, considerable research focuses on understanding the microbial community to benefit modern agriculture. However, many factors influence the plant microbiome. Different agricultural practices such as tillage, drainage, intercropping, rotation, grazing and application of pesticides, fungicides as well as fertilizer can affect microbial diversity dramatically (Peiffer et al., 2013; Kato et al., 2015; Rothenberg et al., 2016; Vukicevich et al., 2016; Jenkins et al., 2017). Soil type, environmental conditions and host genotype also play important roles in shaping the microbiome assemblage. For rice, metagenomic, transcriptomic, proteomic as well as amplicon sequencing approaches used to characterize the microbial community of plants grown in soil have shown that numerous factors including environmental factors, plant age and genotype all greatly influence it's microbiome (Knief et al., 2012; Sessitsch et al., 2012; Edwards et al., 2015). Productivity and health of agricultural systems depend greatly on the functional processes carried out by the plant-associated microbial community (Buyer et al., 1999; Hacquard, 2016).

However, it is conceivable that plants maintain a core microbiome independent from soil type, environment, host genotype, agricultural management and other factors. The concept of a core microbiome was first proposed for the human microbiome and has been further expanded to plant-associated microbes (Engelbrektson et al., 2012; Shade and Handelsman, 2012). These core microorganisms constitute a conserved subset of microbes that likely play important roles for host plants as well as for the surrounding microbial communities (Engelbrektson et al., 2012; Huse et al., 2012).

Moreover, there are also limited studies regarding microbiome variation along different life stages of plants. Reproduction is an important stage, and seeds usually contain a high diversity of microbes that can be transmitted vertically across generations (Bragina et al., 2013; Hodgson et al., 2014; Truyens et al., 2015; Shahzad et al., 2018). Seed germination is a complex process, during which the initially dormant seeds undergo physiological state changes (Ofek et al., 2011). Investigation of the microbiome temporal shift from seed to seedling as well as spatial shift from root to shoot and from tissue surface to interior may help to shed light on the interactions between the host and the associated microbiome.

The primary objective of this project was to identify the microbiome associated with rice shoots and roots and compare them with the microbiome associated with rice seeds. Furthermore, we wanted to illustrate the effect of rice tissue compartment, genotype, growth location and harvest year in shaping the microbial community. Finally, the core microbiome related with rice seedlings was also expected to be revealed. To achieve these goals, we characterized the microbial biodiversity of rice seedlings, both in shoot and root tissue, derived from seeds germinated in axenic conditions. Microorganisms associated with different rice seedling compartments (surface and endosphere of shoots and roots) were characterized by amplicon sequence of 16S for bacteria and ITS for fungi. Rice seeds from

different geographic cultivation areas of different rice genotype in different harvest years were used in this study (**Supplementary Table 1**). The composition and population structure in seedling and root compartments were compared to those of previously published data for the seeds and seed compartments (Eyre et al., 2019). Finally, core bacterial and fungal taxa were identified.

MATERIALS AND METHODS

Rice Seeds

Rice seeds were obtained from Dr. Yulin Jia, USDA Dale Bumpers National Rice Research Center, Stuttgart, Arkansas. Six different *japonica* rice seeds representing two rice varieties (M202 and Katy) were collected from two locations: research fields at the Dale Bumpers (DB) Research Center and the University of Arkansas (UA) in 2 years (2013 and 2014) (see Eyre et al., 2019). Seeds were enclosed in envelopes (50 g for each type of seeds) and sent through standard mail. They were stored dry at 4°C after received.

Rice Seedling Growth

Sand (100 mL) and distilled water (40 mL) were poured into each square plant culture vessels (SPL Life Science, Incu Tissue) and autoclaved. After cooling, rice seeds were embedded into the sand and vessels sealed with 3M medi-pore tape. Each vessel contained 5–6 rice seeds and for each rice type 4 replicates were grown. Vessels were placed in an incubator at 26/20°C under a 14 h light/10 h dark cycle for 3 weeks during which time rice seeds germinated and grew to 3–4 leaf seedlings (Ding et al., 2012).

Seedling Compartments Sample Collection

For rice seedlings, shoots and roots were separated and put into sterile 50 mL falcon centrifuge tubes using sterile tweezers and scissors. Each falcon tube contained 3–6 shoots or roots from the germinated rice seeds (root samples were first manually shaken before placing into falcon tubes in order to remove the loosely associated sand). Then 20 mL of sterile distilled water was added. The tubes were vortexed for 2 min to remove any adhering microbes, and the liquid was collected. Tubes were vortexed two more times, followed by three 1-min sonication with sterile water using a sonication probe (Microson Ultrasonic Cell Disruptor model XL2000, Misonix Incorporated New York, United States, output 7 watts) to remove tightly adhering microbes. Liquid extracts were pooled together based on different seed types to form the shoot and root surface compartment samples. The remaining shoot and root tissue were washed two more times by sonication and then placed separately in sterile tubes.

After preliminary confirmation and evaluation for bacteria and fungi existing in the four seedling compartments by plate culturing, samples for genomic DNA extraction were then processed. To extract DNA from shoot and root surface fractions (all replicates were used for DNA extraction and combined), the liquid extracts were centrifuged at 12,000 rpm for 15 min and the supernatant was removed from the pellets. Respective pellets represented the shoot surface and root surface compartments. Pellets were collected and stored at −20°C until DNA extraction (Bulgarelli et al., 2012; Engelbrektson et al., 2012; Bulgarelli et al., 2015). For shoot and root endosphere DNA samples, the remaining shoot tissue and root tissue after washing by sonication (above) were stored at −20°C until DNA extraction (Engelbrektson et al., 2012; Bulgarelli et al., 2013).

DNA Extraction

Whole genomic DNA was extracted from the 24 different samples. The pellet collected from "shoot surface," "root surface" samples as well as the shoot and root tissue were placed separately in sterile mortar and pestles. Liquid nitrogen was added. Samples were thoroughly ground and DNA was extracted using the "Wizard Genomic DNA Purification Kit" by Promega (Madison, WI, United States) following the provided instructions (Fadrosh et al., 2014). DNA quality and concentration were checked using the NanoDrop spectrophotometer (model ND-1000, Thermo Fisher Scientific, Waltham, MA, United States).

16S V3-V4 and ITS1 PCR Amplification

The amplification was carried out using primers modified from Fadrosh et al. (2014). For bacteria, a region of approximately 460 bp encompassing the V3 and V4 hypervariable regions of the 16S rRNA gene was targeted (IlluminaF: 5′-CCTACGGGNGGCWGCAG-3′ and IlluminaR: 5′-GACTACHVGGGTATCTAATCC-3′) (Klindworth et al., 2013)[1]. For fungi, the primers were used to amplify 291 ± 58 bp ITS1 region (ITS1F: 5′-CTTGGTCATTTAGAGGAAGTAA-3′ and ITS2R: 5′-GCTGCGTTCTTCATCGATGC-3′) (White et al., 1990; Gardes and Bruns, 1993; Usyk et al., 2017). Overhang adapters were added to primers for compatibility with the Nextera Index Kit (Illumina, San Diego, CA, United States).

Two stages of PCR were then conducted as described in Eyre et al. (2019). Specific index pairs were assigned to each sample following the manufacturer's user manual. Bacterial 16S amplicon and fungal ITS amplicon coming from same sample shared the identical barcode for Mi-Seq sequencing. All 48 amplicon products (24 for bacteria and 24 for fungi) were quantified using a Bioanalyzer (Agilent 2200 TapeStation, CA, United States). Amplicons were diluted and pooled together at equimolar concentrations to ensure equal proportions of the bacterial and fungal amplifications. The prepared samples were submitted to the Genomic Sciences Laboratory at North Carolina State University for "Illumina MiSeq 300 bp Paired-End Sequencing" (Illumina, San Diego, CA, United States).

Sequencing Data Analysis

Sequencing data obtained from the Illumina MiSeq runs was demultiplexed at the sequencing center for the 24 different samples (**Supplementary Table 1**) based on the barcode sequences attached to each sample. FastQC v0.11.8[2] was then used to visualize the quality of raw sequences. Reads for

[1]http://support.illumina.com/downloads/16s_metagenomic_sequencing_library_preparation.html

[2]https://www.bioinformatics.babraham.ac.uk/projects/fastqc/

each sample were further separated as bacterial and fungal sequences using a custom Python script based on the different primer sequences used for 16S and ITS amplification. The R package "DADA2" was then used to generate the amplicon sequence variants (ASVs) table (Callahan et al., 2016). Through "DADA2," the demultiplexed "fastq" files for each sample were filtered, trimmed and dereplicated to discern the error rates. Forward/reverse reads were merged together, and chimeras were removed from the whole set. The ASVs table was generated and sequences were then assigned to taxonomy through DADA2. "SILVA reference database" (version 132) (Wang et al., 2007; Quast et al., 2012)[3] was used for 16S amplicon data "assignTaxonomy" function. For fungal taxonomy, the general "fasta" release files from "UNITE ITS database" was used (Version 18.11.2018)[4]. Singletons were removed before subsequent analysis.

Data Exploration and Statistical Analysis

Based on output from the "DADA2" package, statistical analysis was performed using different R packages (R version 3.5.2)[5]. "VennDiagram" package was used to show the distribution of unique ASVs among different samples (Schwenk, 1984). Alpha-diversity analysis was conducted using "alpha" function from R package "microbiome" (Lahti and Shetty, 2018). Different index value of alpha diversity was obtained while Shannon, Chao1 and InverseSimpson index were plotted through "ggplot2" (Wickham, 2016) -based R package "ggpubr" (Kassambara, 2018). Function "stat_compare_means" from "ggpubr" was used for *T*-test between groups. "Ordinate" function from package "Phyloseq" was used for the Principal coordinate analysis (PCoA) and default distance Bray was applied. "Plot_ordination" function from package "ggplot2" (Wickham, 2016) was used to build the plot. For the summarization of samples taxonomic composition, microbial genomics module of QIAGEN CLC Genomics Workbench 20.0[6] was used to build the sunburst figures. Taxa with at least 1% of the total reads were then extracted and used to summarize the distribution of taxa across different tissue compartments using R package "Phyloseq" (McMurdie and Holmes, 2013). Package "ggplot2" was used for bar chart plotting. Function "subset_taxa," "get_taxa" and "sample_sums" from package "Phyloseq" were used to extract taxa of interest and get read abundance from taxa of interest as well as sample of interest. Unpaired *T*-test and ANOVA analysis were carried out to compare taxa abundance among groups using Prism Graphpad software[7]. For further insight into the microbial distribution pattern across rice tissue compartments, data from seeds and seedlings were combined and taxa presenting more than 0.1% of the total reads were extracted, normalized and subjected to K-means clustering. The distance matrices were made by using the "vegdist" function in R package "Vegan" (Oksanen, 2015) and the clusters were then generated by hierarchical agglomerative clustering (function "hclust") using complete linkage. This multivariate clustering analysis was used to reveal similar groupings of taxa as cluster patterns in the dataset across tissues. The taxa included in these clusters are shown in **Supplementary Tables 5, 6**. In the end, core members of the microbial communities were extracted using R package microbiome (Lahti and Shetty, 2018) with 100% representation (i.e., present in all 6 samples within a group, seedling samples were grouped based on the 4 compartments). When compare seedlings data with previous seeds data (Eyre et al., 2019), ASV table from seedlings data was combined with ASV table from early published seeds data and then subjected to corresponding analysis.

[3]https://zenodo.org/record/1172783#.XvQNAmpKjfA

[4]https://unite.ut.ee/repository.php

[5]https://www.r-project.org/; https://rstudio.com/

[6]https://digitalinsights.qiagen.com

[7]https://www.graphpad.com/scientific-software/prism/

RESULTS

Changes of Microbial Members in the Rice During Shoot and Root Growth

The number of reads before and after quality control and the number of ASVs per sample as well as per tissue compartment are shown in **Supplementary Tables 1, 2**. After quality control, 18,308,731 total raw reads were separated, trimmed and filtered to yield 4,101,915 bacterial reads and 5,917,486 fungal reads, respectively. With the exception of the root surface sample from fungi, the number of high-quality reads per tissue compartment after quality control ranged between 955,602–2,496,917.

Distribution of unique ASVs as having more than one read in any of seedling tissue compartments was summarized firstly to reveal a broad picture of the microbial members within the rice. The Venn diagrams shown in **Figures 1A,C–E** showed the distribution of bacterial members within different rice seedling compartments (shoot_endosphere, shoot_surface, root_endosphere, and root_surface). Examination of the 4 seedling compartments revealed a total of 966 unique ASVs (**Figure 1A**). More ASVs were found in root tissue (887) than in shoots (282). For both the root and shoot tissues, the number of ASVs was slightly higher in the surface samples (680) compared to the endosphere (575). In addition, for both the surface and endosphere sample, the root contained more ASVs than shoot samples (Root surface: 592 vs. Shoot_surface: 268; Root endosphere: 543 vs. Shoot endosphere 133). Overall, 640 (66.3%) of the ASVs were uniquely found only in single seedling compartments: 298 (30.8%) out of all ASVs were only found in root surface sample; 273 (28.3%) for the root endosphere; 60 (6.2%) for the shoot surface and 9 (0.9%) for the shoot endosphere. Of the total 966 microbial ASVs, only 89 (9.2%) were shared by all 4 seedling compartments.

Based on our previously published data (Eyre et al., 2019), a total of 296 ASVs were detected in the rice seeds. Comparison of the rice seeds and seedling data sets revealed 91 ASVs were shared, representing 30.7% of those present in the seeds (7.8% of total ASVs) as shown in **Figure 1C**. When the seedling data sets were separated into shoots and roots, 54 ASVs were shared by rice seeds, shoots and roots (**Figure 1D**). However, 88 of the 91 were shared between roots and seeds, whereas 57 of the 91 were shared between shoots and seeds. On the

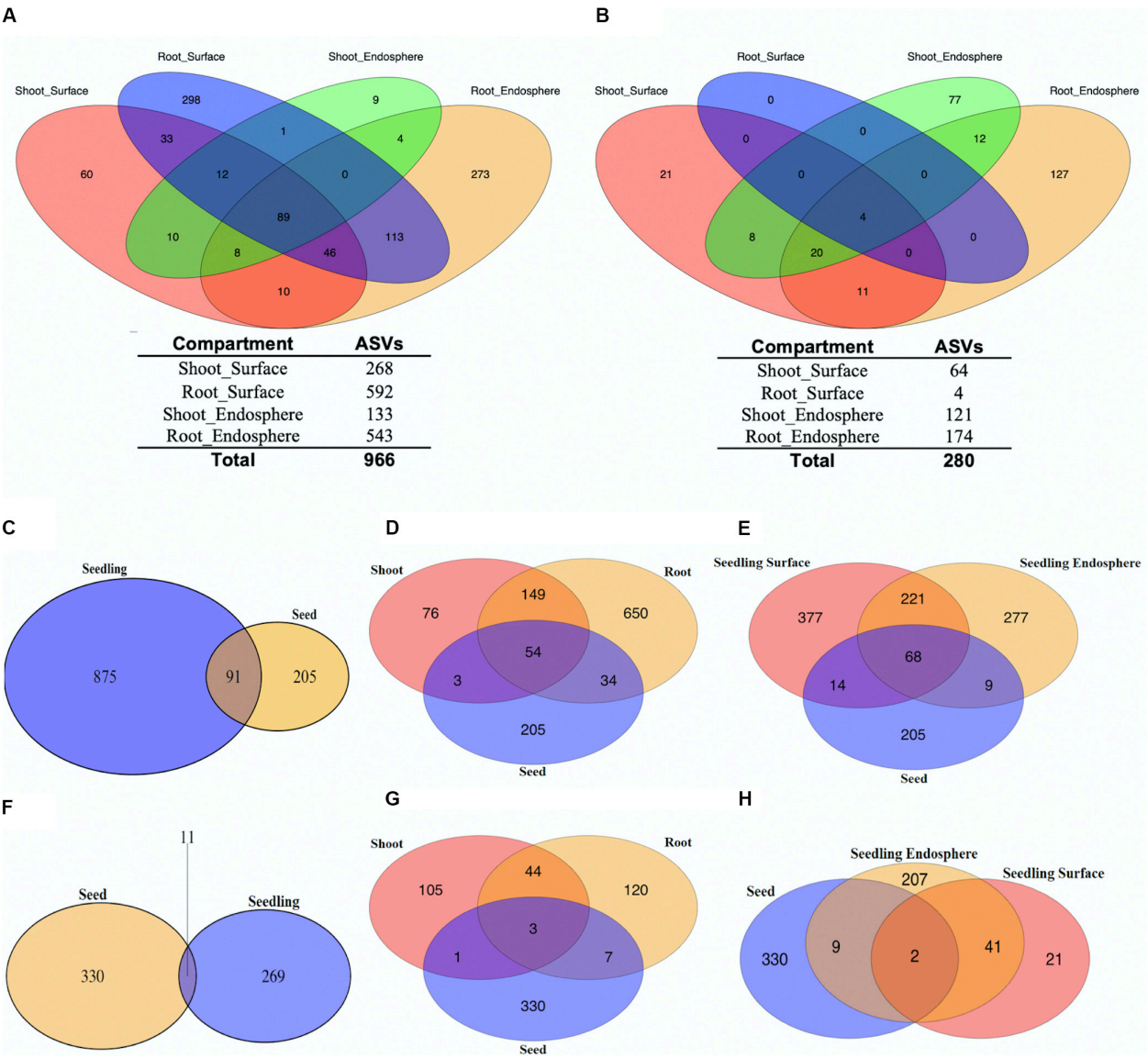

FIGURE 1 | Relationships between unique ASVs by tissue and tissue compartments. Distribution of unique bacterial **(A)** and fungal **(B)** ASVs separated by seedling compartment. The number of unique ASVs found in each seedling compartment are shown below the Venn Diagram (Pink: Shoot_Surface; Blue: Root_Surface; Green: Shoot_Endosphere; Yellow: Root_Endosphere). Unique ASVs in seedlings compared to unique ASVs in seeds **(C–H)**. Distribution of unique bacterial **(C–E)** and fungal **(F–H)** ASVs separated by tissue compartments.

other hand, the shared ASVs only represented 9.9% (88 out of 887) of root ASVs while representing 20.2% (57 out of 282) of shoot ASVs. Seedling samples were further separated to seedling surface and seedling endosphere. Inspection revealed that 68 ASVs were shared between rice seeds, seedling surface and seedling endosphere samples, whereas 82 and 77 of seed ASVs were shared with seedling surface and seedling endosphere samples, respectively. The shared ASVs accounted for 12.1% (82 out of 680) of total seedling surface ASVs and for seedling endosphere, the shared ASVs account for 13.4% (77 out of 575). In sum, from the perspective of the seed, a greater number of the bacterial seed microbiome was retained by the root than the shoot, but these seed derived microbes showed little preference for being retained in the seedling surface or endosphere compartments.

To better understand the microbiome dynamics from seeds to seedlings, additional analyses were performed using the four different seed compartments: outer husk, husk, outer grain and grain (Eyre et al., 2019; see **Supplementary Figure 1**). From outer surface to inner grain, the number of shared ASVs among seed compartments and seedling samples decreased, consistent with the observation that the number of ASVs decreased in rice seeds from outer surface to inner grain (Eyre et al., 2019). From the perspective of seed compartments, 43.6% (85/195)

of outer husk ASVs were shared with seedling samples (roots and shoots); 39.0% (57 out of 146) of husk ASVs were shared with seedlings; 41.2% (35 out of 85) of outer grain ASVs were shared while only 18.9% (7 out of 37) of grain ASVs were shared (**Supplementary Figures 1A–D**). Similar patterns were observed when comparing seed compartments with seedling surface and endosphere compartments (**Supplementary Figures 1E–H**). Thus, overall, although the outer husk contributed the most ASVs to the seedling microbiome, there appeared to be little preference based on proportion regarding which seed compartment contributed predominantly to the seedling microbiome, with the possible exception of the grain which contributed the fewest and lowest proportion.

For the fungal dataset, 280 ASVs in total were detected for rice seedlings (**Figure 1B**). Overall, 225 (80.4%) of the ASVs were found to be uniquely associated with specific seedling compartments: 21 (7.5%) ASVs were only found in shoot surface sample; 0 (0%) for root surface; 77 (27.5%) were found specific for shoot endosphere and 127 (45.4%) for root endosphere. Only 4 of the total 280 ASVs were shared by all 4 seedling samples, all of which were found on the root surface. The low number of ASVs found on the root surface preclude any further general inferences regarding the effect of organs (root/shoot) and location (surface/endosphere) impacting the fungal communities.

Seedling fungal data were then compared with previous rice seeds data (Eyre et al., 2019) where 341 fungal ASVs were detected (**Figures 1F–H**). Only 11 ASVs were shared, representing 1.8% of the total ASVs (3.2% of seed data set). Similar to the bacterial analysis, seedling samples were then separated by shoots and roots: 3 ASVs which represents 0.5% of total were shared by rice seeds, shoots and roots. During germination, 10 [out of 341 (2.9%)] of seeds ASVs were shared with root samples while 4 [out of 341 (1.2%)] of seeds ASVs were shared with shoot samples. Moreover, the shared ASVs represented 5.8% (10 out of 174) of root ASVs and represented 2.6% (4 out of 153) of shoot ASVs. Seedling samples were further separated to seedling surface and seedling endosphere. Only 2 ASVs which represent 0.3% of total were shared by rice seeds, seedling surface and endosphere samples. During germination, the 11 [out of 341 (3.2%)] seed ASVs were shared with seedling endosphere samples while only 2 [out of 341 (0.6%)] were shared with seedling surface samples. The shared ASVs accounted for 4.2% (11 out of 259) of total seedling endosphere ASVs. For the seedling surface, the shared ASVs accounted for 3.1% (2 out of 64). Additional analyses were conducted using the four seed compartments: outer husk, husk, outer grain and grain to better understand the microbiome shift from seeds to seedlings (**Supplementary Figure 2**). From the outer surface to inner interior, 2.7% (7 out of 262) of outer husk ASVs were shared with seedling samples; 3.6% (4 out of 112) of husk ASVs were shared with seedlings; 3.4% (7 out of 211) of outer grain ASVs were shared and 7.6% (5 out of 66) of grain ASVs were shared. Overall, even though the number of fungal ASVs commonly associated with seeds and seedlings was low, each seed compartment contributed fairly evenly to the seedling microbiome, which were predominantly located in the root and endophyte tissues.

Diversity and Driving Factors of Microbial Communities

To evaluate diversity of microbial communities associated with rice seedlings, alpha diversity was calculated across samples grouped to different compartments, years, genotypes and locations (**Figure 2** and **Supplementary Tables 3, 4**). Alpha diversity provides information regarding species richness (ASV abundance) and diversity within single samples. For rice seedlings, associated bacteria were more diverse than associated fungi. Moreover, root samples were more diverse than shoot samples while the surface samples were more diverse than the endosphere samples (except for fungi associated with shoot surface compartment). Analysis of the combined seeds data with seedlings data indicated that bacteria associated with seedlings were more diverse than those associated with seeds while fungi associated with seed samples were slightly more diverse than those associated with seedlings. Other factors including genotype, location and year also had minimal effect on diversity.

To better understand the impact of different factors (seedling compartment, harvesting year, harvesting location, rice genotype) on the microbial community, Principal Coordinates Analysis (PCoA) was used to explore the internal relationships of those variables (**Figures 3A,B**). For bacterial and fungal datasets of rice seedlings, PCoA plots showed that samples generally clustered together based on different tissue compartments, indicating distinct communities. However, when samples were grouped based on different harvesting year, location or rice genotype, no obvious clusters were evident (**Supplementary Figures 5, 6**). Seeds data were also combined with seedling data and subjected to PCoA analysis (**Figures 3C,D**). Microbiome community (both bacterial and fungal) associated with rice seeds were very distinct from those associated with seedlings. For rice seeds, consistent with previous publication (Eyre et al., 2019), the grain compartment formed the most distinct bacterial grouping. Inspection of the fungal PCoA in seeds samples revealed that the grain, outer grain, and outer husk tissues formed distinct groupings with the husk overlapping all three. Though the seedling sample did not show clear community patterns in the combined analysis with the seed data, when the seed data was removed, the bacterial community possessed by shoot samples was different from root samples and the microbiome associated with plant surface was distinct from the plant endosphere (as shown in **Figures 3C,D**).

Taxon Composition of Microbial Communities

To better understand changes in microbial communities during germination, CLC workbench (Microbial genomics module) was used to visualize taxa proportions for comparing seeds and seedlings. For bacteria (**Figure 4A** and **Supplementary Figure 3**), Proteobacteria (87%) and Actinobacteria (12%) composed the entire seeds bacterial community. Though Proteobacteria were also dominant for seedlings (63%), reduced Actinobacteria (4%) were detected along with emerging Bacteroidetes (29%) and Firmicutes (3%). In addition, during germination the abundance of Gammaproteobacteria increased compared to seeds (from 0.9

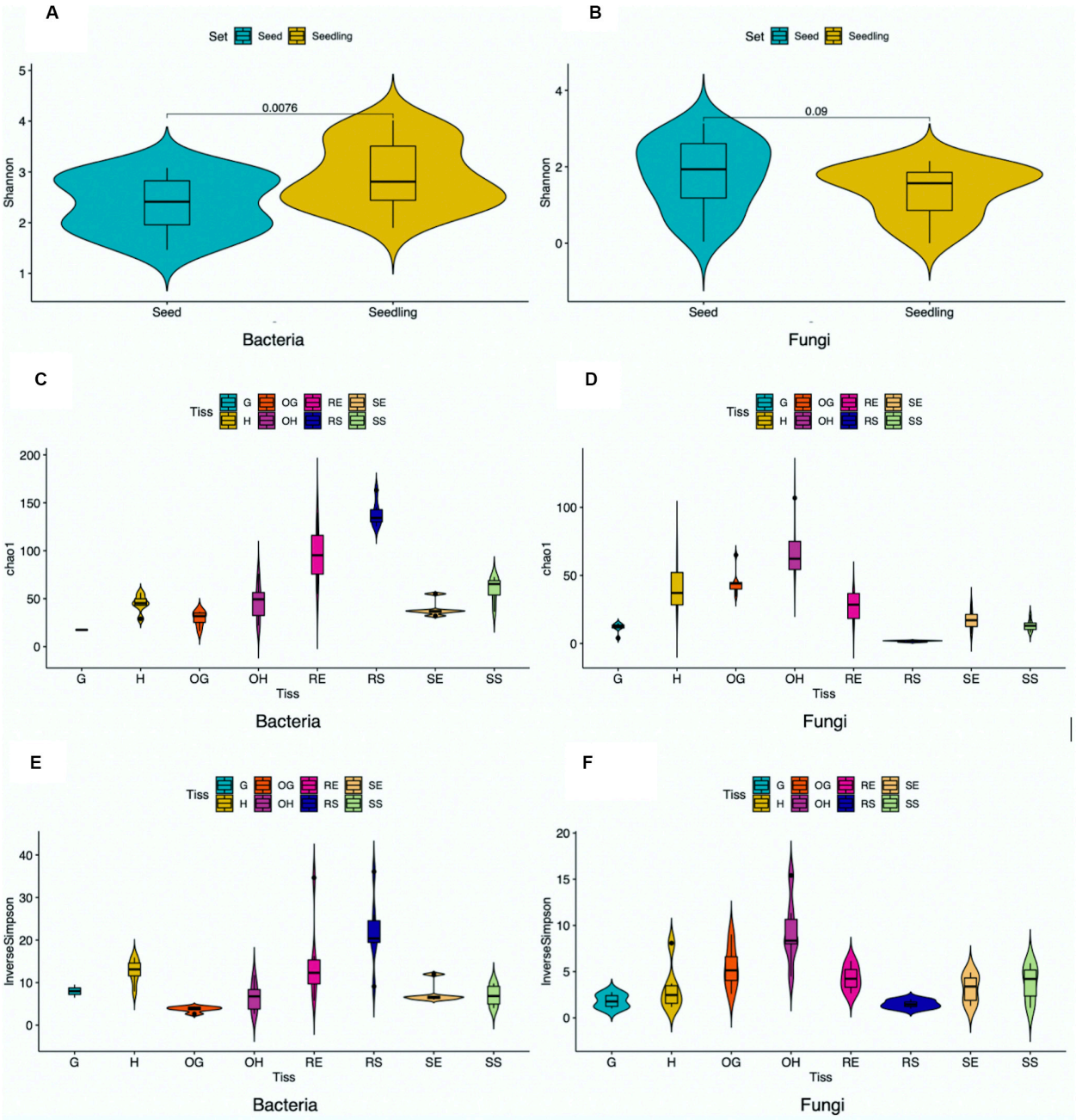

FIGURE 2 | Alpha diversity of bacterial **(A,C,E)** and fungal **(B,D,F)** ASVs within samples pooled based on tissue compartments. Unpaired *t*-test was performed, and *P*-values were added in **(A,B)** (G, Grain; H, Husk; OG, Outer Grain; OH, Outer Husk; RE, Root Endosphere; RS, Root Surface; SE, Shoot Endosphere; SS, Shoot Surface).

to 30.9%, $P = 0.0259$) where Alphaproteobacteria were prevalent (86% in seeds).

A total of 247 taxonomic classifications primarily at the genus level were detected for the combined seed and the seedling datasets. Nineteen taxa were identified representing 91.5% of the total reads (**Figure 4B**, seeds data and seedlings data combined). Of those taxa, 12 were from proteobacteria (4 Alphaproteobacteria and 8 Gammaproteobacteria), 3 were from Bacteroidetes, 3 Actinobacteria and 1 Firmicutes. Considering the seedling samples, 16 taxa were present in the combined data set (taxa *Curtobacterium*, *Microbacterium*, *Enterobacteriaceae,* and *Atlantibacter* were absent from seedlings while *Luteibacter* was included). Taxon composition of the root endosphere was similar as the root surface, except for increased abundance of *Pseudomonas* (from 1.3 to 10%, $P = 0.0028$), *Massilia* (from 2.8 to 10.4%, $P = 0.0206$) and reduced abundance of *Herbaspirillum*

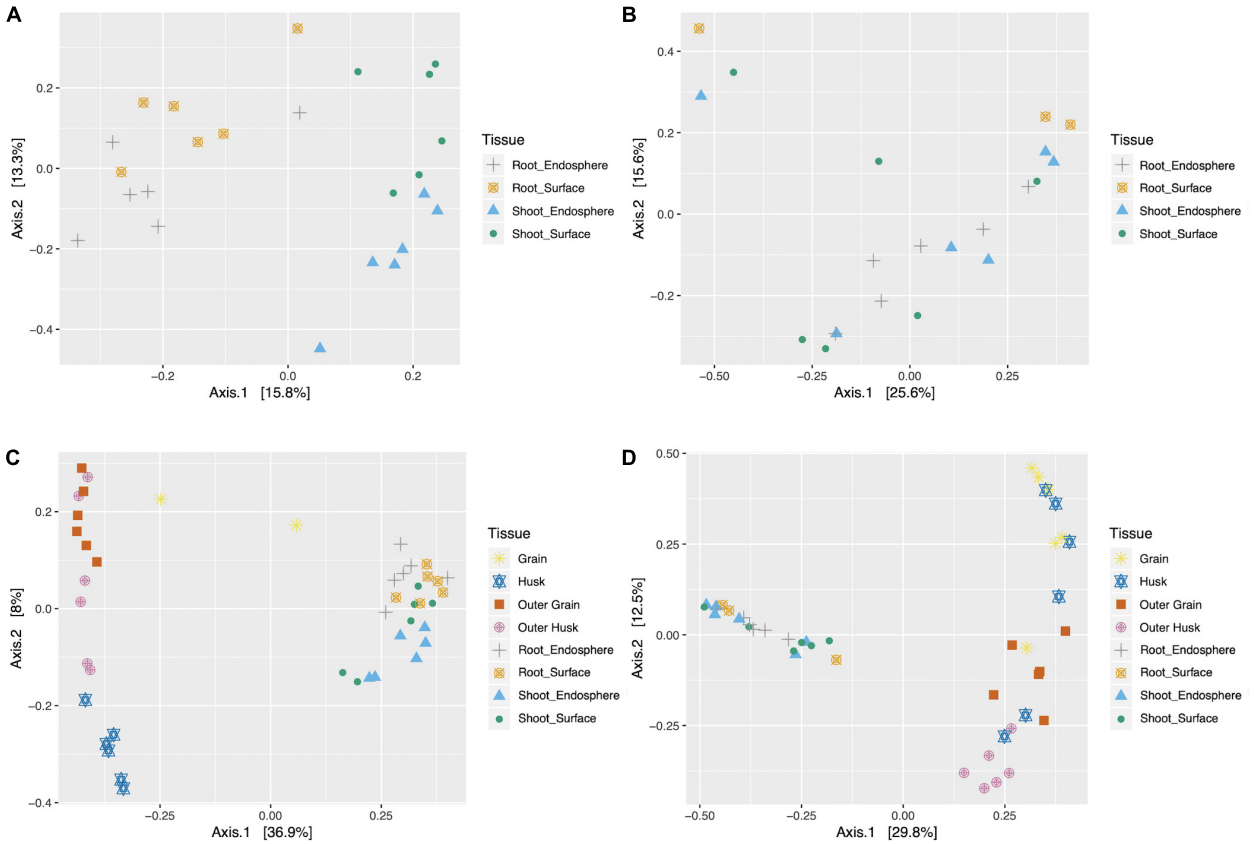

FIGURE 3 | Bacterial and fungal Principal coordinate analysis (PCoA) for seedlings **(A,B)** and combined data (seeds and seedlings data) **(C,D)**. PCoA was performed on the bacterial **(A,C)** and fungal **(B,D)** samples, colorized by different tissue compartment (G, Grain; H, Husk; OG, Outer Grain; OH, Outer Husk; RE, Root Endosphere; RS, Root Surface; SE, Shoot Endosphere; SS, Shoot Surface).

(from 21.4 to 5.2%, P = 0.0250). The shoot sample contained a greater abundance of Actinobacteria (8.5% for shoot and 0.6% for root, $P = 0.0009$) and *Aureimonas* (3.8% for shoot and 0.2% for root, $P = 0.001$) than roots. The shoot endosphere sample was richest in *Pantoea* and least rich for *Bacteroidetes*. In contrast, in the shoot surface sample, Gammaproteobacteria (from 57.4 to 13.6%, $P < 0.0001$) were reduced while Bacteroidetes (from 3.8 to 39.3%, $P < 0.0001$) increased. When examined based on ASV abundance distribution (**Figure 4C**) rather than read abundance, compared to read abundance bar plot, *Methylobacterium* increased in seedlings and *Actinobacteria* increased in root samples. For the shoot endosphere sample, *Bacteroidetes* ASVs were highly prominent.

With respect to seeds, 8 taxa were included in the 19 taxa in the combined seeds and seedlings dataset. These observations were similar to previous findings using the seeds data alone, where 9 taxa were identified whose abundance were higher than 1% of total reads with the addition of *Franconibactor* (Eyre et al., 2019). Moreover, the taxon composition in rice seedlings was distinct from seed samples. *Curtobacterium* and *Microbacterium* from Actinobacteria were consistently present for all tissue compartments, however, the abundance was reduced in seedling samples compared to rice seed (*Curtobacterium* from 8 to 0.4%, $P < 0.0001$; Microbacterium from 2 to 1%, $P = 0.0281$). A similar pattern was also observed for 4 genera from Alphaproteobacteria, which were very prominent in seeds. In contrast, compared to seed samples, members from Gammaproteobacteria, Bacteroidia and Firmucutes were abundant in seedlings and represented 47.4% of the total reads.

For fungi, the seed and seedling communities were comprised of Ascomycota and Basidiomycota (**Figure 5A**). Tremellomycetes (from 27.9 to 28.13%, $P > 0.05$) and Cytobasidiomycetes (from 1.5 to 0.9%, $P > 0.05$) were the most abundant taxa for Basidiomycota and their total proportion remained unchanged during germination (for seedling samples, 99% ASVs from Tremellomycetes could not be assigned to a specific genus while in Cytobasidiomycetes, genus *Occultifur* emerged to be dominant as the genus *Symmetrospora* became undetectable). In contrast, for Ascomycota, the abundance of Sordariomycetes increased dramatically (from 3.5 to 54%, $P < 0.0001$) while Dothideomycetes were reduced (from 66.5 to 17%, $P < 0.0001$) in seedlings. It should also be noted that for Sordariomycetes, *Fusarium* became prevalent in seedlings compared to seeds where *Nigrospora* was the primary (**Supplementary Figure 4**).

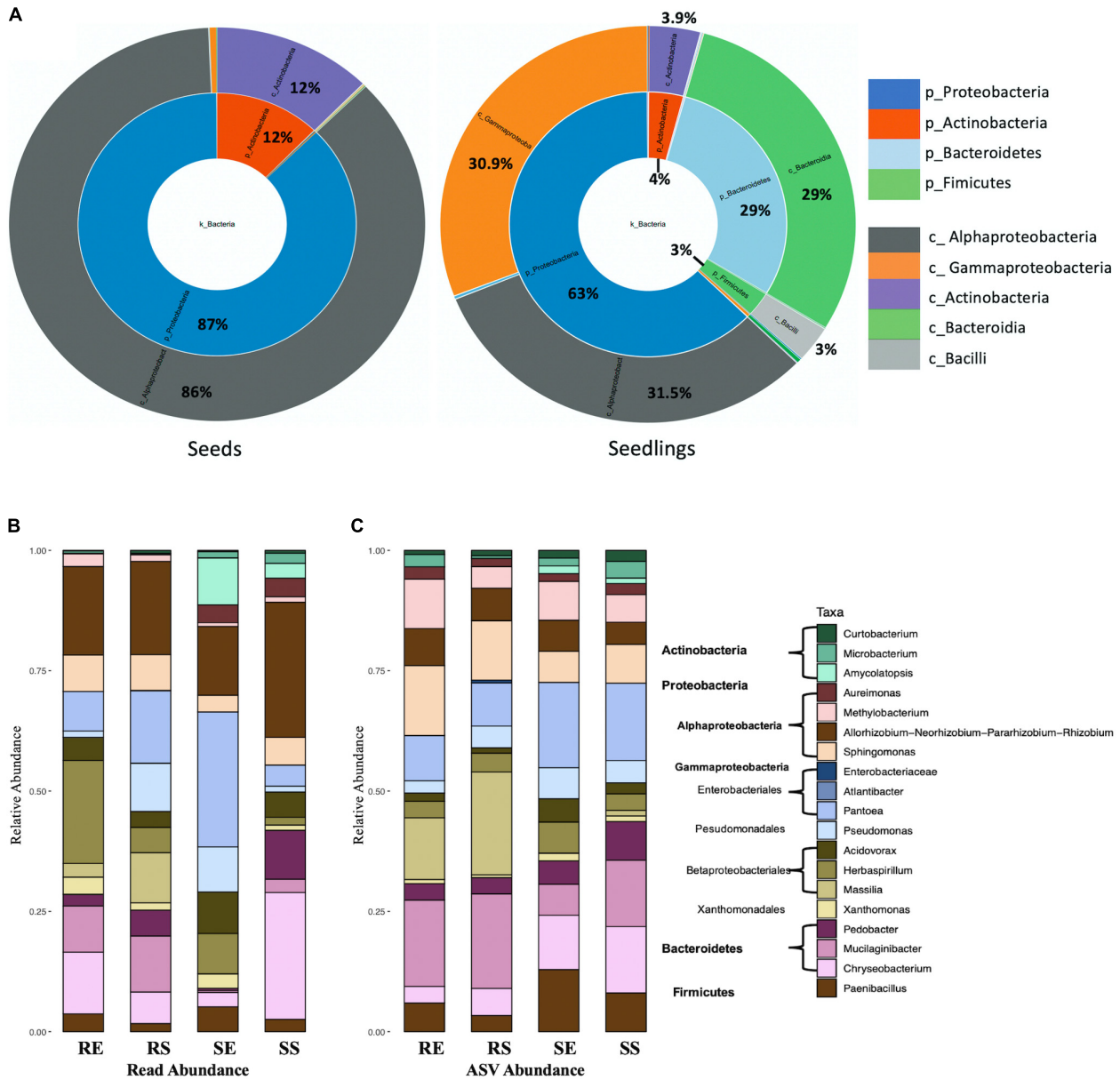

FIGURE 4 | Bacterial taxon composition for microbial communities. Comparison of seeds and seedlings data at phylum and order level **(A)**. Bacterial genera bar graphs based on reads abundance **(B)** and ASVs abundance **(C)** for 4 different tissue compartments (Top genera with > 1% total reads). Tissue compartment: RE, Root Endosphere; RS, Root Surface; SE, Shoot Endosphere; SS, Shoot Surface.

In the fungal data, 159 taxonomic classifications primarily at the genus level were detected for rice seedlings. Similar to bacteria, fungal taxa with at least 1% of the reads (13 genera representing 90.6% of total reads) from seeds and seedlings dataset were examined (**Figure 5B**, combined seeds and seedlings dataset). Overall, taxa assigned to *Fusarium* and *Tremellales* accounted for 74.8% (**Figure 5B**) of the whole seedling taxon composition. However, compared to other seedling samples, Tremellales (0.5% for root endosphere, ANOVA P = 0.0024) and all Basidiomycota (0.6% for root endosphere, ANOVA P = 0.0021) were poorly represented in the root endosphere compartment (**Figure 5B**). Inspection based on ASV abundance rather than read abundance, revealed that *Occultifur* which only represented 0.15 and 0.04% of reads in root endosphere and shoot surface compartments showed higher relative ASV abundance. Furthermore, in the root endosphere, based on reads Basidiomycota accounted for less than 2% of the root endosphere reads, whereas they accounted for ∼25% of ASV abundance (**Figure 5C**).

With regards to changes in fungal taxa during seedling development, taxa *Pleosporales* (from 42.3 to 7.9%, $p < 0.0001$),

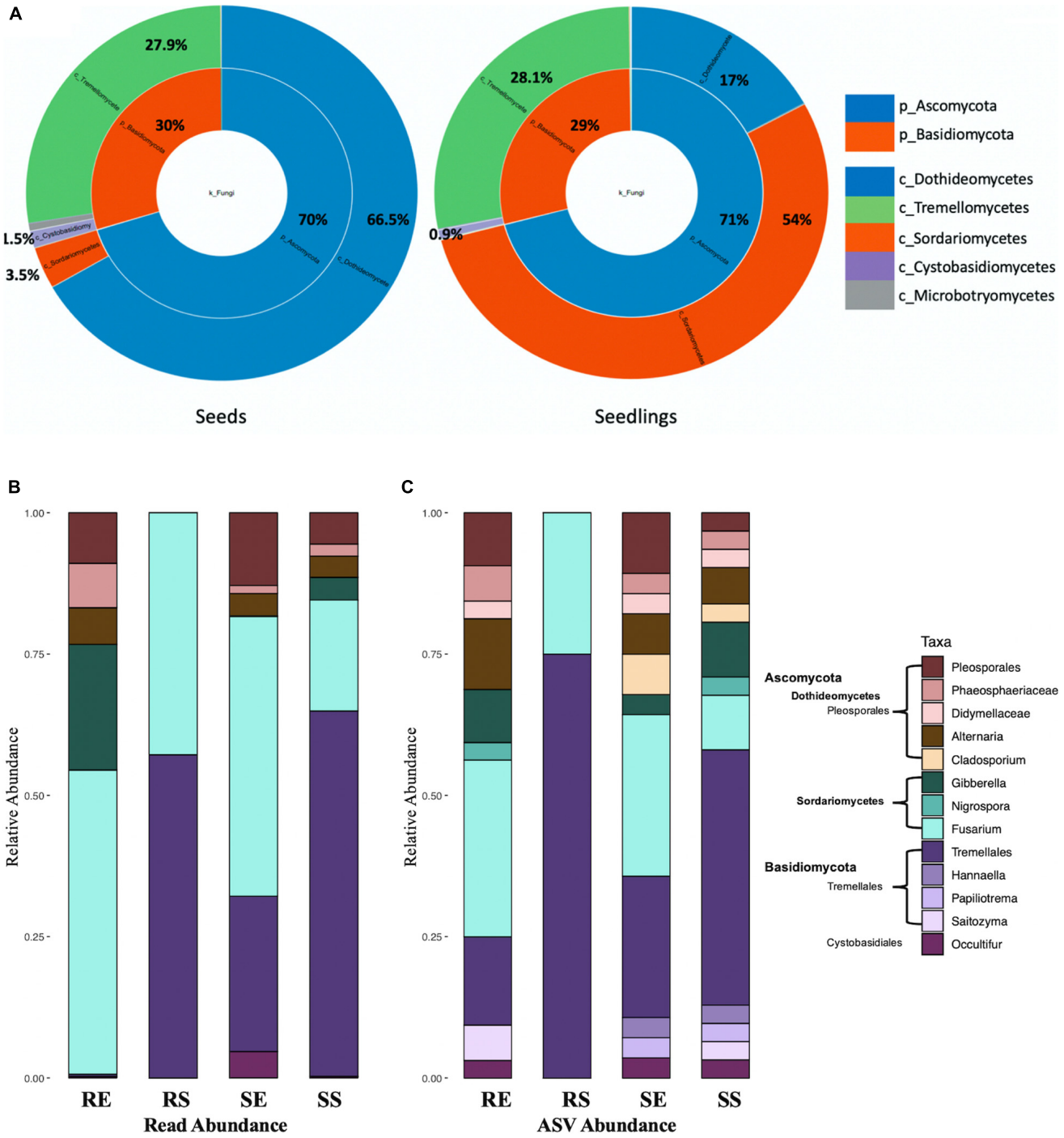

FIGURE 5 | Fungal taxon composition for microbial communities. Comparison of seeds and seedlings data at phylum and order level **(A)**. Fungal genera bar graphs based on reads abundance **(B)** and ASVs abundance **(C)** for 4 different tissue compartments (Top genera with > 1% total reads). Tissue compartment: RE, Root Endosphere; RS, Root Surface; SE, Shoot Endosphere; SS, Shoot Surface.

Didymellaceae (from 11.1% to non-detectable), *Alternaria* (from 13.9 to 4%, P = 0.0072) and *Cladosporium* (from 4.5% to non-detectable) diminished while *Fusarium* (from non-detectable to 41.6%) and Tremellales (from 4.8 to 34.2%, P = 0.0002) increased. Although only 4 fungal ASVs were found on the root surface they were predominantly Tremellales. Taxa such as *Papiliotrema*, *Saitozyma*, *Hannaella*, *Nigrospora*, *Gladosporium* and Didymellaceae showed increased relative ASV abundance when compared to read abundance (**Figures 5B,C**).

Microbiome Patterns Across Rice Tissue Compartments

Multivariate clustering analysis showed the bacterial data was assigned to 6 clusters across tissue compartments (**Figure 6A** and **Supplementary Figure 7**). Taxa assigned to cluster B were found predominately in the root endosphere which contained 28.6% of the total root endosphere reads. Members of this cluster were primarily from the Proteobacteria. Taxa in cluster E were abundant in root surface compartments representing 20.1% of the root surface reads. Members of clusters B, E, and F, which were prominent in seedlings, were largely absent from seeds. Cluster A, which was made up of 10 taxa including *Rhizobium*, *Paenibacillus*, *Pedobacter,* and *Microbacterium,* was prominent in both seed and seedling compartments. Taxa in cluster C were dominant in seeds, particularly in grain and husk samples and included taxa *Cautobacterium*, *Kineococcus* as well as *Methylobacterium*. Similar to Cluster C, Cluster D also contained taxa dominant in seeds compartments such as *Brevundimonas*, *Sphingomonas,* and *Roseomonas*.

K-means clustering was also applied to the fungal dataset and provided 6 clusters across 8 tissue compartments (**Figure 6B** and **Supplementary Figure 7**). Taxa in cluster A were dominant in the root endosphere sample and were all Ascomycota. They represented 34.5% of total ASV reads in this compartment. Six taxa in cluster E including *Saitozyma* and *Nigrospora* were found mostly in outer husk compartment representing 33.7% of outer husk reads. Half of them were Ascomycota while the other half were Basidiomycota. Cluster C (*Occultifur* and *Fusarium* included) contained taxa that were in high abundance in the root samples and shoot endosphere compartment. They were largely absent from seeds. A similar pattern was found for Cluster B, which contained taxa absent from seeds but abundant for root surface and shoot samples. Furthermore, this cluster had modest representation in the outer husk and outer grain compartments of seeds. Only one taxon was present in Cluster B: Tremellales. Cluster F was primarily restricted to seeds and carried taxa that were most abundant in outer grain and outer husk compartment such as *Hannaella* and *Phaeosphaeria*. Of note, members of Cluster D, which included *Alternaria* and *Curvularia* taxa were detected in all seed compartments but highest in grain.

Identification of a Core Microbiome

In total, 25 bacterial taxa and 8 fungal taxa were identified as core members in one or more seedling compartments. The bacterial core represented 90.0% of the bacterial total reads, while the fungal core represented 61.3% of the fungal total reads. From the perspective of ASVs, the ASVs identified in the bacterial core represented 42.2% (494/1171) of the bacterial ASVs, while the fungal core represented 21.8% (133/610) of the fungal ASVs.

Considering the bacterial core (**Table 1**), 10 taxa were detected in all samples of the root endosphere; 21 taxa for root surface samples; 11 taxa for shoot endosphere samples and 15 for shoot surface. Genera including *Allorhizobium*, *Sphingomonas, Methylobacterium, Aureimonas, Pantoea,* and *Xanthomonas* were consistently detected as core for all four tissue compartments. Less prevalent taxa such as *Microbacteriaceae* and *Rhizobiaceae* were only absent for root endosphere samples; *Mucilaginibacter* and *Paenibacillus* were consistently detected as core except for shoot endosphere samples. *Curtobacterium*, *Pseudomonas,* and *Chryseobacterium* were only identified in surface samples.

For the fungal core (**Table 2**), 7 taxa were detected in all samples of the root endosphere; 1 taxon for root surface samples; 3 taxa for shoot endosphere samples and 2 for shoot surface. *Fusarium* was consistently detected except for the shoot surface samples and Pleosporales was consistently detected except for the root surface samples. *Alternaria* was only detected in the endosphere sample. For the Basidiomycota, only Occultifur in the root endosphere and Ustilaginaceae in the shoot surface were found. Ascomycota such as Didymellaceae, Phaeosphaeriaceae and *Clonostachys* were also identified as core for the root endosphere.

Core microbiome found in seedlings were compared to those found in seed samples (Eyre et al., 2019). Generally, the seed bacteria core was a subset of the seedling except for Franconibacter found in grain. Genera of *Methylobacterium, Aureimonas, Rhizob*ium and *Sphingomonas* were consistently detected in seed and seedling samples. However, for the fungal core, genera contained in seedlings were a subset of those contained in seed samples except for *Clonostachys* and Ustilaginaceae. Pleosporales, *Alternaria*, Didymellaceae, Phaeosphaeriaceae and Occultifur were dominant in seed samples while *Fusarium* was only detected as core in the outer husk.

DISCUSSION

Microbes can colonize different plant compartments and prosper on the outer surfaces as well as inside plant tissues (Turner et al., 2013; Berg et al., 2014). In this study, we first explored the microbiome associated with rice seedlings derived exclusively from seeds. For the seedling bacterial data, fewer ASVs were detected in endosphere samples than surface samples. This may due to physical as well as biochemical barriers that restrict microbes from colonizing inside plants. In addition, the roots harbored more ASVs than shoots, which may be a result of the soil facilitating microbial growth within roots. However, a similar pattern was not seen in the fungal data where limited ASVs were detected in the root surface sample. Perhaps the method of sample collection of roots, which involved gentle shaking to remove debris and the method used for amplicon production may explain the small number of ASVs in fungal data. Overall, the percentage of shared ASVs in the 4 seedling compartments was low both for fungi and bacteria, suggesting tissue compartment as the driving factor of microbial communities. The PCoA analysis further confirmed this conclusion.

When compared to the previous seed data (Eyre et al., 2019), shared bacterial as well as fungal ASVs constituted a low proportion of the whole, indicating ASV composition in the seeding is very different from seeds and development plays an important role in proliferation of the rice associated microbiomes. This was evident from the PCoA analysis. The high proportion of seedling specific bacterial and fungal ASVs may

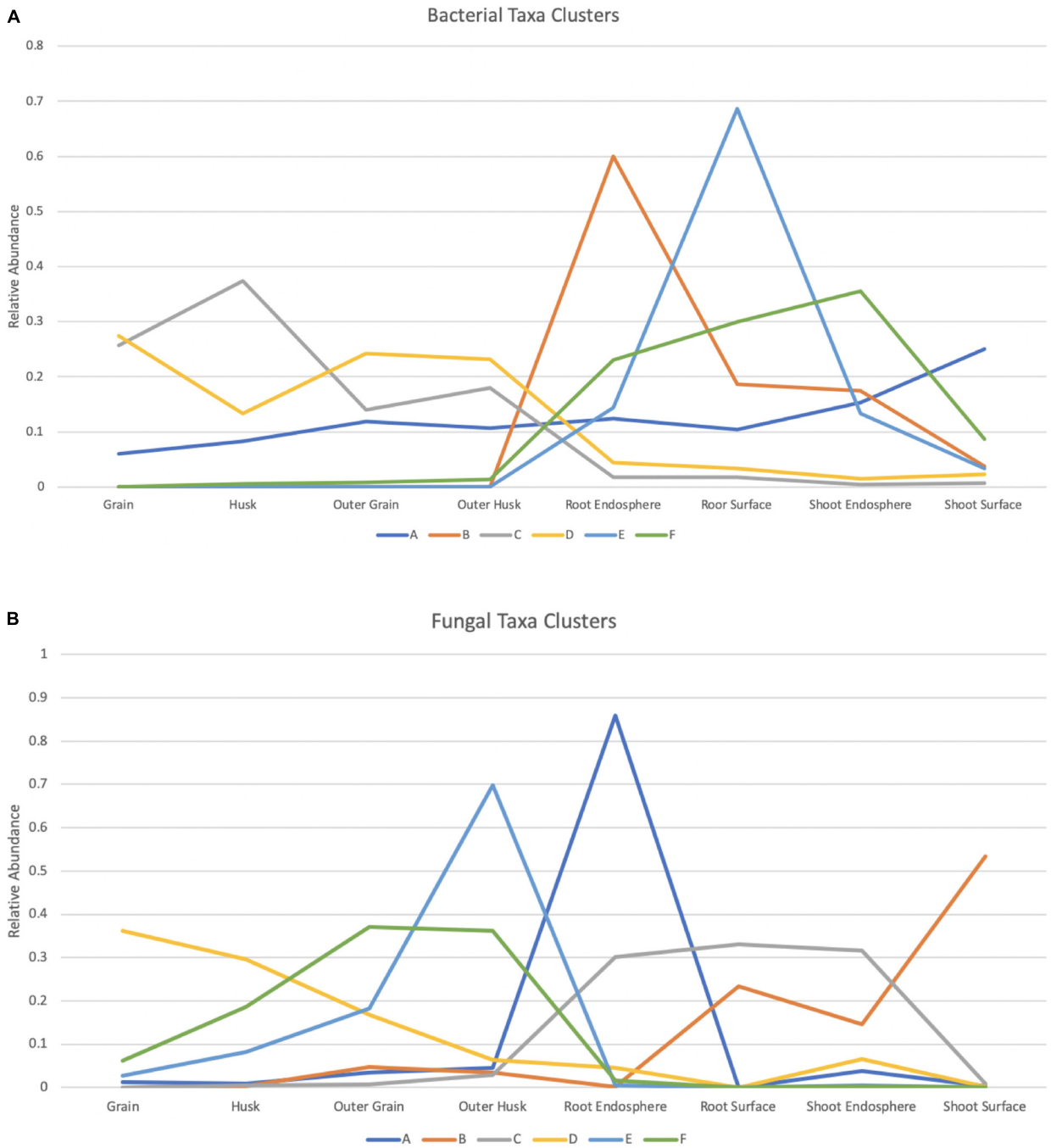

FIGURE 6 | Bacterial and fungal taxa clusters (Microbial taxa with > 0.1% total read abundance). Clustering of the normalized relative abundance values for bacterial **(A)** and fungal **(B)** taxa. Node values represent the average of the normalized abundance values within a cluster for each of the tissue compartments, A–F represent the 6 clusters summarized from the data and taxa included in each cluster can be found in **Supplementary Tables 5, 6**.

due to the nutrient rich environment provided by soil and/or nutrients released from seedlings during germination. As such, rare microbes, possibly existing as fungal and bacterial spores in the seed prospered in the seedlings and were identified as unique ASVs (Darrasse et al., 2010; Huang et al., 2016; Johnston-monje et al., 2016; Shade et al., 2017). However, it is also formally possible that the seedling ASVs from sand result from DNA contamination, present even in sterile sand.

For the bacterial microbiome, the ASV pool of the grain contributed the least ASVs to the seedling while the outer husk contributed the most. This may imply some valuable function associated with the outer husk compartment, whereby

TABLE 1 | Bacterial core seedling microbiome.

	RE	RS	SE	SS
Actinobacteria		Microbacterium (–, **1**, 1, 1)	Amycolatopsis (–, –, **1**, 1)	Amycolatopsis (–, –, 1, **1**)
		Microbacteriaceae (–, **1**, 1, 1)	Microbacterium (–, 1, **1**, 1)	Microbacterium (–, 1, 1, **1**)
		Curtobacterium (–, **1**, –, –)	Microbacteriaceae (–, 1, **1**, 1)	Microbacteriaceae (–, 1, 1, **1**)
		Kineococcus (–, **1**, –, –)		Curtobacterium (–, –, –, **1**)
		Quadrisphaera (–, **1**, –, –)		
Proteobacteria Alphaproteobacteria	Allorhizobium (**2**, 2, 1, 1)	Allorhizobium (2, **2**, 1, 1)	Allorhizobium (1, 1, **1**, 1)	Allorhizobium (1, 1, 1, **1**)
	Sphingomonas (**4**, 2, 1, 1)	Sphingomonas (2, **7**, 2, 2)	Sphingomonas (1, 2, **2**, 2)	Sphingomonas (1, 2, 2, **2**)
	Methylobacterium (**4**, 2, 1, 1)	Aureimonas (–, **2**, 1, 1)	Aureimonas (–, 1, **1**, 1)	Aureimonas (–, 1, 1, **1**)
	Novosphingobium (**1**, 1, –, –)	Rhizobiaceae (–, **1**, 1, 1)	Rhizobiaceae (–, 1, **1**, 1)	Rhizobiaceae (–, 1, 1, **1**)
	Aureimonas (**1**, –, –, –)	Methylobacterium (2, **3**, 1, 1)	Methylobacterium (1, 1, **1**, 1)	Methylobacterium (2, 1, 1, **2**)
		Novosphingobium (1, **1**, –, –)		
		Roseomonas (–, **2**, –, –)		
		Belnapia (–, **1**, –, –)		
Proteobacteria Gammaproteobacteria	Pantoea (**2**, 2, 1, 1)	Pantoea (2, **6**, 1, 1)	Pantoea (1, 1, **1**, 1)	Pantoea (1, 1, 1, **1**)
	Xanthomonas (**1**, 1, 1, 1)	Luteibacter (–, **1**, –, –)	Xanthomonas (1, 1, **1**, 1)	Xanthomonas (1, 1, 1, **1**)
	Herbaspirillum (**1**, –, –, –)	Pseudomonas (–, **3**, –, 2)	Cupriavidus (–, –, **1**, –)	Pseudomonas (–, 2, –, **2**)
		Massilia (–, **1**, –, –)		
		Xanthomonas (1, **1**, 1, 1)		
Bacteroidetes	Mucilaginibacter (**3**, 2, –, 1)	Chryseobacterium (–, **1**, –, –)		Chryseobacterium (–, –, –, **1**)
		Mucilaginibacter (2, **3**, –, 1)		Mucilaginibacter (1, 1, –, **1**)
Firmicutes	Paenibacillus (**1**, 1, –, 1)	Paenibacillus (1, **3**, –, 1)		Paenibacillus (1, 1, –, **1**)

The bacterial ASVs and their representative taxa shared between all samples of a seedling compartment. The numbers in parentheses represent the number of ASVs belonging to the bacterial taxa that are shared with other compartments in order according to the header. The bold number represents the number of ASVs belonging to the compartment of interest (RE, Root Endosphere; RS, Root Surface; SE, Shoot Endosphere; SS, Shoot Surface).

microbes are recruited from the parent plant during growth and development and may confer some benefit to rice growth. When the seeds germinate, those microbiome from the outer husk are thus recruited again to favor rice seedlings. Microbes from the grain compartment on the other hand, may be highly specialized and do not thrive as robustly as epiphytes during seedling growth due to unsuitable living environment and resource limitations (James et al., 2002; Compant et al., 2010; Turner et al., 2013).

The total number of fungal ASVs of seeds and seedlings were similar. However, the amount of shared ASVs between seeds and seedlings were extremely low. This may be result of the methods used for sample collection, amplicon production and two independent sequencing data processing for seed and seeding. Alternatively, germination and development play a major role in establishment of the seedling fungal community. Many fungi found in the seeds may be opportunistic saprophytes

TABLE 2 | Fungal core seedling microbiome.

	RE	RS	SE	SS
Ascomycota	Pleosporales		Pleosporales	Pleosporales
Dothideomycetes	(**1**, –, 1, 1)		(1, –, **2**, 1)	(1, –, 2, **1**)
	Alternaria		Alternaria	
	(**2**, –, 1, –)		(2, –, **1**, –)	
	Didymellaceae			
	(**1**, –, –, –)			
	Phaeosphaeriaceae			
	(**1**, –, –, –)			
Ascomycota Sordariomycetes	Fusarium	Fusarium	Fusarium	
	(**1**, 1, 1, –)	(1, **1**, 1, –)	(1, 1, **2**, –)	
	Clonostachys			
	(**1**, –, –, –)			
Basidiomycota	Occultifur			Ustilaginaceae
	(**1**, –, –, –)			(–, –, –, **1**)

The fungal ASVs and their representative taxa shared between all samples of a seedling compartment. The numbers in parentheses represent the number of ASVs belonging to the bacterial taxa that are shared with other compartments in order according to the header. The bold number represents the number of ASVs belonging to the compartment of interest (RE, Root Endosphere; RS, Root Surface; SE, Shoot Endosphere; SS, Shoot Surface).

and are readily lost and fall to levels below our limits of detection during seedling growth (Afkhami and Rudgers, 2008; Márquez et al., 2012). Nevertheless, the outer husk and outer grain compartments contributed the most fungal ASVs to the seedling which may be due to the high diversity of fungi associated with those two compartments (**Figure 2**).

Different field conditions and agricultural activities alter the microbial community (Buyer et al., 1999; Hacquard, 2016), as may genetic differences of host plants (Peiffer et al., 2013). In this study, it was the tissue compartment that proved to be the principal driving factor of microbial community. This discovery also suggested that there may be core microbiome consistently associating with rice plants regardless of location, genotype and harvesting time. Additional studies using rice representing more diverse genotypes from more growing locations and harvesting years would be needed to confirm conclusions obtained in this study. In fact, little is known about the mechanisms for microbial community build up. More knowledge is needed regarding the interaction between host and microbiome as well as interaction among different microbial communities (Lau and Lennon, 2011; Cordero and Datta, 2016; Henry et al., 2016).

Taxa composition of tissue compartments revealed here are consistent with previous studies related to microbiome communities associated with plants (Fischer et al., 2012; Lundberg et al., 2012; Sessitsch et al., 2012; Vorholt, 2012; Bodenhausen et al., 2013; Bulgarelli et al., 2013; Schlaeppi and Bulgarelli, 2015). Similar taxon compositions were detected in the root endosphere and on the root surface, indicating that both of those two compartments inherited similar microbial taxa from seeds. However, some differences were noted, indicating that the endosphere may impose some selection mechanisms. More *Pseudomonas* and *Massilia* accumulated in root endosphere rather than on the root surface and those microbes are strongly linked to plant growth promotion. It is noteworthy that ASV abundance was also analyzed in addition to read abundance. In a number of instances, taxa showed dramatic differences in read abundance compared to their taxonomic (ASV) abundance. For those who had lower proportion of read abundance but higher ASV abundance such as *Actinobacteria* in roots, it may suggest a higher evolution potential for this specific taxon. On the contrary, for the *Rhizobium* genus from Alphaproteobacteria, ASV abundance in seedlings was lower than read abundance, suggesting ASVs detected in this genus are quite conservative.

A point worth highlighting is that though not detected in seeds, Bacteroidetes (29%) and Firmicutes (3%) were detected in seedling samples. It is likely that Bacteroidetes and Firmicutes exist in the seed samples in the first place, but the amount of those bacteria fell below our limits of detection in seeds. Previous research had identified Bacteroidetes and Firmicutes associated with rice seeds (Okunishi et al., 2005; Mano et al., 2006; Zhang et al., 2019). The reason they were identified elsewhere may be because they were either isolated bacteria from culturable colonies (Okunishi et al., 2005; Mano et al., 2006) or larger sample amounts for gDNA were used (Zhang et al., 2019). Also, the rice varieties and rice growing conditions were different from our studies, which may have enhanced these taxa in seeds. It is likely that during the process of rice germination, rich nutrients either from rice shoots and roots or soil facilitate the thriving of Bacteroidetes and Firmicutes. Moreover, those bacteria may promote rice growth, generating a mutualism interaction with rice (Urai et al., 2008; Madhaiyan et al., 2010a; Köberl et al., 2011). In fact, considerable microbiome research has revealed a close relationship of rice plants with Bacteroidetes and Firmicutes (Mano and Morisaki, 2008; Raweekul et al., 2016; Lu et al., 2018), consistent with our findings for seedlings.

Proteobacteria, which predominated root endosphere compartments (Cluster B **Figure 6A**), likely represent specific root endophytes. Other Proteobacteria dominated the root surface (Cluster E), indicating those bacteria live in association with roots and were not selected as endophytes. Cluster A

revealed taxa found in the seed that remained in the seedling compartments. This cluster is made up of *Paenibacillus*, *Acidovorax, Pedobacter, Rhizobium, Microbacterium,* and others. It is not known how exactly these taxa are selected, but they may be of particular interest.

From the fungal clustering analysis, taxa were generally found to be associated with specific compartments. Taxa enriched in the root endosphere sample (Cluster A) were identified as Ascomycota. *Gibberella* which can infect rice and produce gibberellin was present in this cluster and gibberellin is a growth hormone promoting cell elongation, flower formation and seedling growth (Cerdá-Olmedo et al., 1994; Zainudin et al., 2008). Furthermore, members of the genus *Clonostachys* found in this cluster have been developed as biological control agents (Xue, 2003; Jensen et al., 2004; Rodríguez et al., 2011).

An important goal of this work was to define a core microbiome of rice for both bacteria and fungi as these may represent microbes that confer beneficial properties. A number of core bacteria were identified, such as *Rhizobium-Allorhizobium-Pararhizobium-Neorhizobium* that can fix nitrogen and colonize inside plant tissue. These microbes have been also found colonizing roots of non-legume crops such as wheat, barley, maize and rice and could be used as biofertilizer through bio-inoculating with crop seeds (Boddey et al., 1995; Webster et al., 1997; Yanni et al., 1997; Gutierrez-Zamora and Martinez-Romero, 2001; Lupwayi et al., 2004; Chi et al., 2005; Da et al., 2011; Ren et al., 2011; Mousavi et al., 2014, 2015). Species from the genus *Pantoea* (Monier and Lindow, 2005) have been found as part of the epi- and endophyte flora of various plant hosts. They are considered to be phosphate-solubilizing microorganisms (PSMs) and may be valuable to solubilize inorganic phosphates (Son et al., 2006; Coutinho and Venter, 2009). *Pseudomonas, Bacillus*, and *Enterobacter* are also known as PSMs (Raj et al., 1981; Laheurte and Berthelin, 1988). A rice endophyte *Pantoea agglomerans* YS19 was further demonstrated to have nitrogen-fixing activity, producing phytohormones that can improve rice biomass and affect allocations of host photosynthates (Feng et al., 2006). This species was also found to have anti-disease properties that protect pear and apple from *B. cinerea*, *Penicillium expansum,* and *Rhizopus stolonifer* (Nunes et al., 2001; Nunes et al., 2002). Furthermore, *P. agglomerans* may also regulate water content of wheat rhizosphere by improving soil aggregation (Amellal et al., 1998). Siderophores and hydrocyanic acid (HCN) are produced by *Pantoea* which may help with ion absorption and disease control (Selvakumar et al., 2008). However, this genus also contains species that can cause disease on a wide range of host crops as well as human beings (Brenner et al., 1984; Coutinho and Venter, 2009; Kido et al., 2010).

Sphingomonas, also detected as a core bacteria occurs in a diverse range of environments, are metabolically flexible and can consume environmental contaminants (Miyauchi et al., 1999; Aylward et al., 2013). Members of this genus can remediate heavy metals and decompose various pesticides (Miller et al., 2010; Liu et al., 2014). *Sphingomonas* sp. LK11 alleviates salinity stress in *Solanum pimpinellifolium* (Khan et al., 2017). *Sphingomonas panaciterrae* sp. nov. was demonstrated to promote plant growth through production of indole-3-acetic acid (IAA) (Sukweenadhi et al., 2015). They were also shown to protect *Arabidopsis thaliana* against bacterial pathogens (Innerebner et al., 2011). Another core bacteria *Paenibacillus* genus have a broad host range and have been demonstrated to have properties such as nitrogen fixation, bioremediation, and promoting plant growth through production of phytohormones including auxin, indole and phenolic compounds. They can also combat plant pathogens and pests by producing antibiotics (Gardener, 2004; Lal and Tabacchioni, 2009; Govindasamy et al., 2010). *P. polymyxa* can enable host drought tolerance (Shiao and Huang, 2001) as well as confer "Induced systemic resistance" (ISR) in *Arabidopsis* through the emission of volatile organic compounds (VOCs) (Lee et al., 2012).

Other core genera, including members of the genus *Mucilaginibacter* are known to have plant-growth-promoting properties and some species have been isolated from dried rice straw in addition to soil samples (Pankratov et al., 2007; Urai et al., 2008; An et al., 2009; Jeon et al., 2009; Luo et al., 2009; Baik et al., 2010; Madhaiyan et al., 2010b). *Methylobacterium* species were shown to promote plant growth through producing different phytohormones and have been isolated from various plants (Kutschera, 2007). They were also known to solubilize calcium phosphate and fix nitrogen (Subhaswaraj et al., 2017). Bacterial species from *Xanthomonas* and *Pseudomonas* may cause plant disease in some circumstances while other species of *Pseudomonas* can also promote plant growth (Cole et al., 2015; Park et al., 2015). Given their known properties, it is likely many of the core bacteria described here have potential to be developed as biologicals for modern agriculture.

Examination of the core fungi associated with rice seedlings, revealed several genera with known biological properties, including members of the *Alternaria* genus. These fungi are ubiquitous in the environment and commonly act as opportunistic plant pathogens (Al-Hatmi et al., 2016). More than 100 plant species can be infected by *Alternaria* species which can cause leaf spot and other diseases (Rotem, 1994). However, some *Alternaria* species also have biocontrol potential against other plant diseases. *A. zinniae, A. eichhornia,* and *A. cassiae* are commercially available for weed control (Walker and Sciumbato, 1979; Walker, 1980; Aneja and Singh, 1989; Babu et al., 2002). *Occultifur* species are basidiomycetous yeasts and usually use plant leaves and soil as important and interrelated habitats (Khunnamwong et al., 2015, 2017). Some species have been reported as mycoparasites, whereas one species has been reported as a saprophyte (Roberts, 1997; Khunnamwong et al., 2015). Members of the family *Didymellaceae* inhabit a wide range of ecosystems (Chen et al., 2017) and most of them are plant pathogens of a wide range of hosts (Aveskamp et al., 2008, 2010; Chen et al., 2015), however, they also comprises several species recognized as endophytic, fungicolous and lichenicolous fungi (Yang et al., 1994; Sullivan and White, 2000; Hawksworth, 2003; Hawksworth and Cole, 2004; Diederich et al., 2007; Schoch et al., 2009). Those core fungi also can be candidates for biocontrol uses.

Filamentous core fungi from the genus *Fusarium* are widely distributed in plants, soil, water and are abundant members

of the soil microbial community. Most species are harmless while some species can cause diseases of plants as well as animals. Many products from agriculturally important crops can be contaminated by *Fusarium* spp., which can be of concern because of highly toxic metabolites produced by some species (Rippon, 1982; Walsh and Dixon, 1996; Nowicki et al., 2012). Most species from the order *Pleosporales* are harmless saprobes while there are also species associated with plants as parasites, epiphytes and endophytes (Zhang et al., 2009). The corresponding ASVs could be only assigned to *Pleosporales* at the order level rather than species level, indicating further research is needed to accurately characterize the role of fungi in this order. Fungi from *Gibberella* can infect rice and produce gibberellin, a plant hormone promoting cell elongation, flower formation and seedling growth (Cerdá-Olmedo et al., 1994; Zainudin et al., 2008). *Clonostachys rosea f. rosea* from the *Clonostachys* genus is a plant endophyte and has been used as a biological pest control agent against fungi such as *B. cinerea* as well as nematodes (Toledo et al., 2006; Zhang et al., 2008).

In this research, we identified and characterized the microbiome associated with rice seedlings in a sterile environment. However, the main purpose for this research is to understand the dynamics of microbiota shift from rice seeds to seedlings. Seed-borne microbes is of great interest to researchers because those microbes can be vertically transmitted to next generation (Barret et al., 2015; Cope-Selby et al., 2017; Mitter et al., 2017; Shahzad et al., 2018). During the transmission, phyto-beneficial bacteria and fungi inherited from seeds can promote seedling growth as well as mitigate plant stress damage (Mitter et al., 2017; Shahzad et al., 2018). Knowledge about the microbiota shift from rice seeds to rice seedlings can help uncover what microbes have been transmitted vertically and how well they proliferate. Transmitted microbes showing high abundance in seedlings have great potential to be selected by rice as phyto-beneficial microbes. This will further instruct microbiome inoculant engineering to benefit modern agriculture. The use of a sterile environment to monitor shifts in microbiome populations has been used in other studies (Hardoim et al., 2012; Huang et al., 2016; Mitter et al., 2017; Torres-Cortés et al., 2018). However, there is limited data about how rice seed-borne microbes change during the development process. Our research provides the first detailed description of dynamic microbiota shifts from rice seeds to rice seedlings. Rice seeds of different genotype harvested from different locations at different time allowed us to gain novel insight into these population shifts and the core microbiome associated with seedlings tissue compartments. Further experiments with more varieties and sources of seeds are needed to confirm and extend our findings as well as additional studies to compare population shifts of seeds planted in natural soils.

An initial comparison between our findings and other datasets collected from natural conditions revealed some consistent patterns. Edwards and colleagues (Edwards et al., 2018) found tissue compartments and rice development age were more important factors shaping microbiome than growth location. Wang and colleagues detected more diverse bacterial ASVs in roots than stems while fungal ASVs were more diverse in stems than roots (Wang et al., 2016). Although the identified microbiome varied somewhat between different experimental set ups, similarities in the distribution of phyla are apparent, in line with our key findings. For example, Proteobacteria, Actinobacteria, Bacteroidetes and Firmicutes were consistently detected as bacterial phyla associated with rice while Ascomycota and Basidiomycota were found to be dominant fungal phyla (Mano and Morisaki, 2008; Edwards et al., 2015; Bertani et al., 2016; Raweekul et al., 2016; Venkatachalam et al., 2016; Lu et al., 2018; Thapa et al., 2018).

In sum, this study addressed the question of what happens to microbes present in seeds during seedling germination and how are they distributed to above and below ground tissues. Their retention (and loss) and distribution patterns during seedling growth also provides some insight into why they are there. Because the productivity and health of agricultural systems depend greatly upon the functional processes carried out by the plant-associated microbiome, to further examine the question "what are they doing there?" will need further functional analysis of these core microbes. If their function is beneficial and given they are core, they may be persistent and represent valuable biologicals.

The findings of this research support the hypothesis that the process of germination changes the microbial community inherited from seeds and partitions it into the above and below ground tissues. Certain microbes remain associated with specific tissue compartment and accumulate there to build a core microbiome. Most importantly, the effect of rice genotype, growth location and harvest year are not as strong a driving force as tissue compartment on shaping the microbial community. The common core microbiome of rice seedlings revealed by this study offer promise that we can develop and apply universal microbial inoculant to benefit global rice production.

DATA AVAILABILITY STATEMENT

The datasets presented in this study can be found in online repositories. The names of the repository/repositories and accession number(s) can be found at: https://www.ncbi.nlm.nih.gov/, SAMN14836377–SAMN14836424.

AUTHOR CONTRIBUTIONS

MW and YO carried out the plant samples preparation, gDNA extraction, and amplicon library preparation. MW, AE, and MT analyzed the sequencing data. MW wrote the manuscript draft under the review and supervision of MT and RD. All authors conceived and planned the research.

FUNDING

The authors thank the North Carolina State University (NCSU) Plant Soil Microbial Community Consortium and the NCSU College of Agriculture and Life Sciences for supporting this research.

ACKNOWLEDGMENTS

Special thanks to Dr. Yulin Jia from USDA Dale Bumpers National Rice Research Center for providing the rice seeds 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.2020.559728/full#supplementary-material

REFERENCES

Afkhami, M. E., and Rudgers, J. A. (2008). Symbiosis lost : imperfect vertical transmission of fungal endophytes in grasses. *Am. Nat.* 172. 3, 405–416. doi: 10.1086/589893

Al-Hatmi, A. M. S., Meis, J. F., and de Hoog, G. S. (2016). Fusarium: molecular diversity and intrinsic drug resistance. *PLoS Pathog.* 12:e1005464. doi: 10.1371/journal.ppat.1005464

Amellal, N., Burtin, G., Bartoli, F., and Heulin, T. (1998). Colonization of wheat roots by an exopolysaccharide-producing Pantoea agglomerans strain and its effect on rhizosphere soil aggregation. *Appl. Environ. Microbiol.* 64, 3740–3747. doi: 10.1128/aem.64.10.3740-3747.1998

An, D. S., Yin, C. R., Lee, S. T., and Cho, C. H. (2009). *Mucilaginibacter daejeonensis* sp. nov., isolated from dried rice straw. *Int. J. Syst. Evol. Microbiol.* 59, 1122–1125. doi: 10.1099/ijs.0.003384-0

Aneja, K. R., and Singh, K. (1989). *Alternaria alternata*(fr.)keissler, a pathogen ofwaterhyacinth with biocontrol potential. *Trop. Pest Manage.* 35, 354–356. doi: 10.1080/09670878909371402

Aveskamp, M. M., de Gruyter, J., Woudenberg, J. H. C., Verkley, G. J. M., and Crous, P. W. (2010). Highlights of the didymellaceae: a polyphasic approach to characterise phoma and related pleosporalean genera. *Stud. Mycol.* 65, 1–60. doi: 10.3114/sim.2010.65.01

Aveskamp, M. M., Gruyter, J., and de Cours, P. W. (2008). Biology and recent developments in the systematics of phoma, a complex genus of major quarantine significance. *Fungal Divers.* 31, 1–18. doi: 10.1109/CDC.2010.5717258

Aylward, F. O., McDonald, B. R., Adams, S. M., Valenzuela, A., Schmidt, R. A., Goodwin, L. A., et al. (2013). Comparison of 26 sphingomonad genomes reveals diverse environmental adaptations and biodegradative capabilities. *Appl. Environ. Microbiol.* 79, 3724–3733. doi: 10.1128/AEM.00518-13

Babu, R. M., Sajeena, A., Seetharaman, K., Vidhyasekaran, P., Rangasamy, P., Som Prakash, M., et al. (2002). Host range of *Alternaria alternata* – A potential fungal biocontrol agents for waterhyacinth in India. *Crop Prot.* 21, 1083–1085. doi: 10.1016/S0261-2194(02)00076-5

Baik, K. S., Park, S. C., Kim, E. M., Lim, C. H., and Seong, C. N. (2010). *Mucilaginibacter rigui* sp. nov., isolated from wetland freshwater, and emended description of the genus *Mucilaginibacter*. *Int. J. Syst. Evol. Microbiol.* 60, 134–139. doi: 10.1099/ijs.0.011130-0

Barret, M., Briand, M., Bonneau, S., Préveaux, A., Valière, S., Bouchez, O., et al. (2015). Emergence shapes the structure of the seed microbiota. *Appl. Environ. Microbiol.* 81, 1257–1266. doi: 10.1128/aem.03722-14

Berendsen, R. L., Pieterse, C. M. J., and Bakker, P. A. H. M. (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

Bertani, I., Abbruscato, P., Piffanelli, P., Subramoni, S., and Venturi, V. (2016). Rice bacterial endophytes: isolation of a collection, identification of beneficial strains and microbiome analysis. *Environ. Microbiol. Rep.* 8, 388–398. doi: 10.1111/1758-2229.12403

Boddey, R. M., Oliveira, O. C., De, Urquiaga, S., Reis, V. M., Olivares, E. L., et al. (1995). Biological nitrogen fixation associated with sugar cane and rice : contributions and prospects for improvement. *Plant Soil* 174, 195–209. doi: 10.1007/978-94-011-0053-3_9

Bodenhausen, N., Horton, M. W., and Bergelson, J. (2013). Bacterial communities associated with the leaves and the roots of *Arabidopsis thaliana*. *PLoS ONE* 8:e56329. doi: 10.1371/journal.pone.0056329

Bragina, A., Cardinale, M., Berg, C., Berg, G., Schmid, M., and Zentrum, H. (2013). Vertical transmission explains the specific *Burkholderia* pattern in *Sphagnum* mosses at multi-geographic scale. *Front. Microbiol.* 4:394. doi: 10.3389/fmicb.2013.00394

Brenner, D. J., Fanning, G. R., Leete Knutson, J. K., Steigerwalt, A. G., and Krichevsky, M. I. (1984). Attempts to classify herbicola group-*Enterobacter agglomerans* strains by deoxyribonucleic acid hybridization and phenotypic tests. *Int. J. Syst. Bacteriol.* 34, 45–55. doi: 10.1099/00207713-34-1-45

Bulgarelli, D., Garrido-Oter, R., Münch, P. C., 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., Rott, M., Schlaeppi, K., Ver Loren van Themaat, E., Ahmadinejad, N., et al. (2012). Revealing structure and assembly cues for Arabidopsis root-inhabiting bacterial microbiota. *Nature* 488, 91–95. doi: 10.1038/nature11336

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

Buyer, J. S., Roberts, D. P., and Russek-Cohen, E. (1999). Microbial community structure and function in the spermosphere as affected by soil and seed type. *Can. J. Microbiol.* 45, 138–144. doi: 10.1139/w98-227

Callahan, B. J., McMurdie, P. J., Rosen, M. J., Han, A. W., Johnson, A. J. A., and Holmes, S. P. (2016). DADA2: high-resolution sample inference from Illumina amplicon data. *Nat. Methods* 13, 581–583. doi: 10.1038/nmeth.3869

Cerdá-Olmedo, E., Fernández-Martín, R., and Ávalos, J. (1994). Genetics and gibberellin production in *Gibberella fujikuroi*. *Antonie Van Leeuwenhoek* 65, 217–225. doi: 10.1007/BF00871950

Chen, Q., Hou, L. W., Duan, W. J., Crous, P. W., and Cai, L. (2017). Didymellaceae revisited. *Stud. Mycol.* 87, 105–159. doi: 10.1016/j.simyco.2017.06.002

Chen, Q., Zhang, K., Zhang, G., and Cai, L. (2015). A polyphasic approach to characterise two novel species of Phoma (Didymellaceae) from China. *Phytotaxa* 197, 267–281. doi: 10.11646/phytotaxa.197.4.4

Chi, F., Shen, S., Cheng, H., Jing, Y., Yanni, Y. G., and Dazzo, F. B. (2005). Ascending migration of endophytic rhizobia, from roots to leaves, inside rice plants and assessment of benefits to rice growth physiology. *Appl. Environ. Microbiol.* 71, 7271–7278. doi: 10.1128/AEM.71.11.7271

Cole, B. J., Fletcher, M., Waters, J., Wetmore, K., Blow, M. J., Deutschbauer, A. M., et al. (2015). *Genetic Control of Plant Root Colonization by the Biocontrol Agent, Pseudomonas fluorescens.* Available online at: https://escholarship.org/uc/item/5hb1c4p1 (accessed January 10, 2019).

Compant, S., Clément, C., and Sessitsch, A. (2010). Plant growth-promoting bacteria in the rhizo- and endosphere of plants: their role, colonization, mechanisms involved and prospects for utilization. *Soil Biol. Biochem.* 42, 669–678. doi: 10.1016/j.soilbio.2009.11.024

Cope-Selby, N., Cookson, A., Squance, M., Donnison, I., Flavell, R., and Farrar, K. (2017). Endophytic bacteria in *Miscanthus* seed: implications for germination, vertical inheritance of endophytes, plant evolution and breeding. *Gcb Bioenergy* 9, 57–77. doi: 10.1111/gcbb.12364

Cordero, O. X., and Datta, M. S. (2016). Microbial interactions and community assembly at microscales. *Curr. Opin. Microbiol.* 31, 227–234. doi: 10.1016/j.mib.2016.03.015

Coutinho, T. A., and Venter, S. N. (2009). Pantoea ananatis: an unconventional plant pathogen. *Mol. Plant Pathol.* 10, 325–335. doi: 10.1111/j.1364-3703.2009.00542.x

Cruz-Hernández, A., Tomasini-Campocosio, A., Pérez-Flores, L. J., Fernández-Perrino, F. J., and Gutiérrez-Rojas, M. (2013). Inoculation of seed-borne fungus in the rhizosphere of *Festuca arundinacea* promotes hydrocarbon removal and pyrene accumulation in roots. *Plant Soil* 362, 261–270. doi: 10.1007/s11104-012-1292-6

Da, W., Wang, E., Chen, W., Sui, X., Zhang, X., Liu, H., et al. (2011). *Rhizobium herbae* Sp. nov. and *Rhizobium giardinii* related bacteria, minor

microsymbionts of various wild legumes in China. *Int. J. Systemat. Evol. Microbiol.* 61, 1912–1920. doi: 10.1099/ijs.0.024943-0

Darrasse, A., Darsonval, A., Boureau, T., and Durand, K. (2010). Transmission of plant-pathogenic bacteria by nonhost seeds without induction of an associated defense reaction at emergence. *Appl. Environ. Microbiol.* 76, 6787–6796. doi: 10.1128/AEM.01098-10

Diederich, P., Kocourkova, J., Etayo, J., and Zhurbenko, M. (2007). The lichenicolous *Phoma* species (coelomycetes) on *Cladonia*. *Lichenol* 39, 153–163. doi: 10.1017/S0024282907006044

Ding, B., Bellizzi, M. D. R., Ning, Y., Meyers, B. C., and Wang, G.-L. (2012). HDT701, a histone H4 deacetylase, negatively regulates plant innate immunity by modulating histone H4 acetylation of defense-related genes in rice. *Plant Cell* 24, 3783–3794. doi: 10.1105/tpc.112.101972

Edwards, J., Johnson, C., Santos-Medellín, C., Lurie, E., Podishetty, N. K., Bhatnagar, S., et al. (2015). Structure, variation, and assembly of the root-associated microbiomes of rice. *Proc. Natl. Acad. Sci. U.S.A.* 112, E911–E920. doi: 10.1073/pnas.1414592112

Edwards, J. A., Santos-Medellín, C. M., Liechty, Z. S., Nguyen, B., Lurie, E., Eason, S., et al. (2018). Compositional shifts in root-associated bacterial and archaeal microbiota track the plant life cycle in field-grown rice. *PLoS Biol.* 16:e2003862. doi: 10.1371/journal.pbio.2003862

Engelbrektson, A., Kunin, V., Engelbrektson, A., Kunin, V., Glavina, del Rio, T., et al. (2012). Defining the core *Arabidopsis thaliana* root microbiome. *Nature* 488, 86–90. doi: 10.1038/nature11237

Eyre, A. W., Wang, M., Oh, Y., and Dean, R. A. (2019). Identification and characterization of the core rice seed microbiome. *Phytobiomes J.* 3, 148–157. doi: 10.1094/pbiomes-01-19-0009-r

Fadrosh, D. W., Ma, B., Gajer, P., Sengamalay, N., Ott, S., Brotman, R. M., et al. (2014). An improved dual-indexing approach for multiplexed 16S rRNA gene sequencing on the Illumina MiSeq platform. *Microbiome* 2, 1–7. doi: 10.1186/2049-2618-2-6

Feng, Y., Shen, D., and Song, W. (2006). Rice endophyte *Pantoea agglomerans* YS19 promotes host plant growth and affects allocations of host photosynthates. *J. Appl. Microbiol.* 100, 938–945. doi: 10.1111/j.1365-2672.2006.02843.x

Fischer, D., Pfitzner, B., Schmid, M., Simões-Araújo, J. L., Reis, V. M., Pereira, W., et al. (2012). Molecular characterisation of the diazotrophic bacterial community in uninoculated and inoculated field-grown sugarcane (*Saccharum* sp.). *Plant Soil* 356, 83–99. doi: 10.1007/s11104-011-0812-0

Gardener, B. B. M. (2004). Ecology of *Bacillus* and *Paenibacillus* spp. in Agricultural Systems. *Phytopathology* 94, 1252–1258. doi: 10.1094/PHYTO.2004.94.11.1252

Gardes, M., and Bruns, T. D. (1993). ITS primers with enhanced specificity for basidiomycetes-application to the identification of mycorrhizae and rusts. *Mol. Ecol.* 2, 113–118. doi: 10.1111/j.1365-294x.1993.tb00005.x

Govindasamy, V., Senthilkumar, M., Magheshwaran, V., Kumar, U., Bose, P., Sharma, V., et al. (2010). "Bacillus and *Paenibacillus* spp.: potential PGPR for sustainable agriculture," in *Plant Growth and Health Promoting Bacteria*, ed. D. K. Maheshwari, (Berlin: Springer Berlin Heidelberg), 333–364. doi: 10.1007/978-3-642-13612-2_15

Gutierrez-Zamora, M. L., and Martinez-Romero, E. (2001). Natural endophytic association between *Rhizobium etli* and maize (*Zea mays* L.). *J. Biotechnol.* 91, 117–126. doi: 10.1016/s0168-1656(01)00332-7

Hacquard, S. (2016). Disentangling the factors shaping microbiota composition across the plant holobiont. *New Phytol.* 209, 454–457. doi: 10.1111/nph.13760

Hardoim, P. R., Hardoim, C. C., Van Overbeek, L. S., and Van Elsas, J. D. (2012). Dynamics of seed-borne rice endophytes on early plant growth stages. *PLoS ONE* 7:e30438. doi: 10.1371/journal.pone.0030438

Hawkins, H., Johansen, A., and George, E. (2000). Uptake and transport of organic and inorganic nitrogen by arbuscular mycorrhizal fungi. *Plant Soil* 226, 275–285.

Hawksworth, D. L. (2003). The lichenicolous fungi of Great Britain and Ireland: an overview and annotated checklist. *Lichenologist* 35, 191–232. doi: 10.1016/S0024-2829(03)00027-6

Hawksworth, D. L., and Cole, M. S. (2004). Phoma fuliginosa sp. nov., from *Caloplaca trachyphylla* in Nebraska, with a key to the known lichenicolous species. *Lichenol* 36, 7–13. doi: 10.1017/s0024282904013982

Henry, C. S., Bernstein, H. C., Weisenhorn, P., Taylor, R. C., Lee, J., Zucker, J., et al. (2016). Microbial community metabolic modeling : a community data-driven network reconstruction. *J. Cell. Physiol.* 231, 2339–2345. doi: 10.1002/jcp.25428

Hodgson, S., Cates, C., De, Hodgson, J., Morley, N. J., Sutton, B. C., et al. (2014). Vertical transmission of fungal endophytes is widespread in forbs. *Ecol. Evol.* 4, 1199–1208. doi: 10.1002/ece3.953

Huang, M., Tang, Q., Ao, H., and Zou, Y. (2017). Yield potential and stability in super hybrid rice and its production strategies. *J. Integr. Agric.* 16, 1009–1017. doi: 10.1016/s2095-3119(16)61535-6

Huang, Y., Kuang, Z., Wang, W., and Cao, L. (2016). Exploring potential bacterial and fungal biocontrol agents transmitted from seeds to sprouts of wheat. *Biol. Control* 98, 27–33. doi: 10.1016/j.biocontrol.2016.02.013

Huse, S. M., Ye, Y., Zhou, Y., and Fodor, A. A. (2012). A core human microbiome as viewed through 16S rRNA sequence clusters. *PLoS ONE* 7:e34242. doi: 10.1371/journal.pone.0034242

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

Izawa, T., and Shimamoto, K. (1996). Becoming a model plant: the importance of rice to plant science. *Trends Plant Sci.* 1, 95–99. doi: 10.1016/s1360-1385(96)80041-0

James, E. K., Gyaneshwar, P., Mathan, N., Barraquio, W. L., Reddy, P. M., Iannetta, P. P. M., et al. (2002). Infection and colonization of rice seedlings by the plant growth-promoting bacterium herbaspirillum seropedicae Z67. *Mol. Plant-Microbe Interact.* 15, 894–906. doi: 10.1094/MPMI.2002.15.9.894

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. *GCB Bioenergy* 9, 591–612. doi: 10.1111/gcbb.12371

Jensen, B., Knudsen, I. M. B., Madsen, M., and Jensen, D. F. (2004). Biopriming of infected carrot seed with an antagonist, clonostachys rosea, selected for control of seedborne Alternaria spp. *Phytopathology* 94, 551–560. doi: 10.1094/phyto.2004.94.6.551

Jeon, Y., Lee, S. S., Chung, B. S., Kim, J. M., Bae, J. W., Park, S. K., et al. (2009). *Mucilaginibacter oryzae* sp. nov., isolated from soil of a rice paddy. *Int. J. Syst. Evol. Microbiol.* 59, 1451–1454. doi: 10.1099/ijs.0.007138-0

Johnston-monje, D., Lundberg, D. S., Lazarovits, G., Reis, V. M., and Raizada, M. N. (2016). Bacterial populations in juvenile maize rhizospheres originate from both seed and soil. *Plant Soil* 405, 337–355. doi: 10.1007/s11104-016-2826-0

Kassambara, A. (2018). ggpubr:"ggplot2" Based Publication Ready Plots. *R package version 0. 1, 7*. Available online at: https://rpkgs.datanovia.com/ggpubr/ (accessed January 15, 2019).

Kato, H., Mori, H., Maruyama, F., Toyoda, A., Oshima, K., Endo, R., et al. (2015). Time-series metagenomic analysis reveals robustness of soil microbiome against chemical disturbance. *DNA Res.* 22, 413–424. doi: 10.1093/dnares/dsv023

Kawahara, Y., de la Bastide, M., Hamilton, J. P., Kanamori, H., Mccombie, W. R., Ouyang, S., et al. (2013). Improvement of the oryza sativa nipponbare reference genome using next generation sequence and optical map data. *Rice* 6, 3–10. doi: 10.1186/1939-8433-6-1

Khan, A. L., Waqas, M., Asaf, S., Kamran, M., Shahzad, R., Bilal, S., et al. (2017). Plant growth-promoting endophyte *Sphingomonas* sp. LK11 alleviates salinity stress in *Solanum pimpinellifolium*. *Environ. Exp. Bot.* 133, 58–69. doi: 10.1016/j.envexpbot.2016.09.009

Khunnamwong, P., Ribeiro, J. R. A., Garcia, K. M., Hagler, A. N., Takashima, M., Ohkuma, M., et al. (2017). *Occultifur plantarum* f.a., sp. nov., a novel cystobasidiomycetous yeast species. *Int. J. Syst. Evol. Microbiol.* 67, 2628–2633. doi: 10.1099/ijsem.0.001988

Khunnamwong, P., Surussawadee, J., Jindamorakot, S., Ribeiro, J. R. A., Hagler, A. N., and Limtong, S. (2015). *Occultifur tropicalis* f.A., sp. nov., a novel cystobasidiomycetous yeast species isolated from tropical regions. *Int. J. Syst. Evol. Microbiol.* 65, 1578–1582. doi: 10.1099/ijs.0.000140

Khush, G. S. (2000). "Strategies for increasing the yield potential of rice," in *Redesigning Rice Photosynthesis to Improve Yield, Proceedings of the Workshop on the Quest to Reduce Hunger: Redesigning Rice Photosynthesis*, eds J. E. Sheehy, P. L. Mitchell, and B. Hardy (Amsterdam: Elsevier), 207–212.

Kido, K., Hasegawa, M., Matsumoto, H., Kobayashi, M., and Takikawa, Y. (2010). Pantoea ananatis strains are differentiated into three groups based on reactions of tobacco and welsh onion and on genetic

characteristics. *J. Gen. Plant Pathol.* 76, 208–218. doi: 10.1007/s10327-010-0230-9

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/gks808

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

Köberl, M., Müller, H., Ramadan, E. M., and Berg, G. (2011). Desert farming benefits from microbial potential in arid soils and promotes diversity and plant health. *PLoS ONE* 6:e24452. doi: 10.1371/journal.pone.0024452

Kutschera, U. (2007). Plant-associated methylobacteria as co-evolved phytosymbionts: a hypothesis. *Plant Signal. Behav.* 2, 74–78. doi: 10.4161/psb.2.2.4073

Laheurte, F., and Berthelin, J. (1988). Effect of a phosphate solubilizing bacteria on maize growth and root exudation over four levels of labile phosphorus. *Plant Soil* 105, 11–17. doi: 10.1007/BF02371137

Lahti, L., and Shetty, S. (2018). *Introduction to the Microbiome R Package*. Available at: https://www.bioconductor.org/packages/release/bioc/vignettes/microbiome/inst/doc/vignette.html (accessed January 15, 2019).

Lal, S., and Tabacchioni, S. (2009). Ecology and biotechnological potential of *Paenibacillus polymyxa*: a minireview. *Indian J. Microbiol.* 49, 2–10. doi: 10.1007/s12088-009-0008-y

Lau, J. A., and Lennon, J. T. (2011). Evolutionary ecology of plant – Microbe interactions : soil microbial structure alters selection on plant traits. *New Phytol.* 192, 215–224. doi: 10.1111/j.1469-8137.2011.03790.x

Lee, B., Farag, M. A., Park, H. B., Kloepper, J. W., Lee, S. H., and Ryu, C. M. (2012). Induced resistance by a long-chain bacterial volatile: elicitation of plant systemic defense by a C13 volatile produced by *Paenibacillus polymyxa*. *PLoS ONE* 7:e48744. doi: 10.1371/journal.pone.0048744

Liu, F., Chi, Y., Wu, S., Jia, D., and Yao, K. (2014). Simultaneous degradation of cypermethrin and its metabolite, 3-phenoxybenzoic acid, by the cooperation of bacillus licheniformis B-1 and *Sphingomonas* sp. SC-1. *J. Agric. Food Chem.* 62, 8256–8262. doi: 10.1021/jf502835n

Lu, T., Ke, M., Peijnenburg, W. J. G. M., Zhu, Y., Zhang, M., Sun, L., et al. (2018). Investigation of rhizospheric microbial communities in wheat, barley, and two rice varieties at the seedling stage. *J. Agric. Food Chem.* 66, 2645–2653. doi: 10.1021/acs.jafc.7b06155

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

Luo, X., Zhang, L., Dai, J., Liu, M., Zhang, K., An, H., et al. (2009). *Mucilaginibacter ximonensis* sp. nov., isolated from Tibetan soil. *Int. J. Syst. Evol. Microbiol.* 59, 1447–1450. doi: 10.1099/ijs.0.005405-0

Lupwayi, N. Z., Clayton, G. W., Hanson, K. G., Rice, W. A., and Biederbeck, V. O. (2004). *Endophytic rhizobia* in barley, wheat and canola roots. *Can. J. Plant Sci.* 84, 37–45. doi: 10.4141/p03-087

Madhaiyan, M., Poonguzhali, S., Kwon, S. W., and Sa, T. M. (2010a). *Bacillus methylotrophicus* sp. nov., a methanol-utilizing, plant-growth-promoting bacterium isolated from rice rhizosphere soil. *Int. J. Syst. Evol. Microbiol.* 60, 2490–2495. doi: 10.1099/ijs.0.015487-0

Madhaiyan, M., Poonguzhali, S., Lee, J. S., Senthilkumar, M., Lee, K. C., and Sundaram, S. (2010b). *Mucilaginibacter gossypii* sp. nov. and Mucilaginibacter gossypiicola sp. nov., plant-growth-promoting bacteria isolated from cotton rhizosphere soils. *Int. J. Syst. Evol. Microbiol.* 60, 2451–2457. doi: 10.1099/ijs.0.018713-0

Mano, H., and Morisaki, H. (2008). Endophytic bacteria in the rice plant. *Microbes Environ.* 23, 109–117. doi: 10.1264/jsme2.23.109

Mano, H., Tanaka, F., Watanabe, A., Kaga, H., Okunishi, S., and Morisaki, H. (2006). Culturable surface and endophytic bacterial flora of the maturing seeds of rice plants (*Oryza sativa*) cultivated in a paddy field. *Microbes Environ.* 21, 86–100. doi: 10.1264/jsme2.21.86

Márquez, S. S., Bills, G. F., Herrero, N., and Zabalgogeazcoa, I. (2012). Non-systemic fungal endophytes of grasses. *Fungal Ecol.* 5, 289–297. doi: 10.1016/j.funeco.2010.12.001

McMurdie, P. J., and Holmes, S. (2013). phyloseq: an R package for reproducible interactive analysis and graphics of microbiome census data. *PLoS ONE* 8:e61217. doi: 10.1371/journal.pone.0061217

Mendes, R., Kruijt, M., de Bruijn, I., Dekkers, E., van der Voort, M., Schneider, J. H. M., et al. (2011). Deciphering the rhizosphere microbiome for disease-suppressive bacteria. *Science* 332.6033, 1097–1100. doi: 10.1126/science.1203980

Miller, T. R., Delcher, A. L., Salzberg, S. L., Saunders, E., Detter, J. C., and Halden, R. U. (2010). Genome sequence of the dioxin-mineralizing bacterium *Sphingomonas wittichii* RW1. *J. Bacteriol.* 192, 6101–6102. doi: 10.1128/JB.01030-10

Miransari, M. (2011). Arbuscular mycorrhizal fungi and nitrogen uptake. *Arch. Microbiol.* 193, 77–81. doi: 10.1007/s00203-010-0657-6

Mitter, B., Pfaffenbichler, N., Flavell, R., Compant, S., Antonielli, L., Petric, A., et al. (2017). A new approach to modify plant microbiomes and traits by introducing beneficial bacteria at flowering into progeny seeds. *Front. Microbiol.* 8:11. doi: 10.3389/fmicb.2017.00011

Miyauchi, K., Adachi, Y., Nagata, Y., and Takagi, M. (1999). Cloning and sequencing of a novel meta-cleavage dioxygenase gene whose product is involved in degradation of γ-hexachlorocyclohexane in *Sphingomonas paucimobilis*. *J. Bacteriol.* 181, 6712–6719. doi: 10.1128/jb.181.21.6712-6719.1999

Monier, J. M., and Lindow, S. E. (2005). Aggregates of resident bacteria facilitate survival of immigrant bacteria on leaf surfaces. *Microb. Ecol.* 49, 343–352. doi: 10.1007/s00248-004-0007-9

Mousavi, S. A., Österman, J., Wahlberg, N., Nesme, X., Lavire, C., Vial, L., et al. (2014). Phylogeny of the rhizobium-allorhizobium-*Agrobacterium clade* supports the delineation of *Neorhizobium* gen. nov. *Syst. Appl. Microbiol.* 37, 208–215. doi: 10.1016/j.syapm.2013.12.007

Mousavi, S. A., Willems, A., Nesme, X., de Lajudie, P., and Lindström, K. (2015). Revised phylogeny of rhizobiaceae: proposal of the delineation of *Pararhizobium* gen. nov., and 13 new species combinations. *Syst. Appl. Microbiol.* 38, 84–90. doi: 10.1016/j.syapm.2014.12.003

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

Nowicki, M., Nowakowska, M., Niezgoda, A., and Kozik, E. (2012). Alternaria black spot of crucifers: symptoms, importance of disease, and perspectives of resistance breeding. *Veg. Crop. Res. Bull.* 76, 5–19. doi: 10.2478/v10032-012-0001-6

Nunes, C., Usall, J., Teixidó, N., Fons, E., and Viñas, I. (2002). Post-harvest biological control by *Pantoea agglomerans* (CPA-2) on golden delicious apples. *J. Appl. Microbiol.* 92, 247–255. doi: 10.1046/j.1365-2672.2002.01524.x

Nunes, C., Usall, J., Teixidó, N., and Vias, I. (2001). Biological control of postharvest pear diseases using a bacterium, *Pantoea agglomerans* CPA-2. *Int. J. Food Microbiol.* 70, 53–61. doi: 10.1016/S0168-1605(01)00523-2

Ofek, M., Hadar, Y., and Minz, D. (2011). Colonization of cucumber seeds by bacteria during germination. *Environ. Microbiol.* 13, 2794–2807. doi: 10.1111/j.1462-2920.2011.02551.x

Oksanen, J. (2015). Vegan: an introduction to ordination. *Management* 1, 1–10. doi: 10.1016/b978-0-08-003237-5.50005-5

Okunishi, S., Sako, K., Mano, H., Imamura, A., and Morisaki, H. (2005). Bacterial flora of endophytes in the maturing seed of cultivated rice (*Oryza sativa*). *Microbes Environ.* 20, 168–177. doi: 10.1264/jsme2.20.168

Pankratov, T. A., Tindall, B. J., Liesack, W., and Dedysh, S. N. (2007). *Mucilaginibacter paludis* gen. nov., sp. nov. and *Mucilaginibacter gracilis* sp. nov., pectin-, xylan and laminarin-degrading members of the family *Sphingobacteriaceae* from acidic Sphagnum peat bog. *Int. J. Syst. Evol. Microbiol.* 57, 2349–2354. doi: 10.1099/ijs.0.65100-0

Park, Y. S., Dutta, S., Ann, M., Raaijmakers, J. M., and Park, K. (2015). Promotion of plant growth by *Pseudomonas fluorescens* strain SS101 via novel volatile organic compounds. *Biochem. Biophys. Res. Commun.* 461, 361–365. doi: 10.1016/j.bbrc.2015.04.039

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

Peng, S., Buresh, R. J., Huang, J., Yang, J., Zou, Y., Zhong, X., et al. (2006). Strategies for overcoming low agronomic nitrogen use efficiency in irrigated rice systems in China. *F. Crop. Res.* 96, 37–47. doi: 10.1016/j.fcr.2005.05.004

Quast, C., Pruesse, E., Yilmaz, P., Gerken, J., Schweer, T., Yarza, P., et al. (2012). The SILVA ribosomal RNA gene database project: improved data processing and web-based tools. *Nucleic Acids Res.* 41, D590–D596.

Raj, J., Bagyaraj, D. J., and Manjunath, A. (1981). Influence of soil inoculation with vesicular-arbuscular mycorrhiza and a phosphate-dissolving bacterium on plant growth and32P-uptake. *Soil Biol. Biochem.* 13, 105–108. doi: 10.1016/0038-0717(81)90004-3

Raweekul, W., Wuttitummaporn, S., Sodchuen, W., and Kittiwongwattana, C. (2016). Plant growth promotion by endophytic bacteria isolated from rice (*Oryza sativa*). *Sci. Technol. Asia* 21, 6–17.

Ren, D. W., Wang, E. T., Chen, W. F., Sui, X. H., Zhang, X. X., Liu, H. C., et al. (2011). *Rhizobium herbae* sp. nov. and *Rhizobium giardinii*-related bacteria, minor microsymbionts of various wild legumes in China. *Int. J. Syst. Evol. Microbiol.* 61, 1912–1920. doi: 10.1099/ijs.0.024943-0

Rensink, W. A., and Buell, C. R. (2004). Arabidopsis to rice. Applying knowledge from a weed to enhance our understanding of a crop species. *Plant Physiol.* 1, 622–629. doi: 10.1104/pp.104.040170.622

Richardson, A. E., Barea, 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

Rippon, J. W. (1982). *Medical Mycology; The Pathogenic Fungi and the Pathogenic Actinomycetes*, 2nd Edn. Eastbourne: W.B. Saunders Company.

Roberts, P. (1997). New Heterobasidiomycetes from Great Britain. *Mycotaxon* 63, 195–216.

Rodríguez, M. A., Cabrera, G., Gozzo, F. C., Eberlin, M. N., and Godeas, A. (2011). Clonostachys rosea BAFC3874 as a Sclerotinia sclerotiorum antagonist: mechanisms involved and potential as a biocontrol agent. *J. Appl. Microbiol.* 110, 1177–1186. doi: 10.1111/j.1365-2672.2011.04970.x

Rotem, J. (1994). *The Genus Alternaria: Biology, Epidemiology, and Pathogenicity*. St Paul, MN: American Phytopathological Society Press.

Rothenberg, S. E., Anders, M., Ajami, N. J., Petrosino, J. F., and Balogh, E. (2016). Water management impacts rice methylmercury and the soil microbiome. *Sci. Total Environ.* 572, 608–617. doi: 10.1016/j.scitotenv.2016.07.017

Schlaeppi, K., and Bulgarelli, D. (2015). The plant microbiome at work. *Mol. Plant-Microbe Interact.* 28, 212–217. doi: 10.1094/MPMI-10-14-0334-FI

Schoch, C. L., Crous, P. W., Groenewald, J. Z., Boehm, E. W. A., Burgess, T. I., de Gruyter, J., et al. (2009). A class-wide phylogenetic assessment of *Dothideomycetes*. *Stud. Mycol.* 64, 1–15. doi: 10.3114/sim.2009.64.01

Schwenk, A. J. (1984). Venn diagram for five sets. *Math. Mag.* 57:297. doi: 10.2307/2689606

Selvakumar, G., Kundu, S., Joshi, P., Nazim, S., Gupta, A. D., Mishra, P. K., et al. (2008). Characterization of a cold-tolerant plant growth-promoting bacterium *Pantoea dispersa* 1A isolated from a sub-alpine soil in the North Western Indian Himalayas. *World J. Microbiol. Biotechnol.* 24, 955–960. doi: 10.1007/s11274-007-9558-5

Sessitsch, A., Hardoim, P., Döring, J., Weilharter, A., Krause, A., Woyke, T., et al. (2012). Functional characteristics of an endophyte community colonizing rice roots as revealed by metagenomic analysis. *Mol. Plant-Microbe Interact.* 25, 28–36. doi: 10.1094/MPMI-08-11-0204

Shade, A., and Handelsman, J. (2012). Beyond the venn diagram: the hunt for a core microbiome. *Environ. Microbiol.* 14, 4–12. doi: 10.1111/j.1462-2920.2011.02585.x

Shade, A., Jacques, M.-A., and Barret, M. (2017). Ecological patterns of seed microbiome diversity, transmission, and assembly. *Curr. Opin. Microbiol.* 37, 15–22. doi: 10.1016/j.mib.2017.03.010

Shahzad, R., Khan, A. L., Bilal, S., Asaf, S., and Lee, I. (2018). What is there in seeds? Vertically transmitted endophytic resources for sustainable improvement in plant growth. *Front. Plant Sci.* 9:24. doi: 10.3389/fpls.2018.00024

Shiao, Y., and Huang, Y. (2001). Magnetorheological fluid damper. *Smart Mater. Bull.* 2001:14. doi: 10.1128/AEM.71.11.7292

Shimamoto, K., and Kyozuka, J. (2002). Rice as a model for comparative genomics of plants. *Annu. Rev. Plant Biol.* 53, 399–419.

Son, H. J., Park, G. T., Cha, M. S., and Heo, M. S. (2006). Solubilization of insoluble inorganic phosphates by a novel salt- and pH-tolerant *Pantoea agglomerans* R-42 isolated from soybean rhizosphere. *Bioresour. Technol.* 97, 204–210. doi: 10.1016/j.biortech.2005.02.021

Spiering, M. J., Greer, D. H., and Schmid, J. (2006). Effects of the fungal endophyte, Neotyphodium lolii, on net photosynthesis and growth rates of perennial ryegrass (*Lolium perenne*) are independent of in planta endophyte concentration. *Ann. Bot.* 98, 379–387. doi: 10.1093/aob/mcl108

Subhaswaraj, P., Jobina, R., Parasuraman, P., and Siddhardha, B. (2017). Plant growth promoting activity of pink pigmented facultative methylotroph – Methylobacterium extorquens MM2 on *Lycopersicon esculentum* L. *J. Appl. Biol. Biotechnol.* 5, 042–046. doi: 10.7324/JABB.2017.50107

Sukweenadhi, J., Kim, Y. J., Kang, C. H., Farh, M. E. A., Nguyen, N. L., Hoang, V. A., et al. (2015). *Sphingomonas panaciterrae* sp. nov. a plant growth-promoting bacterium isolated from soil of a ginseng field. *Arch. Microbiol.* 197, 973–981. doi: 10.1007/s00203-015-1134-z

Sullivan, R. F., and White, J. F. (2000). Phoma glomerata as a mycoparasite of powdery mildew. *Appl. Environ. Microbiol.* 66, 425–427. doi: 10.1128/AEM.66.1.425-427.2000

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

Toledo, A. V., Virla, E., Humber, R. A., Paradell, S. L., and Lastra, C. C. L. (2006). First record of *Clonostachys rosea* (Ascomycota: Hypocreales) as an entomopathogenic fungus of *Oncometopia tucumana* and *Sonesimia grossa* (Hemiptera: Cicadellidae) in Argentina. *J. Invertebr. Pathol.* 92, 7–10. doi: 10.1016/j.jip.2005.10.005

Torres-Cortés, G., Bonneau, S., Bouchez, O., Genthon, C., Briand, M., Jacques, M. A., et al. (2018). Functional microbial features driving community assembly during seed germination and emergence. *Front. Plant Sci.* 9:902. doi: 10.3389/fpls.2018.00902

Truyens, S., Weyens, N., Cuypers, A., and Vangronsveld, J. (2015). Bacterial seed endophytes: genera, vertical transmission and interaction with plants. *Environ. Microbiol. Rep.* 7, 40–50. doi: 10.1111/1758-2229.12181

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

Urai, M., Aizawa, T., Nakagawa, Y., Nakajima, M., and Sunairi, M. (2008). *Mucilaginibacter kameinonensis* sp., nov., isolated from garden soil. *Int. J. Syst. Evol. Microbiol.* 58, 2046–2050. doi: 10.1099/ijs.0.65777-0

Usyk, M., Zolnik, C. P., Patel, H., Levi, M. H., and Burk, R. D. (2017). Novel ITS1 fungal primers for characterization of the mycobiome. *mSphere* 2, 1–11. doi: 10.1128/mSphere.00488-17

Venkatachalam, S., Ranjan, K., Prasanna, R., Ramakrishnan, B., Thapa, S., and Kanchan, A. (2016). Diversity and functional traits of culturable microbiome members, including cyanobacteria in the rice phyllosphere. *Plant Biol. (Stuttg)* 18, 627–637. doi: 10.1111/plb.12441

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

Vukicevich, E., Lowery, T., Bowen, P., Úrbez-Torres, J. R., and Hart, M. (2016). Cover crops to increase soil microbial diversity and mitigate decline in perennial agriculture. A review. *Agron. Sustain. Dev.* 36:48. doi: 10.1007/s13593-016-0385-7

Walker, H. L. (1980). *Alternaria Macrospora as a Potential Biocontrol Agent for Spurred Anoda: Production of Spores for Field Studies*. Washington, D.C.: U.S. Dept. of Agriculture, 5.

Walker, H. L., and Sciumbato, G. L. (1979). Evaluation of alternaria macrospora as a potential biocontrol agent for spurred anoda (*Anoda cristata*): host range studies. *Weed Sci.* 27, 612–614. doi: 10.1017/s0043174500045987

Walsh, T. J., and Dixon, D. M. (1996). "Spectrum of mycoses", in *Medical Microbiology*, ed. S. Baron, (Galveston, TX: University of Texas Medical Branch at Galveston), 919–925.

Wang, Q., Garrity, G. M., Tiedje, J. M., and Cole, J. R. (2007). Naïve Bayesian classifier for rapid assignment of rRNA sequences into the new bacterial taxonomy. *Appl. Environ. Microbiol.* 73, 5261–5267. doi: 10.1128/aem.00062-07

Wang, W., Zhai, Y., Cao, L., Tan, H., and Zhang, R. (2016). Endophytic bacterial and fungal microbiota in sprouts, roots and stems of rice (*Oryza sativa* L.). *Microbiol. Res.* 188, 1–8. doi: 10.1016/j.micres.2016.04.009

Webster, G., Gough, C., Vasse, J., Batchelor, C. A., Callaghan, K. J. O., Kothari, S. L., et al. (1997). Interactions of rhizobia with rice and wheat. *Plant Soil* 194, 115–122. doi: 10.1007/978-94-011-5744-5_11

White, T. J., Bruns, T., Lee, S., and Taylor, J. (1990). AmpliFication and direct sequencing of fungal ribosomal RNA genes for phylogenetics. *PCR protoc. Guide Methods Appl.* 18, 315–322. doi: 10.1016/b978-0-12-372180-8.50042-1

Wickham, H. (2016). *Ggplot2: Elegant Graphics for Data Analysis*. Berlin: Springer.

Xue, A. G. (2003). Biological control of pathogens causing root rot complex in field pea using clonostachys rosea strain ACM941. *Phytopathology.* 93, 329–335. doi: 10.1094/phyto.2003.93.3.329

Yang, X., Strobel, G., Stierle, A., Hess, W. M., Lee, J., and Clardy, J. (1994). A fungal endophyte-tree relationship: *Phoma* sp. in Taxus wallachiana. *Plant Sci.* 102, 1–9. doi: 10.1016/0168-9452(94)90017-5

Yanni, Y. G., Rizk, R. Y., Corich, V., Squartini, A., Ninke, K., Orgambide, G., et al. (1997). Natural endophytic association between *Rhizobium leguminosarum* bv. trifolii and rice roots and assessment of its potential to promote rice growth. *Plant Soil* 194, 99–114. doi: 10.1007/978-94-011-5744-5_10

Zainudin, N. A. I., Razak, A. A., and Salleh, B. (2008). Bakanae disease of rice in malaysia and indonesia: etiology of the causal agent based on morphological, physiological and pathogenicity characteristics. *J. Plant Prot. Res.* 48, 475–485. doi: 10.2478/v10045-008-0056-z

Zehr, J. P., Jenkins, B. D., Short, S. M., and Steward, G. F. (2003). Nitrogenase gene diversity and microbial community structure: a cross-system comparison. *Environ. Microbiol.* 5, 539–554. doi: 10.1046/j.1462-2920.2003.00451.x

Zhang, J., Zhang, C., Yang, J., Zhang, R., Gao, J., Zhao, X., et al. (2019). Insights into endophytic bacterial community structures of seeds among various *Oryza sativa* L. rice genotypes. *J. Plant Growth Regul.* 38, 93–102. doi: 10.1007/s00344-018-9812-0

Zhang, L., Yang, J., Niu, Q., Zhao, X., Ye, F., Liang, L., et al. (2008). Investigation on the infection mechanism of the fungus *Clonostachys* rosea against nematodes using the green fluorescent protein. *Appl. Microbiol. Biotechnol.* 78, 983–990. doi: 10.1007/s00253-008-1392-7

Zhang, Q. (2007). Strategies for developing green super rice. *Proc. Natl. Acad. Sci. U.S.A.* 104, 16402–16409. doi: 10.1073/pnas.0708013104

Zhang, Y., Schoch, C. L., Fournier, J., Crous, P. W., de Gruyter, J., Woudenberg, J. H. C., et al. (2009). Multi-locus phylogeny of pleosporales: a taxonomic, ecological and evolutionary re-evaluation. *Stud. Mycol.* 64, 85–102. doi: 10.3114/sim.2009.64.04

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 Microbiology

ORIGINAL RESEARCH
published: 22 October 2020
doi: 10.3389/fmicb.2020.587667

Bacillus velezensis CLA178-Induced Systemic Resistance of *Rosa multiflora* Against Crown Gall Disease

*Lin Chen[1], Xinghong Wang[1], Qinghua Ma[1], Lusen Bian[1], Xue Liu[1], Yan Xu[1], Huihui Zhang[3], Jiahui Shao[3] and Yunpeng Liu[2]**

[1] Experimental Center of Forestry in North China, Chinese Academy of Forestry, Beijing, China, [2] Key Laboratory of Agricultural Microbial Resources Collection and Preservation, Ministry of Agriculture and Rural Affairs, Institute of Agricultural Resources and Regional Planning, Chinese Academy of Agricultural Sciences, Beijing, China, [3] Jiangsu Provincial Key Lab for Organic Solid Waste Utilization, National Engineering Research Center for Organic-based Fertilizers, Nanjing Agricultural University, Nanjing, China

OPEN ACCESS

Edited by:
Christos Zamioudis,
Democritus University of Thrace, Greece

Reviewed by:
Yong-Soon Park,
Chungbuk National University, South Korea
Adriana Fabra,
National University of Río Cuarto, Argentina

***Correspondence:**
Yunpeng Liu
liuyunpeng@caas.cn

Specialty section:
This article was submitted to Microbe and Virus Interactions with Plants, a section of the journal Frontiers in Microbiology

Received: 27 July 2020
Accepted: 28 September 2020
Published: 22 October 2020

Citation:
Chen L, Wang X, Ma Q, Bian L, Liu X, Xu Y, Zhang H, Shao J and Liu Y (2020) Bacillus velezensis CLA178-Induced Systemic Resistance of Rosa multiflora Against Crown Gall Disease. Front. Microbiol. 11:587667. doi: 10.3389/fmicb.2020.587667

Plant growth-promoting rhizobacteria (PGPRs) are able to activate induced systemic resistance (ISR) of the plants against phytopathogens. However, whether and how ISR can be activated by PGPRs in plants of the *Rosa* genus is unclear. The effects of PGPR *Bacillus velezensis* CLA178 and the pathogen *Agrobacterium tumefaciens* C58 on the growth, plant defense-related genes, hormones, and reactive oxygen species (ROS) in the rose plants were compared. Pretreatment with CLA178 significantly reduced crown gall tumor biomass and relieved the negative effects of the C58 pathogen on plant biomass, chlorophyll content, and photosynthesis of roses. Pretreatment of the roots with CLA178 activated ISR and significantly reduced disease severity. Pretreatment with CLA178 enhanced plant defense response to C58, including the accumulation of ROS, antioxidants, and plant hormones. Moreover, pretreatment with CLA178 enhanced C58-dependent induction of the expression of the genes related to the salicylic acid (SA) or ethylene (ET) signaling pathways. This result suggested that SA- and ET-signaling may participate in CLA178-mediated ISR in roses. Additional experiments in the Arabidopsis mutants showed that CLA178 triggered ISR against C58 in the *pad4* and *jar1* mutants and not in the *etr1* and *npr1* mutants. The ISR phenotypes of the Arabidopsis mutants indicated that CLA178-mediated ISR is dependent on the ET-signaling pathway in an NPR1-dependent manner. Overall, this study provides useful information to expand the application of PGPRs to protect the plants of the *Rosa* genus from phytopathogens.

Keywords: induced systemic resistance, plant growth-promoting rhizobacteria, rose, crown gall disease, hormone

INTRODUCTION

The *Rosa* genus consists of woody plants that are grown worldwide due to their importance in horticulture, cosmetics, and medicine (Hassanein, 2010; Nadeem et al., 2015). This genus includes approximately 200 species and 20,000 cultivars. Roses are typical ornamental plants and have been developed as garden plants or for the cut rose market. Rose hips are used in food and medical

applications; rose flowers are also cultivated for use in food and rose oil production (Byrne, 2009). However, most rose species are susceptible to crown gall disease caused by pathogenic *Agrobacterium* strains, such as *Agrobacterium tumefaciens* (other names: *Agrobacterium fabrum* or *Rhizobium radiobacter*; Martí et al., 1999; Gan and Savka, 2018; Diel et al., 2019). Rose plants infected by *A. tumefaciens* develop crown galls on the basal portions of their stems and roots leading to reduced plant growth. Crown gall disease impairs nutrient uptake, growth, and production. Severe disease can cause death of the plants and serious economic losses (López-López et al., 1999).

Plant diseases can be reduced by several methods, such as application of chemical agents, transgenic approaches, and biological control by the beneficial bacteria (Waard et al., 1993; Dong et al., 2007; Liu et al., 2017). The biological control method involving beneficial rhizobacteria is advantageous for protection of the plants from pathogen attack due to environmental safety. Plant growth-promoting rhizobacteria (PGPRs) benefit plants by improving nutrient uptake, promoting plant growth, antagonizing soilborne pathogens, and enhancing plant resistance (Durán et al., 2018; Stringlis et al., 2018b; Pascale et al., 2020). Biocontrol using PGPR strains has been studied in detail in agricultural crops, such as cucumber, maize, wheat, soybean, lettuce, and barley; however, the application of PGPRs in woody plants has not been well developed, and available information on the subject is considerably lacking (Pieterse et al., 2014; Berendsen et al., 2018).

PGPR can prime the plant immune system for rapid response to a broad range of pathogens without direct contact with the pathogens (Glazebrook, 2005; Yi et al., 2013; Stringlis et al., 2018a). This type of resistance is called induced systemic resistance (ISR). Induction of ISR is an efficient means of biocontrol by PGPRs. ISR is long-lasting and continuously protects the plants (Pieterse et al., 2014).

The mechanism of the onset of ISR triggered by PGPR is incompletely understood; however, several stimulators have been proposed, such as flagellin, lipopolysaccharides (LPS), volatile organic compounds (VOCs), and siderophores (Romera et al., 2019). In plants, jasmonic acid (JA)/ethylene (ET) signaling pathways are important for the activation of ISR by PGPRs (Glazebrook et al., 2003; Pieterse et al., 2014). However, in some cases, salicylic acid (SA) signaling pathway is also involved in ISR. For example, ISR in Arabidopsis triggered by *B. cereus* AR156 requires JA/ET and SA signaling pathways (Niu et al., 2011). Most of the studies on ISR were performed in Arabidopsis or crops. However, the signaling pathways involved in ISR may differ between various plant species and microbes (Romera et al., 2019).

Bacillus species are the most widely used PGPR strains for promotion of plant growth and protection of the plants against biotic and abiotic stresses due to their stress tolerance (Nicholson, 2002; Borriss, 2011). *Bacillus velezensis* CLA178 is a beneficial bacterium isolated from the rhizosphere soil of *Rosa multiflora* that can negatively influence the C58 pathogen infection in plants. In this study, CLA178 was shown to activate ISR against crown gall disease in rose. Physiological analysis and evaluation of the expression of the genes related to plant defense in rose were performed in addition to comparison of ISR phenotypes of various Arabidopsis mutants. These results provided insight into the induction process.

MATERIALS AND METHODS

Isolation and Identification of *B. velezensis* CLA178

Bacillus velezensis CLA178 was isolated from the rhizosphere soil of *Rosa multiflora*. Its morphological characteristics were observed on Luria-Bertani (LB) medium (5 g l^{-1} yeast extract, 10 g l^{-1} tryptone, 10 g l^{-1} NaCl; pH 7.0–7.2) agar plates. The 16S rRNA gene of CLA178 was amplified from the CLA178 genome with the universal primers 27F and 1492R. The 16S rRNA gene sequence and genome sequence of the CLA178 strain were submitted to the NCBI GenBank.

The housekeeping gene *rpoB* of CLA178 was compared with the sequences available in the NCBI GenBank. Multiple alignments were performed by CLUSTAL_X software. The phylogenetic trees were constructed with the MEGA 7 software.

Genome Sequencing and Genotypic Characterization

The complete genome sequencing of the CLA178 strain was performed by combining and Illumina MiSeq system and the PacBio RSII high-throughput sequencing technology at Shanghai Personal Biotechnology Co., Ltd., China. The raw data were filtered and trimmed by AdapterRemoval (ver. 2.1.7) and SOAPec (ver. 2.0) (Luo et al., 2012; Schubert et al., 2016). The reads of Illumina MiSeq system were assembled using A5-miseq (ver. 20160825) and SPAdes genome assembler (ver. 3.11.1) with default parameters (Bankevich et al., 2012; Tritt et al., 2012). The reads of PacBio RSII were assembled into contigs using HGAP4 and CANU (Chin et al., 2016; Koren et al., 2017). The contigs obtained by Illumina MiSeq system and PacBio RSII were analyzed collinearly using MUMmer (Delcher et al., 1999). The quality of genome assembly was improved by the Pilon software (Walker et al., 2014).

The relatedness of the genome sequence of CLA178 to the complete genome sequences of related strains was determined based on the average nucleotide identities (ANI). Genome sequences in a pairwise comparison were split into 1,000 bp windows and aligned with nucmer in MUMmer v3.23 (ANIm) (Kurtz et al., 2004). ANI were calculated using JSpecies v1.2.1 (Meier-Kolthoff et al., 2014).

Plant and Growth Conditions

Rosa multiflora 'Innermis' stems were surface-sterilized with 75% (vol:vol) ethanol and then with 2% (vol:vol) NaClO. The surface-sterilized stems were cut into segments and grown in sterile vermiculite with rooting powder or in 1/4 MS media containing 3% sucrose, 0.6% agar, 0.5 mg l^{-1} 6-benzylaminopurine (6-BA), and 0.2 mg l^{-1} naphthaleneacetic acid. *Rosa multiflora* was cultivated at 25°C with a 14 h/10 h light/dark photoperiod.

Seeds of *Arabidopsis thaliana* ecotype Columbia (Col-0) and the *pad4* (Glazebrook et al., 1996), *jar1* (Staswick et al., 1992), *etr1*

(Bleecker et al., 1988), and *npr1* (Cao et al., 1997) mutants were sown in sterile vermiculite. Thirty-day-old seedlings were used for the experiments. *Arabidopsis thaliana* (Arabidopsis) plants were cultivated in a growth chamber at 25°C with a photoperiod of 14 h of light and 10 h of darkness.

Strain Cultivation and Inoculation

Bacillus velezensis CLA178 and *Agrobacterium tumefaciens* C58 (other names: *Agrobacterium fabrum*, *Rhizobium radiobacter*; ATCC 33970^T = ACCC 10055^T; Martí et al., 1999) were cultured at 30°C with shaking at 0.65 *g* (170 rpm, radium = 2 cm) for 10–12 h in LB medium. The cultures were then centrifuged and resuspended in sterile phosphate buffer (PBS, pH 7.0).

To measure the crown gall tumor of the plants, the seedlings were inoculated with PBS or *B. velezensis* CLA178 at a final density of 5×10^6 CFU ml^{-1} medium. On the second day, the stem was infected with the pathogen *A. tumefaciens* C58 at a density of 10^9 CFU ml^{-1} using a sterile needle (Song et al., 2015). Sterile PBS was used as a negative control. The rose and Arabidopsis seedlings were cultivated at 25°C with a 14 h/10 h light/dark photoperiod for 20 days and 14 days, respectively. The ratio of gall diameter/stem diameter (GD/SD), disease incidence, and disease index were calculated based on the analysis of 30 roses per site (five cuttings times 6 replicates per treatment) or 36 Arabidopsis plants per site (6 seedlings times 6 replicates per treatment). The disease index of rose crown gall disease was determined based on the following revised classification of Krastanova et al. (2010): 0 no galls; 1: small galls, $0 < GD/SD < 0.25$; 2: medium galls, $0.25 < GD/SD < 0.75$; 3: large galls, $0.75 < GD/SD < 1.25$; 4: very large galls, $GD/SD > 1.25$. The tumor size of Arabidopsis was determined based on the following disease index: 0: no galls; 1: small galls, $0 < GD/SD < 1$; 2: medium galls, $1 < GD/SD < 1.5$; 3: large galls, $1.5 < GD/SD < 2$; 4: very large galls, $GD/SD > 2$.

Measurement of the Photosynthetic Rate and Chlorophyll

The photosynthetic rate was determined with a portable photosynthesis measurement system (Li-Cor-6400; Li-Cor Inc.). The chlorophyll content of fully expanded leaves was calculated with a chlorophyll meter (SPAD-502 Minolta). These measurements were calculated based on the analysis of six biological replicates.

Measurement of Phytohormones, Reactive Oxygen Species (ROS) and Antioxidants

Surface-sterilized rose seedlings were cultured in a sterile triangular flask containing 1/4 MS medium, and 11-week-old seedlings were treated with CLA178 at a final density of 5×10^6 CFUs ml^{-1} for one day. After infection with C58 for 6, 24, and 48 h, fresh plant stems (0.1 g) were collected and homogenized with 1 ml of PBS (pH 7.0). The homogenate was shaken at 4°C for 1 h and centrifuged. The supernatant was used to measure the content of SA, JA, ET, or ROS with an ELISA kit (Meimian Biotechnology Co., Ltd., Wu et al., 2018; Lin et al., 2020). The catalase and peroxidase activity were determined by the method reported by Chen et al. (2016). These measurements were analyzed based on six independent experiments.

Transcription Analysis

The plant samples were flash-frozen in liquid nitrogen, and the RNA was extracted with a Qiagen RNeasy plant mini kit. The concentration and quality of the RNA were measured with a NanoDrop ND-2000 spectrophotometer. The transcript levels were determined by reverse transcription-polymerase chain reaction using a PrimeScript RT reagent kit (Takara Biotechnology Co.). Quantitative real-time polymerase chain reaction (qRT-PCR) was performed with TB Green™ Premix EX Taq™ (Takara) using an ABI Quantstudio™ 3D digital PCR system (Life Technologies).

The transcription levels were measured using *RmACT* (*ACTIN*) as an internal reference. The following genes were assayed: *RmERF1* (*ETHYLENE-RESPONSIVE TRANSCRIPTION FACTOR 1*), *RmNPR1* (*NON-EXPRESSER OF PATHOGENESIS-RELATED GENES 1*), *RmAOS* (*ALLENE OXIDE SYNTHASE*), *RmMYC2* (encoding the transcription factor MYC2), and *RmPR1-4* (*PATHOGENESIS-RELATED PROTEIN 1-4*) with *RmPR2* encoding β-1,3-glucanase, *RmPR3* encoding basic chitinase, and *RmPR4* encoding a hevein-like protein. The primers for qRT-PCR are listed in **Supplementary Table S1**. The amino acid sequences of the selected genes from *Rosa multiflora* were aligned with the homologous genes from *Arabidopsis thaliana* (**Supplementary Figure S1**). The similarity of amino acid sequences of the selected genes from *R. multiflora* with the homologous genes from *A. thaliana* was analyzed (**Supplementary Table S2**). The specificity of the amplification was verified by melting-curve analysis and agarose gel electrophoresis. Relative transcription levels were calculated using the $2^{-\Delta\Delta CT}$ method based on three biological replicates (Livak and Schmittgen, 2001).

Statistical Analysis

Differences between the treatments were statistically analyzed using analysis of variance (ANOVA) and Duncan's multiple range tests ($P < 0.05$). SPSS version 25.0 was used for statistical analysis (SPSS Inc.).

RESULTS

Identification of *B. velezensis* CLA178

The strain CLA178 with antagonistic activities was isolated form the rhizosphere of healthy plants (*Rosa multiflora*) cultivated in the Fangshan (Beijing, China) nursery in soils that are known to be highly contaminated by pathogenic *A. tumefaciens*. The cells of the CLA178 strain were rod-shaped, motile, and Gram-positive and had the ability to form spores. The colonies on the LB agar were wrinkled. The CLA178 strain was able to grow in LB with 10% NaCl. The 16S rRNA and whole genome sequences of CLA178 were obtained and deposited in GenBank under the accession numbers MT071299 and CP061087, respectively. The circular chromosome map of CLA178 was presented in

Figure 1A. Analyses of the GenBank and EzBioCloud databases revealed that the 16S rRNA gene sequence of CLA178 is closely related to *Bacillus* species. The phylogenetic analysis of the *rpoB* gene indicated that CLA178 belongs to *Bacillus velezensis* (**Figure 1B**). Additionally, the relatedness of the genome sequence of CLA178 to the genome sequence of related *Bacillus* species was determined based on ANI. The ANIm values of CLA178 to the type strain *B. velezensis* CBMB205 was 98.22%. Based on comparative analysis of the ANI values and phylogenetic analysis of *Bacillus* species, CLA178 was classified as *B. velezensis* (Kim et al., 2014; Miller et al., 2016; Fan et al., 2017; Rabbee et al., 2019).

B. velezensis CLA178 Enhances Plant Biomass Under Crown Gall Disease Stress

To determine whether CLA178 is a PGPR, the impact of *B. velezensis* CLA178 on plant growth was measured. *B. velezensis* CLA178 enhanced leaf area and root biomass of rose plants indicating that CLA178 is the plant growth-promoting strain (**Figures 2A–C**). To assess the effect of *B. velezensis* CLA178 on growth of rose under crown gall disease stress, rose plants were preinoculated with CLA178 for one day in the rhizosphere before infection with *A. tumefaciens* C58. The indexes of plant growth and physiology were evaluated. The negative effect of infection of the stem by C58 on rose plants was evaluated at 30 days post inoculation (dpi). The results showed that the fresh root weight and leaf area of rose were significantly decreased after inoculation with *A. tumefaciens* C58 (**Figures 2A–C**). However, preinoculation with CLA178 before infection of the plant with C58 significantly reduced the negative effect of C58 on root biomass and leaf area (**Figures 2A–C**). Preinoculation with CLA178 also restored a reduction in chlorophyll and photosynthesis caused by C58 in rose (**Figures 2D,E**). These results indicate that CLA178 can suppress the negative effect of C58 on rose.

B. velezensis CLA178 Induces Plant Resistance to *A. tumefaciens* C58 Infection

To investigate whether *B. velezensis* CLA178 influences the interaction between rose and the pathogen to suppress the negative effect of C58 on the plants, the crown gall tumors of rose plants caused by C58 were evaluated. At 20 dpi with the *A. tumefaciens* C58 pathogen on the stem, rose plants pretreated with sterile PBS showed typical symptoms of crown gall tumors (**Figure 3A**). Preinoculation of CLA178 before the plant was infected with C58 resulted in a significant reduction in the GD/SD ratio relative to that in the C58 infection without preinoculation (0.25 vs. 0.99; **Figure 3B**). After plants were infected with C58, the disease incidence and disease index of the rose plants preinoculated with CLA178 were significantly lower than those in plants without preinoculation (disease incidence of 23.3 vs. 80% and disease index of 7.5 vs. 57.5%; **Figures 3C,D**). The biocontrol efficacy of CLA178 was 87% (**Figure 3E**). These results indicated that *B. velezensis* CLA178 can induce systemic resistance of rose against crown gall disease independently of direct contact with the pathogen.

Reactive Oxygen Species Content and Antioxidant Activity Induced by the Strains

To analyze the impact of preinoculation of CLA178 on rose resistance to the C58 pathogen, certain physiological indexes were determined. The accumulation of ROS is an important signal involved in the plant immune response (Rojas et al., 2014).

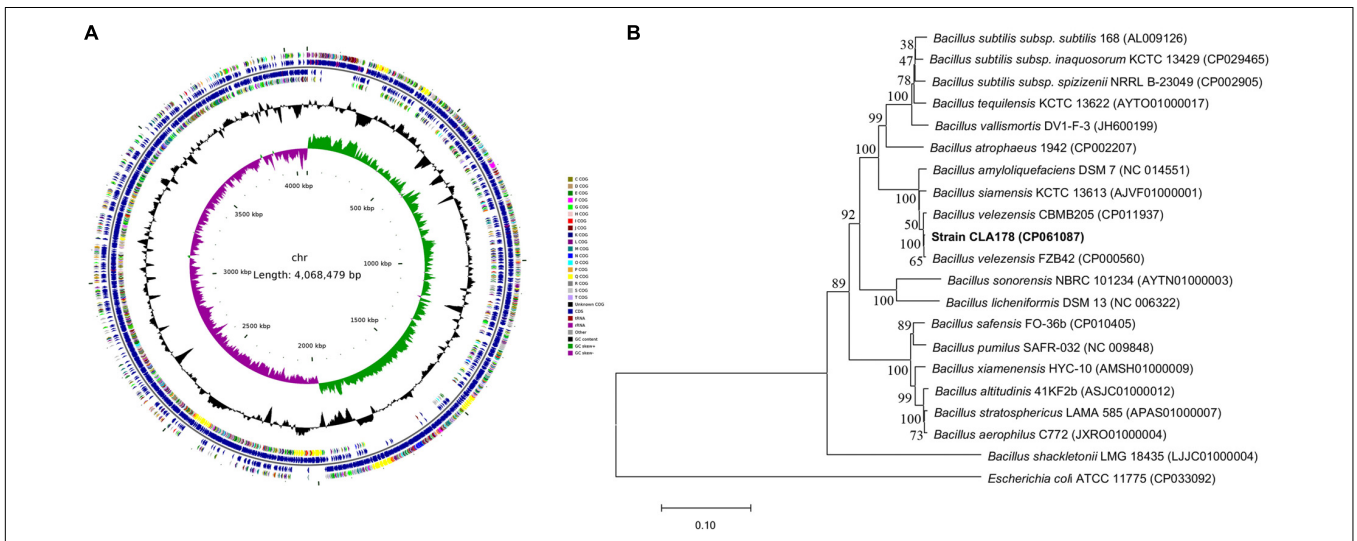

FIGURE 1 | Genomic and phylogenetic structure characteristics of *Bacillus velezensis* CLA178. **(A)** Circular chromosome map of CLA178 was generated by CGView Server. **(B)** Neighbor-joining phylogenetic tree based on *rpoB* gene sequences showed the relationships between strain CLA178 and related taxa. Bootstrap values were determined based on 1000 replications. Bar, 0.1 substitutions per nucleotide position.

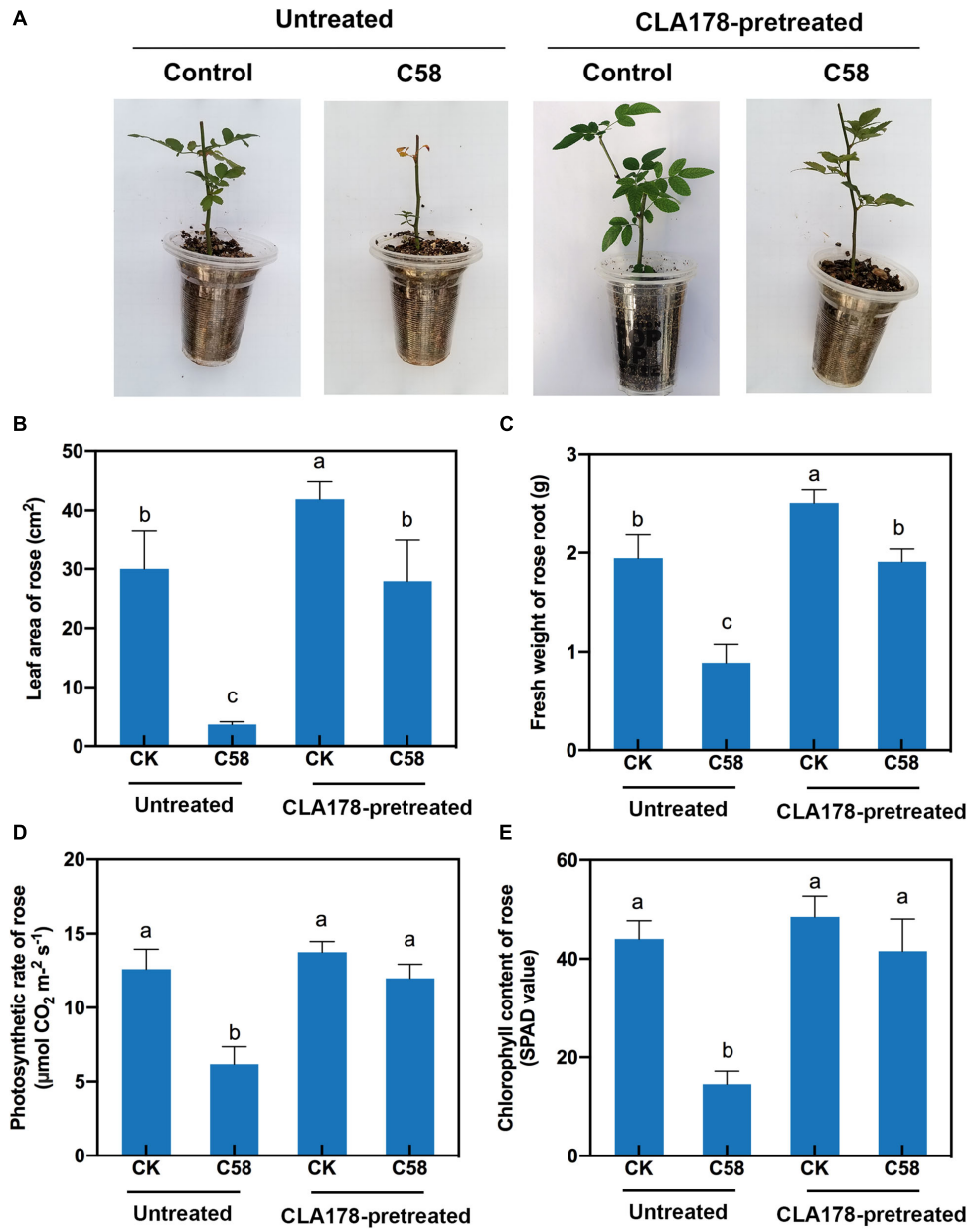

FIGURE 2 | Effect of *Bacillus velezensis* CLA178 on biomass of rose under crown gall disease stress. **(A)** Representative image of rose inoculated with the strains for 30 days. The leaf area **(B)**, root fresh weight **(C)**, photosynthetic rate **(D)**, and chlorophyll content **(E)** of rose were determined. The rose plants were infected with C58 one day after inoculation with CLA178. The untreated and CLA178-pretreated rose plants treated with PBS (control) or infected with the C58 pathogen infection are shown. The values are the mean ± SD. Different letters above the bars indicate significant differences between the treatments (Duncan's least significant difference, $P < 0.05$, $n = 6$).

Pretreatment with CLA178 enhanced the C58-induced ROS accumulation at 6, 24 and 48 h after C58 infection. Thus, pretreatment with CLA178 may enhance plant defense response when the plant was challenged with C58. Additionally, treatment with CLA178 without infection did not induce continuous ROS accumulation in the plant (**Figure 4A**).

Antioxidants are responsible for scavenging excessive ROS, and their activity always corresponds to the ROS content. The results of the assays of antioxidants were similar to the data obtained by the ROS accumulation assay. *Agrobacterium* infection alone induced only a slight increase in CAT activity at 48 h post infection. However, pretreatment with CLA178 significantly enhanced CAT activity at 6, 24 and 48 h after C58 infection (**Figure 4B**). Moreover, in plants pretreated with CLA178, the CAT activity was significantly increased upon C58 infection at 48 hpi (**Figure 4B**). The activity of

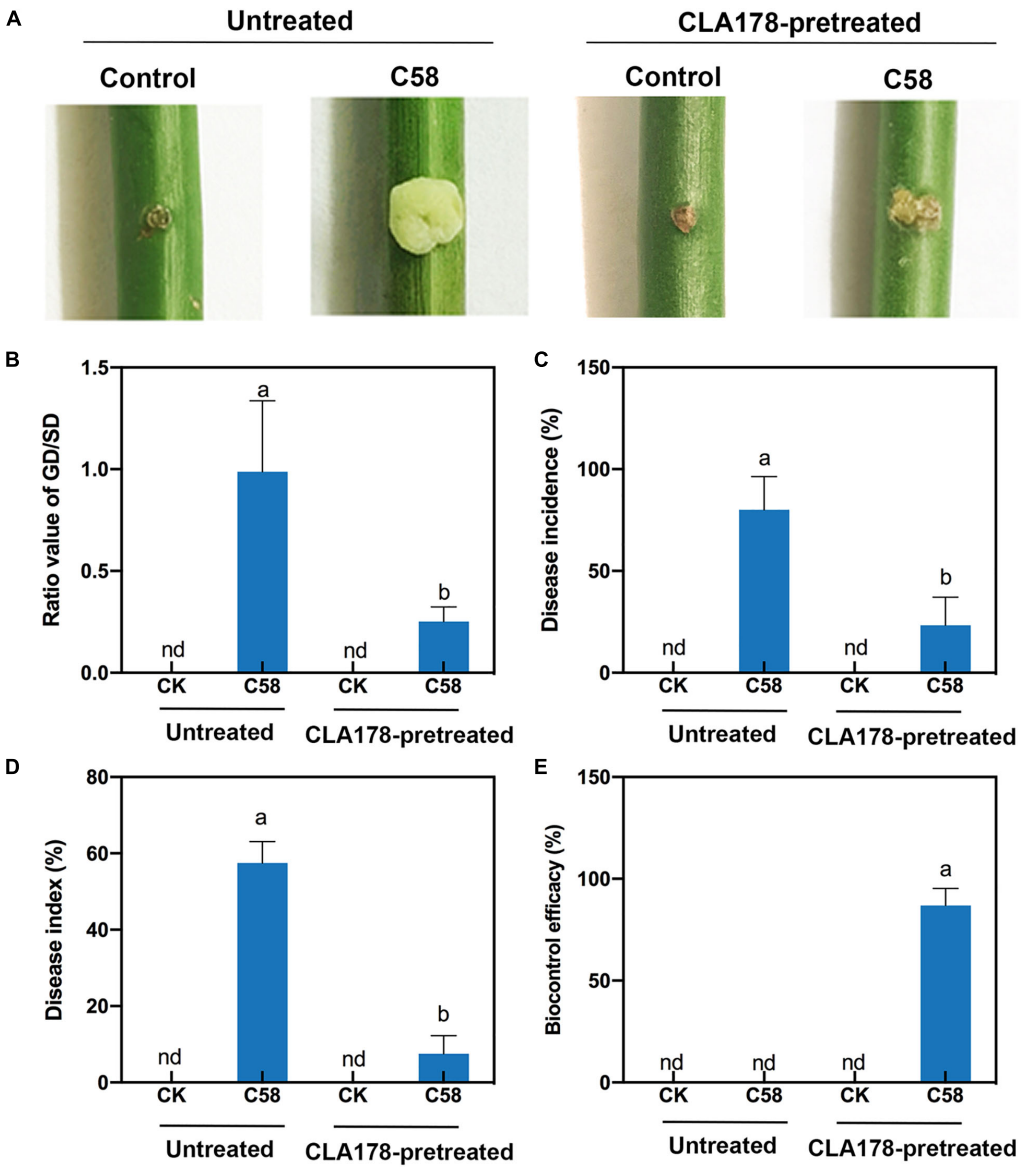

FIGURE 3 | Effect of *Bacillus velezensis* CLA178 on crown gall tumor caused by *Agrobacterium tumefaciens* C58 in rose. **(A)** Representative image of rose inoculated with strains for 20 days. The GD/SD **(B)**, disease incidence **(C)**, disease index **(D)**, and biocontrol efficacy **(E)** in various treatments were measured in 30 plants. The rose plants were infected with C58 one day after inoculation with CLA178. The untreated and CLA178-pretreated rose plants with or without the C58 infection are shown. The values are the mean ± SD. nd, not detected. The same capital letter indicates the same index. Different lowercase letters of each index indicate statistically significant differences between the treatments (Duncan's least significant difference, $P < 0.05$, $n = 30$).

POD was also determined, and no significant differences were observed between various treatments (**Figure 4C**). Overall, our results indicate that CLA178-primed rose plants have enhanced defense response to C58, including ROS accumulation and increased CAT activity.

Phytohormones Induced by *B. velezensis* CLA178

The phytohormones SA, JA, and ET are involved in the defense responses and play important roles in the plant-microbe interactions. To investigate whether these phytohormones are involved in the defense response induced by CLA178, the levels of SA, JA and ET in the plants were measured. The contents of JA and SA in the CLA178-pretreatment group were significantly higher compared with those in the untreated group regardless of C58 infection; however, in CLA178-pretreated and untreated plants, C58 did not strongly influence the contents of JA and SA in the plants (**Figures 5A,B**). Infection with C58 increased the ET content at 6 h and 24 h after the infection in CLA178-pretreated and untreated plants. However, at 48 h after the infection, C58 induced ET accumulation only in CLA178-pretreated plants and not in the untreated plants (**Figure 5C**). This result indicates that

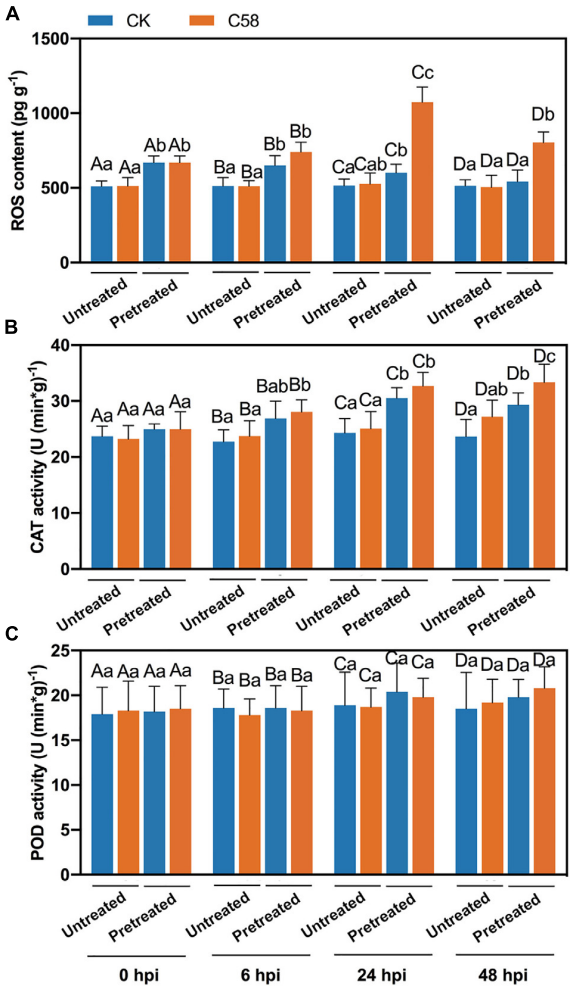

FIGURE 4 | ROS content and CAT and POD activities in rose inoculated with CLA178, C58 or their combination. The plant tissues were harvested at 6, 24, and 48 hpi after *Agrobacterium* infection. ROS content **(A)** and CAT **(B)** and POD activities **(C)** in the plants were determined. "Untreated" and "Pretreated" indicate plants pretreated with PBS and CLA178, respectively. Sterile PBS was used as a control. The values are the mean ± SD. Capital letters indicate the grouping for statistical analysis. Different lowercase letters indicate statistically significant differences between the treatments (Duncan's least significant difference, $P < 0.05$, $n = 6$).

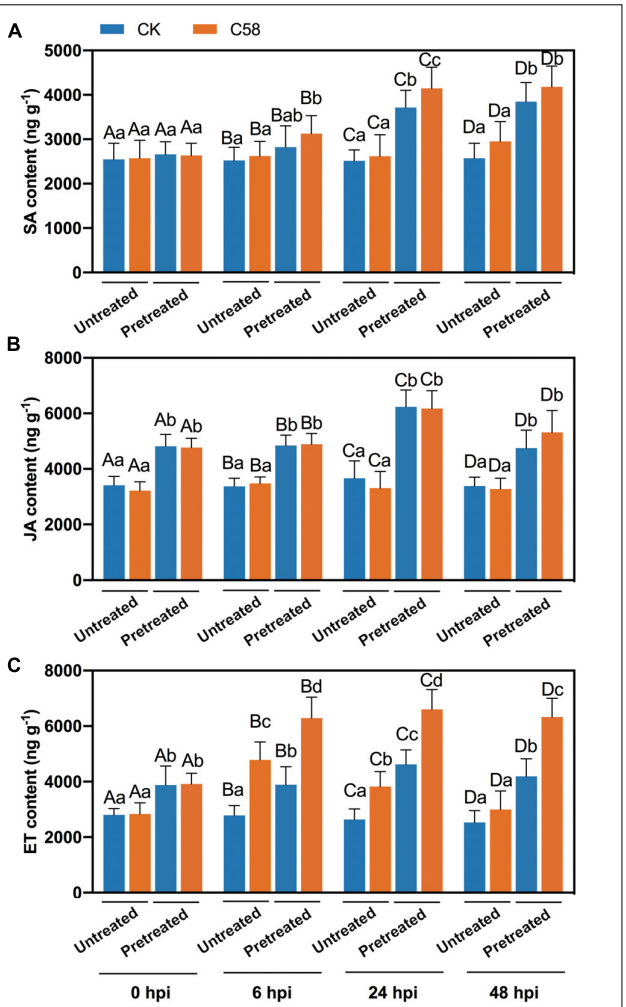

FIGURE 5 | Phytohormone contents in rose. The plant tissues were harvested at 6, 24, and 48 hpi with C58. Salicylic acid **(A)**, jasmonic acid **(B)**, and ethylene contents **(C)** in rose were determined. Sterile PBS was used as a control. The values are the mean ± SD. Capital letters indicate the grouping for statistical analysis. Different lowercase letters indicate statistically significant differences between the plants subjected to various treatments (Duncan's least significant difference, $P < 0.05$, $n = 6$).

ET may play an important role in CLA178-induced plant defense against pathogenic *Agrobacterium*.

Defense-Related Genes Rby *B. velezensis* CLA178

The SA, JA and ET-signaling pathways are important for ISR in the plants. To identify the pathway(s) regulated by CLA178, which may be responsible for CLA178-induced ISR, the transcription of plant genes involved in the SA-, ET- and JA-signaling pathways was measured using qRT-PCR. The descriptions of these genes are provided in **Supplementary Table S1**. *RmPR1* and *RmPR2* are involved in the SA-related pathway. *RmAOS* and *RmMYC2* are involved in the JA-related pathway. *RmERF1* and *RmPR4* are involved in the ET-related pathway. *RmPR3* is involved in the ET- and JA-related pathways. In plants infected with C58, the transcription of these genes was significantly upregulated by CLA178 pretreatment at 6, 24 and 48 hpi (**Figures 6A–C**). In untreated plants, the levels of upregulated genes induced by *Agrobacterium* infection at 6 hpi were higher than those at 24 and 48 hpi. In CLA178-pretreated plants, the transcription of the genes involved in the SA- and ET-related pathways was continuously upregulated at 6, 24, and 48 h post C58 infection; however, the transcription of *RmAOS* and *RmMYC2* involved in the JA-signaling pathway was not induced in plants infected with C58 at 48 hpi (**Figures 6A–C**). These data suggest that the induction of the genes of the SA- and ET-signaling pathways is involved in the CLA178-induced systemic resistance against C58.

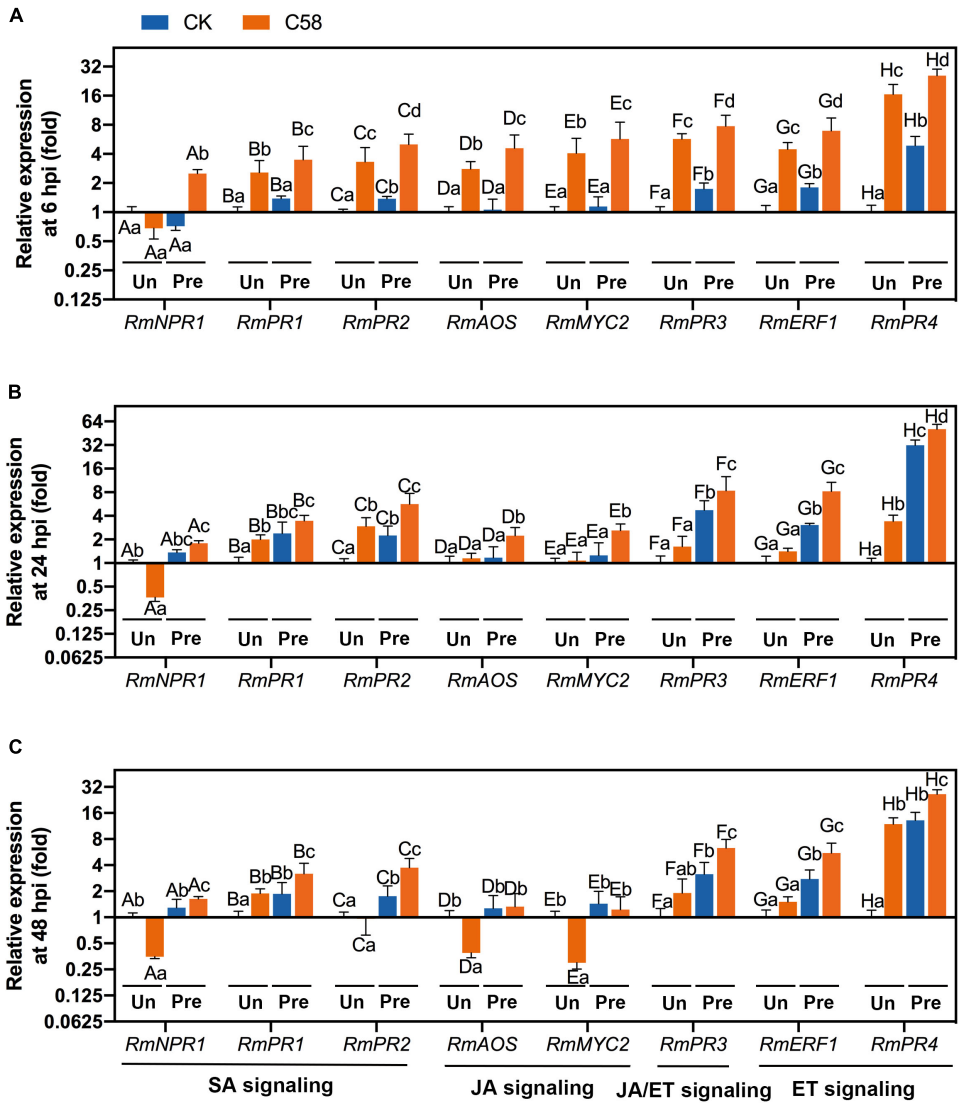

FIGURE 6 | Expression of defense-related genes in rose. At 6 **(A)**, 24 **(B)**, and 48 hpi **(C)** with C58, plant samples were harvested for extraction of RNA. The values indicate the fold-change of the expression levels of each gene in the plants with inoculation relative to control detected by qRT-PCR. All genes were normalized using *ACTIN* as a reference. Expression levels of salicylic acid-related genes and ethylene- or jasmonic acid-related genes were determined in untreated and CLA178-pretreated rose plants treated with PBS or infected with the C58 pathogen. Control plants were treated with sterile PBS. "Un" and "Pre" indicate untreated and CLA178-pretreated plants, respectively. The data are shown as the mean ± SD ($n = 3$). Capital letters indicate the grouping for statistical analysis. Different lowercase letters indicate statistically significant differences between the plants subjected to various treatments (Duncan's least significant difference, $P < 0.05$).

Signaling Pathway Dependence of *B. velezensis* CLA178-Mediated ISR

Then, we assessed whether blocking the signaling pathways disrupts the induction of ISR by CLA178. The resistance of wide-type Arabidopsis Col-0 (WT) and the defense-signaling mutants *pad4* (SA biosynthesis defective mutant phytoalexin deficient 4), *jar1* (JA response mutant), *etr1* (ET response mutant), and *npr1* (non-expresser of PR genes mutant) against C58 infection after induction by CLA178 was compared to confirm our findings. Preinoculation with CLA178 led to a significant reduction in the ratio of GD/SD, disease incidence, and disease index in WT, *pad4*, and *jar1*, but caused no significant reduction in these parameters in the *etr1* and *npr1* mutants at 14 dpi (**Figure 7**). The biocontrol efficacy of CLA178 against crown gall disease in WT and the *jar1* mutant was higher than that in *pad4* (**Figure 7E**). These results indicate that the ET-signaling pathway and NPR1 are necessary for the CLA178-induced ISR in the plants.

DISCUSSION

Crown gall disease is destructive to the production of many plant species of the *Rosaceae* family, such as cherry, peach, and pear trees (Gupta et al., 2010). PGPRs have been reported

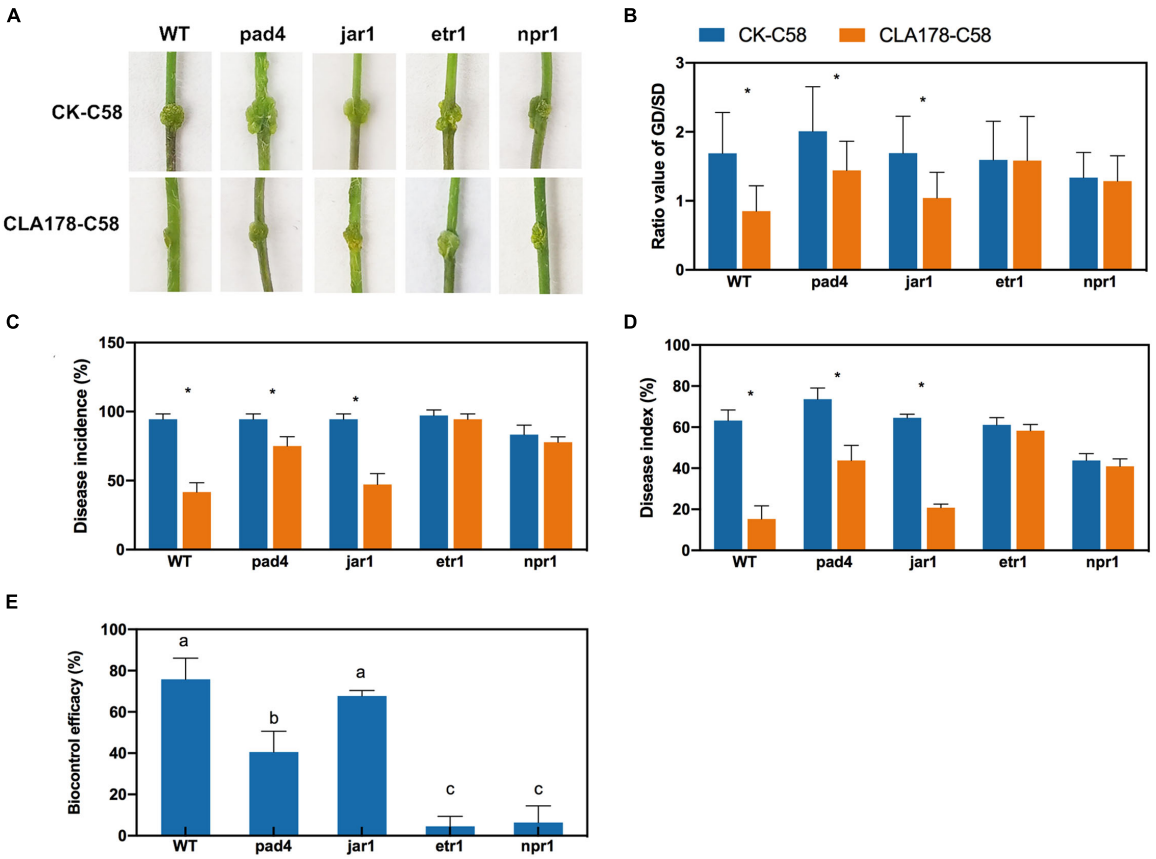

FIGURE 7 | Protection induced by CLA178 against crown gall disease in wide-type and defense-related mutants (*pad4*, *jar1*, *etr1*, and *npr1*) in Arabidopsis. The 30-day-old seedlings were infected with C58 one day after inoculation with CLA178. **(A)** Symptom were observed 14 days after the C58 infection. **(B)** The GD/SD was measured. Disease incidence **(C)**, disease index **(D)**, and biocontrol efficacy **(E)** were calculated. Sterile PBS was used as a control. Asterisks indicate significant differences between the CLA178-treated samples and control according to Student's *t*-test ($P < 0.05$, $n = 36$).

to protect woody plants from pathogen infection by direct antagonism; however, indirect protection of woody plant based on the induction of systemic resistance is poorly understood (Baltruschat et al., 2008; Compant et al., 2013). In this study, preinoculation with *B. velezensis* CLA178 induced rose resistance against the crown gall disease pathogen C58. Pretreatment with CLA178 enhanced an increase in ROS, SA, and ET contents upon C58 infection. The genes of the SA- or ET-signaling pathways were continuously induced by CLA178 pretreatment in rose plants after *Agrobacterium* infection. We hypothesized that CLA178 primes the rose plants for enhanced defense response to pathogenic *A. tumefaciens C58*, and the SA- and ET-signaling pathways may be involved in CLA178-induced ISR in rose. Subsequent experiments showed that CLA178 failed to induce the resistance against C58 in the *npr1* and *etr1* Arabidopsis mutants indicating that the ET-signaling pathway and NPR1 are necessary for CLA178-induced ISR against crown gall disease in Arabidopsis. This study may contribute to the biocontrol of crown gall disease in these plants.

PGPRs can promote rose growth (El-Deeb et al., 2012; Tariq et al., 2016); however, their biocontrol effect has not been evaluated in detail. *Rosa multiflora*, a typical species of the *Rosa* genus with high ornamental and economic value, often suffers from crown gall disease. Crown gall disease in other plants can be suppressed by *Agrobacterium rhizogenes* K84, *Agrobacterium vitis* VAR03-1, *Agrobacterium vitis* E26, *Rahnella aquatilis* HX2, etc., (Wang et al., 2003; Kawaguchi et al., 2008; Guo et al., 2009; Compant et al., 2013). However, most known biocontrol strains used to suppress crown galls are close relatives of the pathogenic "*Agrobacterium*" strains; thus, it is possible that non-pathogenic *Agrobacterium* biocontrol strains acquire virulence plasmids or produce them via a mutation (Mauck et al., 2010). This study is the first to demonstrate that *B. velezensis* CLA178 significantly reduces incidence of crown gall disease in rose by inducing ISR. Moreover, *B. velezensis* is non-pathogenic and environmentally safe to use than other closely related *Agrobacterium* species.

Plant immunity can be triggered by certain beneficial or pathogenic microbes. Oxidative burst is an early event that is always accompanied by MAMP-triggered immunity (MTI) or PAMP-triggered immunity (PTI) (Zamioudis and Pieterse, 2012). However, ROS accumulation in the plants was not increased by C58 in agreement with the data of some previous studies (Lee et al., 2009). In addition to a slight increase in CAT activity observed in plants at 48 hpi, CAT produced by C58 plays

an important role in scavenging ROS produced by the plants in the early stage (Xu and Pan, 2000). The transcription of certain defense-related genes in the plants treated with C58 was minimized 24 hpi. The expression of these genes was suggested to be inhibited by T-DNA or vir proteins (Veena et al., 2003).

To investigate the molecular mechanisms of CLA178 induction of plant resistance to *A. tumefaciens* C58, the transcription of the genes involved in the SA-, JA-, and ET-signaling pathways was determined in rose, and the infection was assayed using related Arabidopsis mutants. The results indicate that the genes involved in the SA- or ET-signaling pathway were continuously induced by *Agrobacterium* in rose plants pretreated with CLA178; however, genes involved in the JA-signaling pathway were not induced. The investigation of gene transcription suggested that the SA- and ET- signaling pathways may be involved in ISR activated by *B. velezensis* CLA178 against crown gall disease in rose. The results obtained using various ISR phenotypes of the defense signaling mutants of Arabidopsis suggest that CLA178-induced ISR against crown gall disease in Arabidopsis is dependent on the ET-signaling pathway in an NPR1-dependent manner. PAD4 plays an important role in the SA-signaling pathway (Tsuda et al., 2008; Dempsey et al., 2011). CLA178 induces weaker ISR in the *pad4* mutant (**Figure 7E**). This result suggests that the SA-signaling pathway may be involved in CLA178-induced ISR.

Phytohormones can influence crown gall disease (Gohlke and Deeken, 2014). The SA content in rose was enhanced by PGPR CLA178 regardless of C58 infection; an increase in SA can repress the conjugal transfer of the Ti plasmid to reduce the virulence of C58 and modulate rhizosphere colonization by specific bacterial families to strengthen the plant immune system (Yuan et al., 2007; Lebeis et al., 2015). The accumulation of ET was observed in rose after CLA178 pretreatment or C58 infection, and ET accumulation was more intense in plants inoculated with a combination of CLA178 and C58. Upon the initiation of infection, ET in combination with indole acetic acid (IAA) is essential for growth of the tumors; however, ET suppresses the *vir* gene expression during the transformation (Lee et al., 2009; Gohlke and Deeken, 2014). The defense-related genes involved in the JA-signaling pathway were significantly influenced by infection with C58; however, the JA content of the rose plants was not significantly increased by C58 (Lee et al., 2009; Gohlke and Deeken, 2014; Song et al., 2015). Some studies demonstrated that the expression of the genes related to the hormone signaling and biosynthesis can be different, and the hormone signaling pathways can be activated by low levels of the hormones (Lee et al., 2009; Pieterse et al., 2014; Song et al., 2015; Wu et al., 2018). The JA content in rose was increased by CLA178 pretreatment; however, the expression of the genes involved in the JA-signaling pathway was not continuously induced by CLA178 in rose plants. Moreover, the *jar1* Arabidopsis mutant was still able to acquire CLA178-induced resistance. These results indicate that JA is not essential for ISR activated by CLA178. Moreover, the JA and ET contents in Arabidopsis leaves were not altered by PGPR *Pseudomonas fluorescens* WCS417r. WCS417r-mediated ISR in Arabidopsis depends on sensitivity to JA and ET (Pieterse et al., 2000). However, in this study, PGPR CLA178 enhanced the levels of these phytohormones in rose. Phytohormone accumulation was also observed in Arabidopsis treated with PGPR *B. amyloliquefaciens* SQR9 (Wu et al., 2018). The difference in the results may be due to different microbial and plant species.

In conclusion, *B. velezensis* CLA178 can suppress the negative effect of C58 on rose and induce systemic resistance against crown gall disease in Arabidopsis via the ET-signaling pathway in an NPR1-dependent manner. This study suggests that application of *B. velezensis* PGPR strains can be used to induce resistance against crown gall disease in woody plants in agroforestry production.

DATA AVAILABILITY STATEMENT

The datasets presented in this study can be found in online repositories. The names of the repository/repositories and accession number(s) can be found below: https://www.ncbi.nlm.nih.gov/, MT071299, https://www.ncbi.nlm.nih.gov/genbank/, CP061087.

AUTHOR CONTRIBUTIONS

LC, YL, and XW conceived and designed this research. LC, YX, LB, HZ, and XL conducted experiments. QM and JS analyzed data. LC and YL wrote the manuscript. All authors read and approved the manuscript.

FUNDING

This work was supported by the Fundamental Research Funds for CAF (CAFYBB2017MA020), National Natural Science Foundation of China (31700548 and 31601826), and the Fundamental Research Funds for the Central Universities (KJQN201744).

ACKNOWLEDGMENTS

We thank Profs. Zhang R.F. (Chinese Academy of Agricultural Sciences, Beijing; Nanjing Agricultural University, Nanjing) for kingly providing us with the seeds of Arabidopsis mutants.

SUPPLEMENTARY MATERIAL

The Supplementary Material for this article can be found online at: https://www.frontiersin.org/articles/10.3389/fmicb.2020.587667/full#supplementary-material

Supplementary Figure 1 | Alignment of amino acid sequences of selected genes from *Rosa* multiflora with the homologous genes from *Arabidopsis thaliana*.

Supplementary Table 1 | Dna primers used in this study.

Supplementary Table 2 | The similarity of amino acid sequences of selected genes from *Rosa multiflora* with the homologous genes from *Arabidopsis thaliana*.

REFERENCES

Baltruschat, H., Fodor, J., Harrach, B. D., Niemczyk, E., Barna, B., Gullner, G., et al. (2008). Salt tolerance of barley induced by the root endophyte *Piriformospora indica is associated with a strong increase in antioxidants*. *New Phytol.* 180, 501–510. doi: 10.1111/j.1469-8137.2008.02583.x

Bankevich, A., Nurk, S., Antipov, D., Gurevich, A. A., Dvorkin, M., Kulikov, A. S., et al. (2012). SPAdes: a new genome assembly algorithm and its applications to single-cell sequencing. *J. Comput. Biol.* 19, 455–477. doi: 10.1089/cmb.2012.0021

Berendsen, R. L., Vismans, G., Yu, K., Song, Y., de Jonge, R., Burgman, W. P., et al. (2018). Disease-induced assemblage of a plant-beneficial bacterial consortium. *ISME J.* 12, 1496–1507. doi: 10.1038/s41396-018-0093-1

Bleecker, A., Estelle, M., Somerville, C., and Kende, H. (1988). Insensitivity to ethylene conferred by a dominant mutation in *Arabidopsis thaliana*. *Science* 241, 1086–1089. doi: 10.1126/science.241.4869.1086

Borriss, R. (2011). *Use of Plant-Associated Bacillus Strains as Biofertilizers and Biocontrol Agents in Agriculture. Bact. Agrobiol. Plant Growth Responses*. Berlin: Springer Berlin Heidelberg, 41–76. doi: 10.1007/978-3-642-20332-9_3

Byrne, D. H. (2009). *Rose Structural Genomics. Genetic Genomics Rosaceae*. New York, NY: Springer, 353–379. doi: 10.1007/978-0-387-77491-6_17

Cao, H., Glazebrook, J., Clarke, J. D., Volko, S., and Dong, X. (1997). The *Arabidopsis* NPR1 gene that controls systemic acquired resistance encodes a novel protein containing ankyrin repeats. *Cell* 88, 57–63. doi: 10.1016/S0092-8674(00)81858-9

Chen, L., Liu, Y., Wu, G., Veronican Njeri, K., Shen, Q., 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

Chin, C. S., Peluso, P., Sedlazeck, F. J., Nattestad, M., Concepcion, G. T., Clum, A., et al. (2016). Phased diploid genome assembly with single-molecule real-time sequencing. *Nat. Methods.* 13, 1050–1054. doi: 10.1038/nmeth.4035

Compant, S., Brader, G., Muzammil, S., Sessitsch, A., Lebrihi, A., and Mathieu, F. (2013). Use of beneficial bacteria and their secondary metabolites to control grapevine pathogen diseases. *BioControl* 58, 1–21. doi: 10.1007/s10526-012-9479-6

Delcher, A. L., Kasif, S., Fleischmann, R. D., Peterson, J., White, O., and Salzberg, S. L. (1999). Alignment of whole genomes. *Nucleic Acids Res.* 27, 2369–2376. doi: 10.1093/nar/27.11.2369

Dempsey, D. A., Vlot, A. C., Wildermuth, M. C., and Klessig, D. F. (2011). Salicylic acid biosynthesis and metabolism. *Arab. B* 9:e0156. doi: 10.1199/tab.0156

Diel, B., Dequivre, M., Wisniewski-Dyé, F., Vial, L., and Hommais, F. (2019). A novel plasmid-transcribed regulatory sRNA, QfsR, controls chromosomal polycistronic gene expression in *Agrobacterium fabrum*. *Environ. Microbiol.* 21: 3063–3075. doi: 10.1111/1462-2920.14704

Dong, S., Tredway, L. P., Shew, H. D., Wang, G. L., Sivamani, E., and Qu, R. (2007). Resistance of transgenic tall fescue to two major fungal diseases. *Plant Sci.* 173, 501–509. doi: 10.1016/j.plantsci.2007.08.002

Durán, P., Thiergart, T., Garrido-Oter, R., Agler, M., Kemen, E., Schulze-Lefert, P., et al. (2018). Microbial interkingdom interactions in roots promote *Arabidopsis survival*. *Cell* 175, 973.e-983.e. doi: 10.1016/j.cell.2018.10.020

El-Deeb, B., Bazaid, S., Gherbawy, Y., and Elhariry, H. (2012). Characterization of endophytic bacteria associated with rose plant (*Rosa damascena trigintipeta*) during flowering stage and their plant growth promoting traits. *J. Plant Interact.* 7, 248–253. doi: 10.1080/17429145.2011.637161

Fan, B., Blom, J., Klenk, H. P., and Borriss, R. (2017). Bacillus amyloliquefaciens, *Bacillus velezensis*, and *Bacillus siamensis* form an "Operational Group *B. amyloliquefaciens*" within the *B. subtilis* species complex. *Front. Microbiol.* 8:22. doi: 10.3389/fmicb.2017.00022

Gan, H. M., and Savka, M. A. (2018). One more decade of *Agrobacterium* taxonomy. *Curr. Top. Microbiol. Immunol.* 418, 1–14. doi: 10.1007/82_2018_81

Glazebrook, J. (2005). Contrasting mechanisms of defense against biotrophic and necrotrophic pathogens. *Annu. Rev. Phytopathol.* 43, 205–227. doi: 10.1146/annurev.phyto.43.040204.135923

Glazebrook, J., Chen, W., Estes, B., Chang, H. S., Nawrath, C., Métraux, J. P., et al. (2003). Topology of the network integrating salicylate and jasmonate signal transduction derived from global expression phenotyping. *Plant J.* 34, 217–228. doi: 10.1046/j.1365-313X.2003.01717.x

Glazebrook, J., Rogers, E. E., and Ausubel, F. M. (1996). Isolation of *Arabidopsis* mutants with enhanced disease susceptibility by direct screening. *Genetics* 143, 973–982. doi: 10.1046/j.1365-2443.1996.d01-265.x

Gohlke, J., and Deeken, R. (2014). Plant responses to *Agrobacterium tumefaciens* and crown gall development. *Front. Plant Sci.* 5:155. doi: 10.3389/fpls.2014.00155

Guo, Y. B., Li, J., Li, L., Chen, F., Wu, W., Wang, J., et al. (2009). Mutations that disrupt either the pqq or the gdh gene of *Rahnella aquatilis* abolish the production of an antibacterial substance and result in reduced biological control of grapevine grown gall. *Appl. Environ. Microbiol.* 75, 6792–6803. doi: 10.1128/AEM.00902-09

Gupta, A. K., Khosla, K., Bhardwaj, S. S., Thakur, A., Devi, S., Jarial, R. S., et al. (2010). Biological control of crown gall on peach and cherry rootstock colt by native *Agrobacterium radiobacter* isolates. *Open Hortic. J.* 3, 1–10. doi: 10.2174/1874840601003010001

Hassanein, A. M. A. (2010). Improved quality and quantity of winter flowering in rose (*Rosa* spp.) by controlling the timing and type of pruning applied in autumn. *World J. Agric. Sci.* 6, 260–267.

Kawaguchi, A., Inoue, K., and Ichinose, Y. (2008). Biological control of crown gall on grapevine and root colonization by nonpathogenic *Agrobacterium vitis* Strain VAR03-1. *Phytopathology* 98, 1218–1225. doi: 10.1094/PHYTO-98-11-1218

Kim, M., Oh, H. S., Park, S. C., and Chun, J. (2014). Towards a taxonomic coherence between average nucleotide identity and 16S rRNA gene sequence similarity for species demarcation of prokaryotes. *Int. J. Syst. Evol. Microbiol.* 64, 346–351. doi: 10.1099/ijs.0.059774-0

Koren, S., Walenz, B. P., Berlin, K., Miller, J. R., Bergman, N. H., and Phillippy, A. M. (2017). Canu: scalable and accurate long-read assembly via adaptive κ-mer weighting and repeat separation. *Genome Res.* 27, 722–736. doi: 10.1101/gr.215087.116

Krastanova, S. V., Balaji, V., Holden, M. R., Sekiya, M., Xue, B., Momol, E. A., et al. (2010). Resistance to crown gall disease in transgenic grapevine rootstocks containing truncated virE2 of *Agrobacterium*. *Transgenic Res.* 19, 949–958. doi: 10.1007/s11248-010-9373-x

Kurtz, S., Phillippy, A., Delcher, A. L., Smoot, M., Shumway, M., Antonescu, C., et al. (2004). Versatile and open software for comparing large genomes. *Genome Biol.* 5:R12. doi: 10.1186/gb-2004-5-2-r12

Lebeis, S. L., Paredes, S. H., Lundberg, D. S., Breakfield, N., Gehring, J., McDonald, M., et al. (2015). Salicylic acid modulates colonization of the root microbiome by specific bacterial taxa. *Science* 349, 860–864. doi: 10.1126/science.aaa8764

Lee, C. -W., Efetova, M., Engelmann, J. C., Kramell, R., Wasternack, C., Ludwig-Muller, J., et al. (2009). Agrobacterium tumefaciens promotes tumor induction by modulating pathogen defense in *Arabidopsis thaliana*. *Plant Cell Online* 21, 2948–2962. doi: 10.1105/tpc.108.064576

Lin, F., Sun, J., Liu, N., and Zhu, L. (2020). Phytotoxicity and metabolic responses induced by tetrachlorobiphenyl and its hydroxylated and methoxylated derivatives in rice (*Oryza sative* L.). *Environ. Int.* 139, 105695. doi: 10.1016/j.envint.2020.105695

Liu, Y., Chen, L., Wu, G., Feng, H., Zhang, G., Shen, Q., et al. (2017). Identification of root-secreted compounds involved in the communication between cucumber, the beneficial *Bacillus amyloliquefaciens*, and the soil-borne pathogen *Fusarium oxysporum*. *Mol. Plant-Microbe Interact.* 30, 53–62. doi: 10.1094/MPMI-07-16-0131-R

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

López-López, M. J., Vicedo, B., Orellana, N., Piquer, J., and López, M. M. (1999). Behavior of a virulent strain derived from *Agrobacterium radiobacter* strain K84 after spontaneous Ti plasmid acquisition. *Phytopathology* 89, 286–292. doi: 10.1094/PHYTO.1999.89.4.286

Luo, R., Liu, B., Xie, Y., Li, Z., Huang, W., Yuan, J., et al. (2012). SOAPdenovo2: an empirically improved memory-efficient short-read de novo assembler. *Gigascience* 1, 18. doi: 10.1186/2047-217X-1-18

Martí, R., Cubero, J., Daza, A., Piquer, J., Salcedo, C. I., Morente, C., et al. (1999). Evidence of migration and endophytic presence of *Agrobacterium tumefaciens* in rose plants. *Eur. J. Plant Pathol.* 105, 39–50. doi: 10.1023/A:1008660500107

Mauck, K. E., De Moraes, C. M., and Mescher, M. C. (2010). Deceptive chemical signals induced by a plant virus attract insect vectors to inferior hosts. *Proc. Natl. Acad. Sci. U.S.A.* 107, 3600–3605. doi: 10.1073/pnas.090719110

Meier-Kolthoff, J. P., Klenk, H. P., and Göker, M. (2014). Taxonomic use of DNA G+C content and DNA-DNA hybridization in the genomic age. *Int. J. Syst. Evol. Microbiol.* 64, 352–356. doi: 10.1099/ijs.0.056994-0

Miller, R. A., Beno, S. M., Kent, D. J., Carroll, L. M., Martin, N. H., Boor, K. J., et al. (2016). *Bacillus wiedmannii* sp. nov., a psychrotolerant and cytotoxic *Bacillus cereus* group species isolated from dairy foods and dairy environments. *Int. J. Syst. Evol. Microbiol.* 66, 4744–4753. doi: 10.1099/ijsem.0.001421

Nadeem, M., Younis, A., Riaz, A., and Lim, K. B. (2015). Crossability among modern roses and heterosis of quantitative and qualitative traits in hybrids. *Hortic. Environ. Biotechnol.* 56, 487–497. doi: 10.1007/s13580-015-0144-8

Nicholson, W. L. (2002). Roles of *Bacillus endospores* in the environment. *Cell. Mol. Life Sci.* 59, 410–416. doi: 10.1007/s00018-002-8433-7

Niu, D. D., Liu, H. X., Jiang, C. H., Wang, Y. P., Wang, Q. Y., Jin, H. L., et al. (2011). The plant growth-promoting rhizobacterium *Bacillus cereus* AR156 induces systemic resistance in *Arabidopsis thaliana* by simultaneously activating salicylate- and jasmonate/ethylene-dependent signaling pathways. *Mol. Plant Microbe Interact.* 24, 533–542. doi: 10.1094/MPMI-09-10-0213

Pascale, A., Proietti, S., Pantelides, I. S., and Stringlis, I. A. (2020). Modulation of the root microbiome by plant molecules: The basis for targeted disease suppression and plant growth promotion. *Front. Plant Sci.* 10:1741. doi: 10.3389/fpls.2019.01741

Pieterse, C. M. J., Van Pelt, J. A., Ton, J., Parchmann, S., Mueller, M. J., Buchala, A. J., et al. (2000). Rhizobacteria-mediated induced systemic resistance (ISR) in *Arabidopsis* requires sensitivity to jasmonate and ethylene but is not accompanied by an increase in their production. *Physiol. Mol. Plant Pathol.* 57, 123–134. doi: 10.1006/pmpp.2000.0291

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

Rabbee, M. F., Sarafat Ali, M., Choi, J., Hwang, B. S., Jeong, S. C., and Baek, K. H. (2019). Bacillus velezensis: a valuable member of bioactive molecules within plant microbiomes. *Molecules* 24, 1–13. doi: 10.3390/molecules24061046

Rojas, C. M., Senthil-Kumar, M., Tzin, V., and Mysore, K. S. (2014). Regulation of primary plant metabolism during plant-pathogen interactions and its contribution to plant defense. *Front. Plant Sci.* 5:17. doi: 10.3389/fpls.2014.00017

Romera, F. J., García, M. J., Lucena, C., Martínez-Medina, A., Aparicio, M. A., Ramos, J., et al. (2019). Induced systemic resistance (ISR) and fe deficiency responses in dicot plants. *Front. Plant Sci.* 10:287. doi: 10.3389/fpls.2019.00287

Schubert, M., Lindgreen, S., and Orlando, L. (2016). AdapterRemoval v2: rapid adapter trimming, identification, and read merging. *BMC Res.* 9:88. doi: 10.1186/s13104-016-1900-2

Song, G. C., Lee, S., Hong, J., Choi, H. K., Hong, G. H., Bae, D. W., et al. (2015). Aboveground insect infestation attenuates belowground *Agrobacterium*-mediated genetic transformation. *New Phytol.* 207, 148–158. doi: 10.1111/nph.13324

Staswick, P. E., Su, W., and Howell, S. H. (1992). Methyl jasmonate inhibition of root growth and induction of a leaf protein are decreased in an *Arabidopsis thaliana* mutant. *Proc. Natl. Acad. Sci. U.S.A.* 89, 6837–6840. doi: 10.1073/pnas.89.15.6837

Stringlis, I. A., Yu, K., Feussner, K., De Jonge, R., Van Bentum, S., Van Verk, M. C., et al. (2018a). MYB72-dependent coumarin exudation shapes root microbiome assembly to promote plant health. *Proc. Natl. Acad. Sci. U.S.A.* 115, E5213–E5222. doi: 10.1073/pnas.1722335115

Stringlis, I. A., Zhang, H., Pieterse, C. M. J., Bolton, M. D., and de Jonge, R. (2018b). Microbial small molecules – weapons of plant subversion. *Nat. Prod. Rep.* 35, 410–433. doi: 10.1039/C7NP00062F

Tariq, U., Riaz, A., Jaskani, M. J., and Zahir, Z. A. (2016). Screening of PGPR isolates for plant growth promotion of Rosa damascena. *Int. J. Agric. Biol.* 18, 997–1003. doi: 10.17957/IJAB/15.0200

Tritt, A., Eisen, J. A., Facciotti, M. T., and Darling, A. E. (2012). An integrated pipeline for de novo assembly of microbial genomes. *PLoS One* 7:e42304. doi: 10.1371/journal.pone.0042304

Tsuda, K., Sato, M., Glazebrook, J., Cohen, J. D., and Katagiri, F. (2008). Interplay between MAMP-triggered and SA-mediated defense responses. *Plant J.* 53, 763–775. doi: 10.1111/j.1365-313X.2007.03369.x

Veena, Jiang, H., Doerge, R. W., and Gelvin, S. B. (2003). Transfer of T-DNA and Vir proteins to plant cells by Agrobacterium tumefaciens induces expression of host genes involved in mediating transformation and suppresses host defense gene expression. *Plant J.* 35, 219–236. doi: 10.1046/j.1365-313X.2003.01796.x

Waard, M. A., Georgopoulos, S. G., Hollomon, D. W., Ishii, H., Leroux, P., Ragsdale, N. N., et al. (1993). Chemical control of plant diseases: problems and prospects. *Annu. Rev. Phytopathol.* 31, 403–421. doi: 10.1146/annurev.py.31.090193.002155

Walker, B. J., Abeel, T., Shea, T., Priest, M., Abouelliel, A., Sakthikumar, S., et al. (2014). Pilon: An integrated tool for comprehensive microbial variant detection and genome assembly improvement. *PLoS One* 9:e112963. doi: 10.1371/journal.pone.0112963

Wang, H. M., Wang, H. X., Ng, T. B., and Li, J. Y. (2003). Purification and characterization of an antibacterial compound produced by *Agrobacterium vitis* strain E26 with activity against *A. tumefaciens*. *Plant Pathol.* 52, 134–139. doi: 10.1046/j.1365-3059.2003.00807.x

Wu, G., Liu, Y., Xu, Y., Zhang, G., Shen, Q., and Zhang, R. (2018). Exploring elicitors of the beneficial rhizobacterium *Bacillus amyloliquefaciens* SQR9 to induce plant systemic resistance and their interactions with plant signaling pathways. *Mol. Plant-Microbe Interact.* 31, 560–567. doi: 10.1094/MPMI-11-17-0273-R

Xu, X. Q., and Pan, S. Q. (2000). An *Agrobacterium catalase* is a virulence factor involved in tumorigenesis. *Mol. Microbiol.* 35, 407–414. doi: 10.1046/j.1365-2958.2000.01709.x

Yi, H. -S., Yang, J. W., and Ryu, C. -M. (2013). ISR meets SAR outside: additive action of the endophyte *Bacillus pumilus* INR7 and the chemical inducer, benzothiadiazole, on induced resistance against bacterial spot in field-grown pepper. *Front. Plant Sci.* 4:122. doi: 10.3389/fpls.2013.00122

Yuan, Z. C., Edlind, M. P., Liu, P., Saenkham, P., Banta, L. M., Wise, A. A., et al. (2007). The plant signal salicylic acid shuts down expression of the vir regulon and activates quormone-quenching genes in *Agrobacterium*. *Proc. Natl. Acad. Sci. U.S.A.* 104, 11790–11795. doi: 10.1073/pnas.0704866104

Zamioudis, C., and Pieterse, C. M. J. (2012). Modulation of host immunity by beneficial microbes. *Mol. Plant-Microbe Interact.* 25, 139–150. doi: 10.1094/MPMI-06-11-0179

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

MINI REVIEW
published: 09 November 2020
doi: 10.3389/fpls.2020.589416

The Role of Secretion Systems, Effectors, and Secondary Metabolites of Beneficial Rhizobacteria in Interactions With Plants and Microbes

*Miriam Lucke†, Mario Gabriel Correa† and Asaf Levy**

Department of Plant Pathology and Microbiology, The Robert H. Smith Faculty of Agriculture, Food and Environment, The Hebrew University of Jerusalem, Rehovot, Israel

OPEN ACCESS

Edited by:
Christos Zamioudis, Democritus University of Thrace, Greece

Reviewed by:
Mika Tapio Tarkka, Helmholtz Centre for Environmental Research (UFZ), Germany
Elena A. Dolgikh, All-Russian Research Institute of Agricultural Microbiology of the Russian Academy of Agricultural Sciences, Russia

***Correspondence:**
Asaf Levy
alevy@mail.huji.ac.il

†These authors have contributed equally to this work

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

Received: *30 July 2020*
Accepted: *14 October 2020*
Published: *09 November 2020*

Citation:
Lucke M, Correa MG and Levy A (2020) The Role of Secretion Systems, Effectors, and Secondary Metabolites of Beneficial Rhizobacteria in Interactions With Plants and Microbes. Front. Plant Sci. 11:589416. doi: 10.3389/fpls.2020.589416

Beneficial rhizobacteria dwell in plant roots and promote plant growth, development, and resistance to various stress types. In recent years there have been large-scale efforts to culture root-associated bacteria and sequence their genomes to uncover novel beneficial microbes. However, only a few strains of rhizobacteria from the large pool of soil microbes have been studied at the molecular level. This review focuses on the molecular basis underlying the phenotypes of three beneficial microbe groups; (1) plant-growth promoting rhizobacteria (PGPR), (2) root nodulating bacteria (RNB), and (3) biocontrol agents (BCAs). We focus on bacterial proteins and secondary metabolites that mediate known phenotypes within and around plants, and the mechanisms used to secrete these. We highlight the necessity for a better understanding of bacterial genes responsible for beneficial plant traits, which can be used for targeted gene-centered and molecule-centered discovery and deployment of novel beneficial rhizobacteria.

Keywords: beneficial bacteria, plant growth promoting bacteria, biocontrol agents, root nodulating bacteria, rhizosphere, effectors, secretion systems

INTRODUCTION

The term rhizosphere was first defined by Hiltner, who described it as the soil compartment influenced by the root (Hiltner, 1904). The rhizosphere differs from the surrounding bulk soil and the plant endophytic compartment in microbial diversity (Hacquard et al., 2015) and its members influence the release of root exudates. Root exudates are responsible for shaping the microbial community structure, including attraction of beneficial microbes (Clark, 1949; Zhalnina et al., 2018; Korenblum et al., 2020). After successfully colonizing plant roots, beneficial microbes secrete proteins and secondary metabolites, relevant for nutrient acquisition, improved plant fitness, and inhibition of pathogen colonization (Pieterse et al., 2014; Bakker et al., 2018; Yu et al., 2019a). Beneficial microbes are subdivided in a coarse manner into plant growth promoting rhizobacteria (PGPR), biocontrol agents (BCAs), and root-nodulating bacteria (RNB; Berendsen et al., 2012). PGPR directly or indirectly induce plant growth *via* secretion of secondary metabolites, which are in turn involved in plant hormone synthesis and nutrient acquisition from soil (Lugtenberg and Kamilova, 2009). RNB are also referred to as biofertilizers. They interact with legume roots as mutualists. Nodules allow the energetically expensive process of nitrogen fixation.

The ammonia produced in the nodules as part of this process is transported into the plant cells in exchange for carbon required for bacterial growth. BCAs or biopesticides in the roots act by eliminating phytopathogens and pests, either indirectly by induction of the plant immune response through induced or acquired systemic resistance, or directly by producing and releasing antimicrobial and pesticidal toxins or by physical niche occupation (Kuc and Tuzun, 1992; Van Wees et al., 1997; Zamioudis and Pieterse, 2012; Bernal et al., 2018).

The effectiveness of beneficial microbes is frequently dependent on secretion systems. Some secretion systems allow translocation of proteins, called effectors, directly from one cell into another without being degraded or utilized by another organism. Other secretion systems and efflux pumps release proteins and secondary metabolites into the medium, respectively. The secreted proteins and metabolites play roles in root colonization, as well as in interactions with the plant immune response and the surrounding prokaryotic and eukaryotic organisms (**Figure 1**; Lugtenberg and Kamilova, 2009; Pieterse et al., 2014; Wu et al., 2018; Jamali et al., 2020).

The goal of this minireview is to describe important bacterial secreted effectors, secondary metabolites and secretion systems which play a role in the interactions of beneficial microbes with plants and surrounding microbes, including bacteria and fungi.

PLANT GROWTH PROMOTING RHIZOBACTERIA

Plant growth promoting rhizobacteria can improve the plant growth in multiple ways. They can indirectly promote growth by forming a biofilm that serves as a protective layer against pathogens or as an enhanced surface for nutrient acquisition from the surrounding soil (Weselowski et al., 2016). They can also produce and secrete growth phytohormones or their intermediates, which directly increase the root surface area, and promote plant development, growth and health (**Figure 1**; Spaepen et al., 2014). Additionally, PGPR increase abiotic stress tolerance in crops.

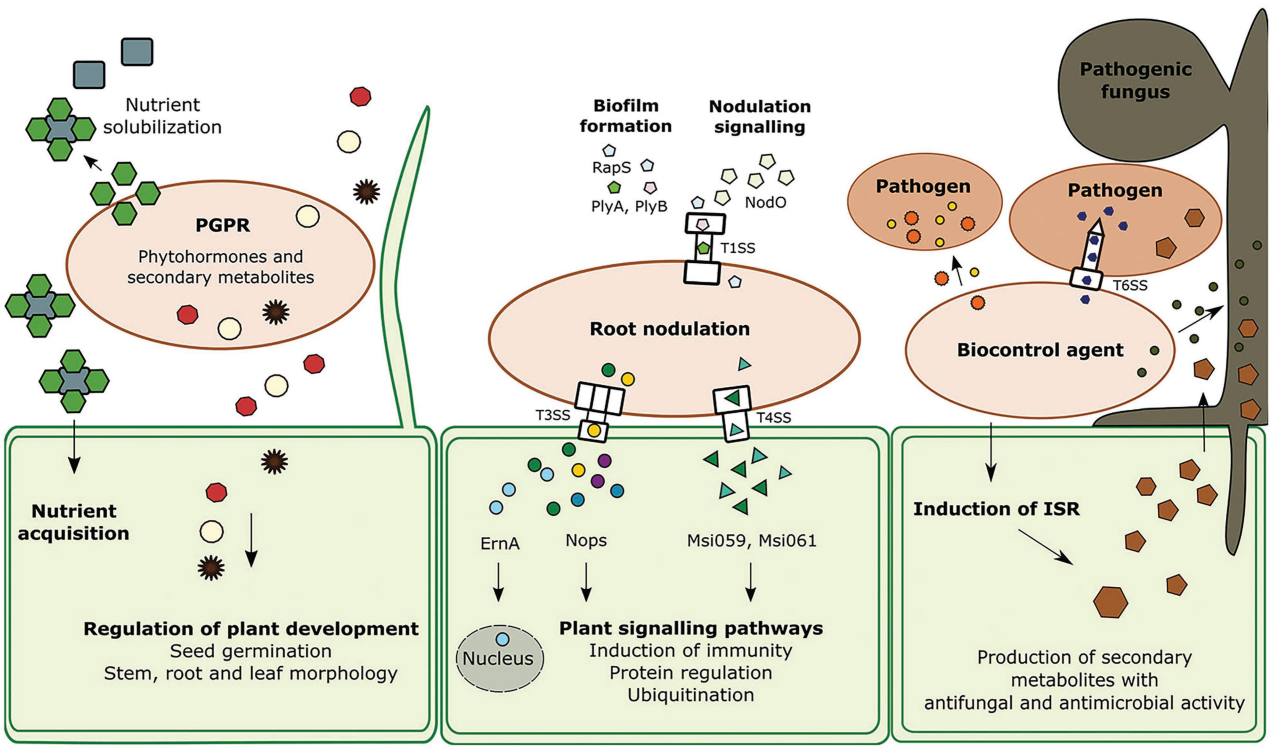

FIGURE 1 | The interaction of the three groups of beneficial bacteria with other species; plant growth promoting rhizobacteria (PGPR), root nodulating bacteria (RNB), and biocontrol agents (BCAs) and their neighboring cells. PGPR produce various secondary metabolites including phytohormones which are regulating several processes in the plant development such as seed germination, stem, leaf, and root morphology. Another feature of PGPR is the solubilization of nutrients. RNB contain several secretion systems that can transport effectors directly into the host cell to regulate certain processes. Type I protein secretion system (TISS) of *Rhizobium leguminosarum bv. Viciae* is responsible for biofilm formation *via* the effectors PlyA, PlyB, and Rhizobium-adhering proteins (RapS). The T1SS is recognizable due to the outer membrane protein TolC. Type III secretion system (T3SS) and type IV secretion system (T4SS) are secreting effectors which can trigger protein regulation and induce plant immune responses. At least one effector travels into the plant cell nucleus. BCAs produce antibacterial and antifungal protein toxins and small molecules. Proteinaceous toxins are transferred through the Type VI secretion system (T6SS), a powerful nanoweapon, into the host cell. Specific antimicrobials can kill phytopathogens like fungi, oomycetes, and bacteria. In addition, biocontrol agents can trigger the plant immunity pathway induced systemic resistance (ISR), which leads to the production of antimicrobials which can eliminate a broad spectrum of organisms.

Plant growth promoting rhizobacteria secrete organic acids and other secondary metabolites that solubilize macronutrients and micronutrients and increase their bioavailability for plants. Nitrogen and phosphorus are two of the essential macronutrients for plant growth. Nitrogen fixation will be discussed in the next section. Phosphorus in soil is highly unavailable for plants. Beneficial microbes mobilize phosphorus *via* organic chelators like citric, gluconic and malic acid. The secretion of these acids leads to a decrease of the soil pH and production of plant bioavailable HPO_4^{2-} (Rodríguez and Fraga, 1999; Ivanova et al., 2006; Yasmin et al., 2009). Some members of the *Bacillus*, *Pseudomonas* and *Enterobacter* genera are very efficient phosphorus solubilizing bacteria and have been shown to improve yield and growth of crops (Jha et al., 2012; Goswami et al., 2014).

Iron is an essential micronutrient for plant growth and development and iron-deficient plants suffer from yellow stripe chlorosis in young leaves (Abadía et al., 2002). The application of *Alcaligenes* 637Ca and *Staphylococcus* MFDCa1 to pear and apple roots, respectively, increased the foliar enzymatic activity of a plant enzyme that is responsible for Fe3+ reduction and helps plants uptake iron under iron deprivation (İpek et al., 2017; Aras et al., 2018). Some *Pseudomonas* spp. PGPR secrete siderophores like carboxylates, catecholate, and hydroxamate for Fe acquisition in *Zea mays*. Siderophores also have antimicrobial properties against the phytopathogens *Rhizoctonia solani* and *Sclerotium rolfs* (Sharma and Johri, 2003; Yu et al., 2011; Scagliola et al., 2016; Trapet et al., 2016; Mullins et al., 2019).

In contrast to the indirect effect of solubilization of nutrients on plant growth, phytohormone production by PGPR directly interfaces with plant growth signaling networks. The five main phytohormones are produced by PGPR: auxins, cytokinins, gibberellins (GAs), ethylene (ET), and abscisic acid. One of the most studied PGPRs is the *Azospirillum* genus. *Azospirillum. brasilense* consists of four different pathways to produce IAA, which is the most common phytohormone from the auxin class. The indole-3-pyruvate pathway produces the highest amount of IAA (Kloepper et al., 1989; Puente et al., 2004). The IAA biosynthesis pathways are well understood in bacteria, but the reason for the existence of those pathways in bacteria is still unknown (Molina et al., 2018). The indole-3-pyruvate pathway is highly dependent on the key enzyme indole-3-pyruvate decarboxylase encoded by the gene *ipdC* (Spaepen et al., 2014). *ipdC* alone is responsible for induction of root hair formation as was shown by laboratory inoculation experiments of *Arabidopsis thaliana* with *A. brasilense* SP245 strain (Cohen et al., 2008; Rivera et al., 2018). However, field experiments are inconsistent and did not reproduce the results (Díaz-Zorita and Fernández-Canigia, 2009). Generally, field experiments inoculated with *Azospirillum* showed an inconsistent increase in grain yield (Dobbelaere et al., 1999; Vande Broek et al., 1999). *Azospirillum* has many features, in addition to auxin production, which could lead to plant growth promotion including nitrogen fixation, siderophore production, and phosphate solubilization. Hence, there might be a growth promotion as an additive or a synergistic combination of the various pathways (Spaepen et al., 2008). The technological progress in the field allows us to combine large-scale ecological studies with a reductionist genetic approach that reveals bacterial genes that promote growth. A recent study using a 185-member bacterial community showed that this community causes *Arabidopsis* root growth inhibition. Interestingly, several strains of the *Variovorax* genus were found to maintain root development. Further genetic approach identified that the *Variovorax* genomes encode an auxin degrading operon that is necessary and sufficient for causing this beneficial phenotype even in such a complex ecosystem (Finkel et al., 2020).

Another example for phytohormone production in rhizobacteria is of the phytohormones gibberellins. GAs are involved in many developmental processes in plants, such as flowering regulation, seed germination, stem and leaf elongation, and pollen maturation (Achard et al., 2007; Ariizumi and Steber, 2007). Biosynthesis of GA was found in many bacteria such as *Bacillus pumilus, Bacillus licheniformis*, and *Leifsonia soli* (Kang et al., 2016; Kim et al., 2017). *Leifsonia soli* SE134 has been shown to enhance plant growth of the GA deficient *Waito-C* rice dwarf mutant cultivar and can extend shoot length, plant weight, and seed germination in cucumber, and tomato under greenhouse conditions, which may be due to GA synthesis (Kang et al., 2014).

Cytokinins are another group of growth-stimulating phytohormones that are responsible for cell division, plant senescence, seed germination, flower and fruit development, and apical dormancy (Akhtar et al., 2020). *Pseudomonas fluorescens* G20-18 and 6–8 strains produces cytokinins (García de Salamone et al., 2001; Pallai et al., 2012). It has been shown that canola inoculated with G20-18 and 6–8 strains had greater root length than the non-inoculated control in a gnotobiotic assay (Pallai et al., 2012). Großkinsky et al. (2016) revealed by constructing various knock-out and gain of function mutants of G20-18, that cytokinins have a protective role against *Pseudomonas syringae* pv. tomato DC3000 and can suppress disease response in *A. thaliana* (Großkinsky et al., 2016).

The aforementioned studies showed that PGPR can secrete multiple molecules which lead to various phenotypes. Exactly which conditions favor release of these beneficial growth-promoting molecules is still poorly understood. Knowing these conditions is important given that abiotic and biotic stressors can affect phytohormone production (Díaz-Zorita and Fernández-Canigia, 2009).

Ethylene is an important plant growth hormone that ameliorates harmful effects of abiotic stress conditions in plants (Glick, 2014). Its precursor is 1-aminocyclopropane-1-carboxylate (ACC). PGPR can improve plant tolerance to abiotic stressors *via* the production of ACC deaminase, which cleaves ET and produces α-ketobutyrate and ammonia. ACC deaminase indirectly counteract saline plant growth inhibitory effects in plants, hence plants are more salt stress tolerant (Orozco-Mosqueda et al., 2019, 2020). *Pseudomonas putida* UW4 carrying the *acdS* gene that encode for ACC deaminase was able to restore 66% of canola shoot fresh mass when grown in cold temperatures under high salt levels. Remarkably, the Δ*acdS* strain yielded only 20% of shoot fresh mass under the same conditions, demonstrating the importance of this single bacterial gene in promoting plant growth (Cheng et al., 2007). Trehalose, a non-reducing disaccharide, is especially induced in bacteria under saline stress and reduces osmotic, ionic and saline stress

responses, by interacting with ABA, volatile compounds and exopolysaccharides (EPS; Avonce et al., 2006). Recently the synergetic effect of trehalose accumulation and ACC-deaminase production has been discovered in *Pseudomonas* sp. UW4 protecting tomato plants under saline stress. The plants were unable to survive the abiotic stress when the UW4 *acdS* and *treS* (trehalose synthesis) genes where knocked out (Orozco-Mosqueda et al., 2019). More detailed information about the synergistic effect of rhizobacteria produced ACC deaminase and plant compounds were reviewed recently by Forni et al. (2017). ACC deaminase also plays a role in synergetic function with other soil living-organisms such as in rhizobacteria for induced nodulation. *Pseudomonas fluorescens* YsS6 promotes the growth of *Rhizobia tropici* CIAT899, leading to an induced growth of *Phaseolus vulgaris*. The plant growth induction was only observed when YsS6 expressed *acdS* (Nascimento et al., 2019).

ROOT-NODULATING BACTERIA

Root-nodulating bacteria have developed an impressive and complex symbiosis with their legume host. One of the first steps in this relationship, is the secretion of flavonoids by the host plant that diffuse across the membrane of the Rhizobia and induce synthesis of the NodD protein which activates transcription of other genes involved in nodulation including Nod factor (NF) production (Wang et al., 2012). NFs are primary signal molecules produced by bacteria and detected by the plant to induce nodule organogenesis (Nelson and Sadowsky, 2015). In addition to NFs, other molecules and proteins mediate other aspects of the rhizobia-legume symbiosis such as root colonization, symbiont recognition and suppression of the plant immune system. To perform all of these tasks, Rhizobia make use of special secretion systems that translocate effectors to their host. These include type I, type III and IV secretion systems (Cianciotto, 2005; Schmeisser et al., 2009; Nelson and Sadowsky, 2015).

Type I protein secretion system (TISS) of *Rhizobium leguminosarum bv. viciae* is encoded by the *prsD* and *prsE* genes. This T1SS is responsible for secretion of the EPS-glycanases PlyA and PlyB (Russo et al., 2006). These enzymes play a key role in biofilm formation; by cleaving the EPS chains they modulate the structure and maturation of the biofilm. Mutations in *prsD* and *prsE* greatly suppress the formation of biofilm on glass surfaces (Russo et al., 2006). Biofilm formation is an important step in root colonization and in symbiotic interaction formation. Once rhizobia attach to root hairs, they aggregate and form a biofilm, which is encased in a structure called a cap that is made of cellulose and EPS (Smit et al., 1987; Downie, 2010). Some proteins such as Rhizobium-adhering proteins (Raps) are required for stability of the cap, and are exported through the PrsDE T1SS (Smit et al., 1987; Russo et al., 2006; Krehenbrink and Allan, 2008; Poole et al., 2018). RapA1 is a calcium-binding Rap located at the cell pole (Poole et al., 2018). RapA1 overexpression in *R. leguminosarum bv. trifolii* R200 increased attachment to red clover roots by up to 5-fold and its overexpression in *Rhizobium etli* enhanced the capability of attachment to common bean roots (Mongiardini et al., 2009; Frederix et al., 2014). TISS also secretes NodO, a well-studied protein from *R. leguminosarum*, that is critical for signaling during nodulation (Finnie et al., 1997).

TolC is an integral membrane protein that is part of the outer membrane component of T1SS. TolC from *Sinorhizobium meliloti* functions in the symbiotic relationship with *Medicago sativa* (Cosme et al., 2008). *S. meliloti tolC* mutant showed an 8-fold reduction in the number of nodules compared with the wild type and presented an ineffective nitrogen fixation in the roots of *M. sativa* (Cosme et al., 2008). TolC may participate in the efflux of antimicrobial compounds produced by the host plant, resistance to osmotic or oxidative stress, polysaccharide biosynthesis, and the secretion of proteins or other molecules relevant for the symbiosis, such as NFs, that can affect directly or indirectly the formation of nodules in the roots of *M. sativa* (Srinivasan et al., 2015; Mergaert, 2018).

Other secretion systems, such as the type III secretion system (T3SS), are employed for effector translocation into the host plants. T3SS is mostly studied for its role in plant disease. The effectors can interfere with plant signaling and plant cell recognition. Transcriptional studies have shown expression of T3SS genes at different stages of the Plant-*Rhizobium* interaction such as root colonization, infection and nodulation. The T3SS of *Bradyrhizobium japonicum* USDA110 is expressed in infection threads and developing nodules of soybean (Zehner et al., 2008). Several T3SS genes of *Rhizobium* sp. *NGR234* are expressed in mature nodules of *Cajanus cajan* and *Vigna unguiculate* (Viprey et al., 1998; Perret et al., 1999; Tampakaki, 2014). Regulatory analyses of the T3SS of *Rhizobium* sp. *NGR234* showed that it is activated after Nod factors generation and its activity continues for at least 24 h (Kobayashi et al., 2004; Marie et al., 2004). These results indicate that effector secretion through T3SS concurs with development of the infection thread. T3SS is strongly regulated after sensing potential plant hosts.

T3SS genes called rhc (*Rhizobium* conserved), encode different nodulation outer proteins (Nops) that can be divided into two groups. The first group is composed of the core components of T3SS pilus that spans the plant cell wall (Saad et al., 2008; Deakin and Broughton, 2009; López-Baena et al., 2016). NopA and NopB are the major and minor subunits, respectively. NopX likely polymerizes to form a transmembrane pore (the translocon) through which other effectors enter the plant cytoplasm (Deakin and Broughton, 2009; López-Baena et al., 2016). The second group is composed of the effectors that are injected through T3SS machinery into the host cytoplasm. Several Sinorhizobial proteins secreted through the T3SS have been identified. These include NopL and NopP that may interfere with plant signaling pathways, as both can be phosphorylated by plant kinases and have shown to be responsible for optimal nodulation of host plants *Flemingia congesta* and *Tephrosia vogelii* (Bartsev et al., 2004; Skorpil et al., 2005; Gourion et al., 2015). NopL was shown to interfere with mitogen-activated protein kinase (MAPK) that is involved in pathogen recognition in both basal plant defense and R-mediated resistance (Pedley and Martin, 2005; Zhang et al., 2011). NopM belongs to the IpaH-SspH-YopM family of effectors found in animal pathogens, which are known

to be involved in targeting nuclei of host cells and ubiquitination process (Bartsev et al., 2004; Skorpil et al., 2005; Rohde et al., 2007). A later study indicated a possible role for NopM as a functional E3 ubiquitin ligase domain in *Rhizobium* sp. strain NG234 (Xin et al., 2012). In the same study it was further mentioned that when expressed in *Nicotiana benthamiana*, NopM reduced reactive oxygen species (ROS) and induced plant defense gene expression (Xin et al., 2012). NopT effector has homology with the avirulence protein AvrPphB of the phytopathogen *P. syringae* and YopT of *Yersinia* spp. which are known to possess a protease activity. NopT mutants of NGR234 affected nodulation either positively (*P. vulgaris* cv. Yudou No. 1; *T. vogelii*) or negatively (*Crotalaria juncea*; Dai et al., 2008). NopM and NopT have shown to have either negative or positive effects in nodulation in a host dependent manner (Dai et al., 2008; Kambara et al., 2009)

Another effector, NopD in *Sinorhizobium fredii* HH103, has been predicted to be a C48 cysteine peptidase (Rodrigues et al., 2007). The C48 cysteine peptidase family contains the protein XopD, a T3SS effector from the plant pathogen *Xanthomonas campestris* (Hotson et al., 2003). It functions *in planta* to target SUMO-conjugated proteins (Hotson et al., 2003). XopD interferes with the plant's ability to regulate the expression of specific proteins (Nelson and Sadowsky, 2015). NopC is a T3SS-dependent effector that lacks homologues in pathogenic bacteria but its function in plants is still unknown (Jiménez-Guerrero et al., 2015). NopJ acts as acetyltransferase that prevents phosphorylation of MAP kinases by acetylating the phosphorylation sites, thereby inactivating the MAP kinases (Mukherjee et al., 2006). Recently, a conserved T3SS effector, ErnA, was described in *Bradyrhizobium* (Teulet et al., 2019). Interestingly, this effector is targeted to the plant nucleus and may bind nucleic acids in the plant nuclei. Gain and loss of function experiments demonstrated the direct involvement of ErnA for nodule formation. All T3SS effectors and their predicted function are described in **Table 1**.

The bacterial type IV secretion systems (T4SS) is a unique system in its ability to transfer large nucleic acid molecules, in addition to proteins, across the cell envelope (Christie and Cascales, 2005; Sgro et al., 2019). Rhizobial T4SS shares strong homology to the VirB/VirD4 subunits found in *Agrobacterium* (Sullivan et al., 2002; Christie et al., 2014). The T4SS in *Agrobacterium tumefaciens*, is used for translocation of both T-DNA and effector proteins (Kuldau et al., 1990; Zupan and Zambryski, 1995). T4SS has been identified in rhizobia such as *Mesorhizobium loti* R7A (Hubber et al., 2007; Miwa and Okazaki, 2017) and *R. etli* CFN42 (Lacroix and Citovsky, 2016). T4SS could be involved in the nodulation process in *Rhizobium* in early stages. *M. loti* T4SS mutants delayed nodulation on *Lotus corniculatus* and allows effective nodulation on *Leucaena leucocephala* (Hubber et al., 2004, 2007). *R. etli* encodes a T4SS locus (*vir*) and is able to mediate transfer and integration of DNA into plant cell genome when provided with a T-DNA (Lacroix and Citovsky, 2016). However, a T-DNA-like sequences in *R. etli* was not identified, suggesting that *Rhizobium*-mediated plant transformation does not occur in nature, although it cannot be ruled out that other *Rhizobium* strains, not yet sequenced, harbor a T-DNA.

Thus far, only two T4SS candidate effector proteins were identified in rhizobia. These are Msi059 and Msi061 from *M. loti* R7A (Nelson and Sadowsky, 2015). Msi059 shares a partial protein sequence similarity to the XopD C48 cysteine peptidase (Rodrigues et al., 2007; Nelson and Sadowsky, 2015). The other T4SS effector Msi061, shares protein similarity with *A. tumefaciens* effector VirF (Tzfira et al., 2004). VirF interacts with the host Skp1 protein to facilitate protein degradation of effector proteins VirE2 and Vip1 leading to unbinding of the T-DNA after entry into the host cell (Tzfira et al., 2004). The specific role of the Msi059 and Msi061 in RNB remains unidentified, but the latest evidence suggests that they modulate protein expression levels *in planta* (Nelson and Sadowsky, 2015).

Type VI Secretion System (T6SS) contractile nanoweapons allows bacteria to inject toxins directly into prey cell membranes, periplasm or cytoplasm, leading to cell growth arrest. In rhizobia, T6SS sequence have been found in several species such as *R. leguminosarum*, *B. japonicum*, *M. loti*, *Sinorhizobium saheli*, and *S. fredii* (Bladergroen et al., 2003). T6SS was related to the prevention of nodulation on *Pisum sativum cv. Rondo* (Bladergroen et al., 2003). Recently, it was reported that *R. etli* Mim1 T6SS mutant produced plants with lower dry weight and smaller nodules than the wild-type strain, suggesting for the first time a positive role of T6SS in Rhizobium-legume symbiosis (Salinero-Lanzarote et al., 2019). The rhizobacterium *Azorhizobium caulinodans* ORS571 utilizes its T6SS to outcompete other strains during infection of its host *Sesbania rostrata* (Lin et al., 2018). However, the researchers could not show involvement in inter-bacterial competition *in vitro*. The nitrogen fixing bacteria *Azoarcus olearius* BH72 encodes two T6SS operons, one of which is strongly up-regulated when nitrogen is absent (Jiang et al., 2019). *Kosakonia* strains are endophytic nitrogen fixers involved in plant growth promotion in rice (Bertani et al., 2016). T6SS of *Kosakonia* KO348 is important for rhizoplane and endosphere colonization but it is not clear exactly how (Mosquito et al., 2019). One possibility is that the microbes use the T6SS to facilitate colonization by inhibiting competitors in the rhizosphere.

Although different secretion systems and effectors have been identified in RNB, their specific role in symbiosis and nodulation is still unclear. Further molecular and biochemical work should be done to characterize the molecular mechanisms leading to secretion of proteins and other molecules and their effects *in planta*.

BIOCONTROL AGENTS

Biocontrol agents secrete a broad spectrum of secondary metabolites and proteins which can serve as antibacterial and antifungal compounds, such as enzymes which are able to degrade different compartments of various organisms (Mullins et al., 2019; Vesga et al., 2020). Some BCAs employ secretion systems to penetrate the neighboring cells and inject toxins into them. *Pseudomonas* spp., and *Bacillus* spp. are two of the most studied organisms in the BCA field. The most important and most studied secondary metabolites are antibiotics such as Phenazines, Phloroglucinols, Dialkylresorcinols, Pyrolnitrin, Pyoluteorin, Mupirocin, Peptide antibiotics, Hyrdogen cyanide, Rhizoxins, and Oxyvinylglycines

TABLE 1 | Summary of all discussed bacteria, predicted function, and secreted molecules in this review. Some molecules are secreted from different bacteria.

Bacterial strain	Molecules	Predicted function	References
Plant growth promoting			
Enterobacter, Bacillus, Pseudomonas	Organic acids	Phosphate solubilization	Jha et al., 2012; Goswami et al., 2014
Pseudomonas spp. GRP3A, PRS9, *Pseudomonas chlororaphis* ATCC 9446	Siderophores	Fe acquisition	Sharma and Johri, 2003; Trapet et al., 2016
Azospirillum brasilense SP245	IAA production	Induction of root hair formation	Cohen et al., 2008; Molina et al., 2018
Leifsonia soli SE	Gibberellin	Induction of plant growth and seed germination	Kang et al., 2014
Pseudomonas fluorescens G20-18	Cytokinins	Suppression of disease resistance, cell elongation	Großkinsky et al., 2016
Root nodulation			
Rhizobium leguminosarum bv. viciae A34	Exopolysaccharide (EPS)-glycanases PlyA and PlyB	Biofilm maturation	Russo et al., 2006; Bogino et al., 2013
Sinorhizobium meliloti	TolC protein	Nodules production	Cosme et al., 2008; Srinivasan et al., 2015; Mergaert, 2018
Rhizobium leguminosarum bv. trifolii R200, *Rhizobium etli*	RapA1	Biofilm formation	Mongiardini et al., 2009; Ho et al., 2014; Poole et al., 2018
Rhizobium leguminosarum spp.	NodO	Signaling for nodulation	Finnie et al., 1997; Krehenbrink and Allan, 2008
Sinorhizobium fredii HH103	NopD	Regulating expression of plant proteins	Hubber et al., 2004; Rodrigues et al., 2007; Nelson and Sadowsky, 2015
Bradyrhizobium japonicum USDA110, *Sinorhizobium fredii* NGR234, HH103, USDA257	NopL	Induction of plant immune response	Pedley and Martin, 2005; Zhang et al., 2011
Bradyrhizobium japonicum USDA110, *Sinorhizobium fredii* NGR234, HH103	NopM	Ubiquitination process	Rohde et al., 2007; Burkinshaw and Strynadka, 2014; Zheng and Shabek, 2017
Rhizobium etli CNPAF512, *Sinorhizobium fredii* NGR234, HH103, USDA257	NopP	Phosphorylated by plant kinases	Bartsev et al., 2004; Skorpil et al., 2005; Gourion et al., 2015
Sinorhizobium fredii NGR234	NopT	Cysteine protease activity	Dai et al., 2008; Kambara et al., 2009; Gourion et al., 2015; Nelson and Sadowsky, 2015
Rhizobium sp. NGR234	NopJ	Inactivates MAP kinases	Mukherjee et al., 2006; Kambara et al., 2009; Gourion et al., 2015
Mesorhizobium loti R7A	Msi059	Regulating expression of plant proteins	Rodrigues et al., 2007; Nelson and Sadowsky, 2015
Mesorhizobium loti R7A	Msi061	Protein degradation of VirE2 and Vip1	Nelson and Sadowsky, 2015
Bradyrhizobium strain ORS3257	ErnA	An unknown function in the plant nucleus	Teulet et al., 2019
Biocontrol			
Pseudomonas spp., *Bacillus* spp.	Antibiotics	Virulence against phytopathogens	Guilleroux and Osbourn, 2004; Daval et al., 2011; Cao et al., 2018
Pseudomonas fluorescens Pf29Apr	DAPG	Downregulation of pathogenic enzymes	Daval et al., 2011
Pseudomonas fluorescens MFE01	T6SS related- toxins	Virulence against phytopathogens	Decoin et al., 2014
Pseudomonas brassocaecearum Q8r1–96	RopAA, RopB, RopM, DAPG	Induction of plant immune responses	Mavrodi et al., 2011
Bacillus subtilis BBG111	Cyclic lipopeptides (CLCPs)	Induction of plant immune responses	Ongena et al., 2005; García-Gutiérrez et al., 2013; Farace et al., 2015
Bacillus velezensis	Lipopeptide compounds	Antifungal	Cao et al., 2018

(Raaijmakers et al., 2002; Weller, 2007; Mavrodi et al., 2011). *Bacillus velezensis* strains isolated from tomato rhizosphere strongly inhibit growth of *Ralstonia solanacearum* and *Fusarium oxysporum* under both laboratory and greenhouse conditions (Cao et al., 2018). This is done by production of different lipopeptide compounds whose production is stimulated during the BCA interaction with *R. solanacearum*. Recently, a survey of bacteria isolated from the phyllosphere of *A. thaliana* revealed novel antibiotics, with possible novel modes of actions (Helfrich et al., 2018). Antibiotics can be identified *via* HPLC and then tested for their antagonistic effect against different pathogens (Shahid et al., 2017). Many toxins (proteins or secondary metabolites) which are produced by beneficial bacteria have been studied beyond their antimicrobial/antifungal activity. For example, the BCA *P. fluorescens* Pf29Arp downregulates relevant pathogenicity enzymes (laccasses, exogluanases, and mitogen-activated kinases) in the fungus *Gaeumannomyces graminis var. tritici*, the causing agent of take-all disease (Guilleroux and Osbourn, 2004; Daval et al., 2011). Other assays can be used for profiling secondary metabolites, such as the use of LC-MS on crude extracts from BCA strains, *in silico* screening of antagonistic potential on pathogenic genes, and finally *in vitro* screening against specific pathogens (Jinal and Amaresan, 2020). *Burkholderia ambifaria*, a biocontrol agent was screened for its antimicrobial metabolites which led to detection of Cepacin A *via* LC-MS. Mutants for Cepain A production in *B. ambifaria* have a significantly reduced inhibition activity against *Pythium ultimum* in a pea infection model (Mullins et al., 2019).

Disease-suppressive soils prevent establishment of pathogens or lead to minor plant disease. The Raaijmakers group was able to demonstrate the involvement of beneficial bacteria from Burkholderiaceae family in disease-suppressive activity against *R. solani* (Chapelle et al., 2016; Carrión et al., 2018). They isolated representative Burkholderiaceae strains and uncovered genes involved in *in vitro* and *in situ* antifungal activity *via* the production of sulfurous volatile compounds (Carrión et al., 2018). Recently, they showed that an endophytic consortium of *Chitinophaga* and *Flavobacterium* consistently inhibited *Rhizoctonia solani* infection (Carrión et al., 2019). Moreover, they showed that the fungal infection enriched the root metagenome for chitinase genes and candidate biosynthetic gene clusters that likely produce antifungals. Finally, site-directed mutagenesis revealed a new NRPS-PKS gene cluster from *Flavobacterium* that is essential for disease suppression by the consortium. This is a fine example of how years of research revealed first specific BCA strains and later on their molecular mechanism that underlies a reproducible root microbiome that mediated plant protection.

As discussed already in the root nodulation section, Gram-negative bacteria can be equipped with different secretion systems. The T6SS translocates toxins into the neighboring cells that are killed if they do not have the matching immunity protein (Hood et al., 2010). T6SS genes were found, for examples, in *P. fluorescens* strain MFE01. Different T6SS effectors are injected by this strain. However, those toxins are not virulent against eukaryotic cells, but against a broad spectrum of pathogenic bacteria (Decoin et al., 2014). Bernal and colleges identified in *P. putida* KT2440 three T6SS clusters and 10 T6SS effector-immunity pairs. One of the T6SS loci is responsible to bactericidal activity against phytopathogens *in vitro* and in *planta* on *N. benthamiana*, although the *in planta* effect was mild (Bernal et al., 2017).

Biocontrol agents can also induce plant responses by secreting secondary metabolites. Often this results in an induction of the plant immune response called induced systemic resistance (ISR), which is regulated by the plant hormones jasmonic acid (JA) and ET (Berendsen et al., 2012; Pieterse et al., 2014). ISR is a response which is known to be triggered by rhizobacteria and leads to secretion of antimicrobial secondary metabolites from plants (Pieterse et al., 2012; Yu et al., 2019b). *Pseudomonas fluorescens* Q8r1–96 contains T3SS effectors RopAA, RopB, and RopM. In *N. benthamiana* these effectors suppress two plant immune pathways after leaf infection with *P. syringae* DC3000; the hypersensitive response and the production of reactive oxygens species (Mavrodi et al., 2011). Q8r1-96 also produces DAPG, which suppresses the take-all disease in wheat (Brazelton et al., 2008; Mavrodi et al., 2011; Kwak et al., 2012; Yang et al., 2020). *Bacillus* spp. is a well-established ISR elicitor. *Bacillus subitilis* BBG111 releases cyclic lipopeptides (CLPs), which are magnifying the plant microbe-associated molecular patterns (MAMPs) triggered immunity (MTI). The MTI recognizes microbe derived compounds, such as flagellins, lipopolysaccharides, and chitin that trigger the ISR pathway in rice against *R. solani* (Chandler et al., 2015; Lastochkina et al., 2019). This induction of ISR does not necessarily lead to the resistance against one phytopathogen since it is not species-specific (Chandler et al., 2015). *Bacillus* spp. and *Pseudomonas* spp. increase ISR in different kinds of crops (tomato, melon, and bean) against different organisms including fungi, bacteria, and nematodes (Ongena et al., 2005; García-Gutiérrez et al., 2013; Farace et al., 2015). The induction of ISR is very powerful, however its broad-spectrum activity may lead to killing of beneficial bacteria.

Often the combination of both PGPR and BCA can ensure both plant protectiveness and growth induction. Both traits can be tested *in vitro* on specific media. Liu et al. (2017) screened 196 PGPR strains based on their disease suppression for broad-spectrum antagonistic activity. In a second screen selected strains were tested for PGPR traits *in vitro*. For example, nitrogen fixation was tested on nitrogen-free semisolid medium and phosphate solubilization on media with different phosphate sources. In advanced screens, the PGPR strains were tested in planta for biological control of multiple plant diseases and most of them significantly reduced at least two tested diseases. Gene encoding antimicrobials were predicted but have not been experimentally validated (Liu et al., 2017).

The root nodules are also sites of active antimicrobial production. *Brevibacillus brevis* is an accessory species which resides near dominant rhizobia species. An untargeted *in planta* metabolomics study of this strain led to identification of nonribosomal peptides, Britacidin and gramicidin. Sequencing of the strain's genome led to assignment of these antimicrobials to their cognate biosynthetic gene clusters. It is yet unknown whether these antimicrobials are used in competition between the natural nodule microbiome or protect it from pathogen infection (Hansen et al., 2020).

DISCUSSION

Much research has been conducted regarding PGPR, BCA, and RNB and many effectors are known and are not mentioned in this review. Despite the knowledge of those secreted molecules, their functionality *in planta* remains unclear (Bai et al., 2020; Kumar and Dubey, 2020; Zhou et al., 2020). The importance of the RNB secretion system in nodule formation and symbiosis between rhizobia and legumes is known; however, direct interactions of effectors and plant proteins and the specific processes regulated by the effectors are not understood. Many hypotheses have been postulated but were not confirmed experimentally (Sachs et al., 2018). High-quality ecological studies revealed the function of specific rhizobacteria in protecting plants against bacterial, fungal, and oomycete pathogens but did not reveal the compounds responsible for this effect (Duran et al., 2018; Kwak et al., 2018). High-density transposon screens coupled with in planta phenotyping can uncover the genes responsible for these antagonistic phenotypes. Another approach that should be applied is systematic gain of function approach to uncover the secondary metabolome encoded by the biosynthetic gene clusters of beneficial rhizobacteria. This can be done by using large scale operon cloning, induction of operons in organisms such as *Escherichia coli*, and applying the lysates on plants to couple microbial metabolites with beneficial functions.

We believe that genetic, metagenomics, transcriptomics, proteomics (secretome), and metabolomics analyses should increase our knowledge about the effectors and small molecules injected by rhizobacteria into the host, nearby pathogens, or released into the surrounding soil (Levy et al., 2018a). Identification of the specific genes, proteins and molecules responsible for growth promotion and protection against pathogens will allow a more accurate identification of beneficial strains and engineering of plant supportive microbiomes. We think that the entire field will gain important basic and applied insights by moving from identification of beneficial strains through extensive phenotype screening toward molecule-centered or gene-centered phenotypic associations. Identification of new genes and molecules that underlie a beneficial phenotype will allow accurate discovery of novel beneficial strains based on their genetic and chemical features identified from metagenome and metabolome surveys. Downstream functional analysis such as random mutagenesis of beneficial microbes coupled with identification of phenotypes *in planta*, protein binding assays to identify the binding partners of effectors in plant cells, or cell-based assays to show translocation of effectors into plants could improve molecular understanding of beneficial bacterial interaction with plants. Specifically, very little is known on the interaction of proteins and small molecules from beneficial microbes with the different branches of the plant immune system.

In addition to the lack of functional studies revealing the molecular basis for a beneficial microbial phenotype in plants, the understanding of bacteria communities in soil is also very partially understood. Recently, more studies include synthetic communities, revealing that certain assemblies of rhizobacteria are having a positive influence on plant fitness and health (Finkel et al., 2017; Helfrich et al., 2018). Sequenced and annotated genomes of those bacterial communities are available, however functional analysis lags behind (Finkel et al., 2017; Helfrich et al., 2018; Levy et al., 2018a,b).

AUTHOR CONTRIBUTIONS

All authors listed have made a substantial, direct and intellectual contribution to the work, and approved it for publication.

FUNDING

ML is supported by the Landtagsstipendium from the Ministry of Science, Research and Arts from Baden-Württemberg in Germany. MC is supported by a scholarship from IFARHU. AL is supported by Alon Fellowship of the Israeli council of higher education. This research was supported by the Israel Science Foundation (Grant No. 1535/20).

ACKNOWLEDGMENTS

We thank Omri Finkel and Alexander Martin Geller for critical reading and editing of the manuscript.

REFERENCES

Abadía, J., López-Millán, A. F., Rombolà, A., and Abadía, A. (2002). Organic acids and Fe deficiency: a review. *Plant Soil* 241, 75–86. doi: 10.1023/A:1016093317898

Achard, P., Liao, L., Jiang, C., Desnos, T., Bartlett, J., Fu, X., et al. (2007). DELLAs contribute to plant photomorphogenesis. *Plant Physiol.* 143, 1163–1172. doi: 10.1104/pp.106.092254

Akhtar, S. S., Mekureyaw, M. F., Pandey, C., and Roitsch, T. (2020). Role of cytokinins for interactions of plants with microbial pathogens and pest insects. *Front. Plant Sci.* 10:1777. doi: 10.3389/fpls.2019.01777

Aras, S., Arıkan, Ş., İpek, M., Eşitken, A., Pırlak, L., Dönmez, M. F., et al. (2018). Plant growth promoting rhizobacteria enhanced leaf organic acids, FC-R activity and Fe nutrition of apple under lime soil conditions. *Acta Physiol. Plant.* 40:120. doi: 10.1007/s11738-018-2693-9

Ariizumi, T., and Steber, C. M. (2007). Seed germination of GA-insensitive sleepy1 mutants does not require RGL2 protein disappearance in arabidopsis. *Plant Cell* 19, 791–804. doi: 10.1105/tpc.106.048009

Avonce, N., Mendoza-Vargas, A., Morett, E., and Iturriag, G. (2006). Insights on the evolution of trehalose biosynthesis. *BMC Evol. Biol.* 6, 1–15. doi: 10.1186/1471-2148-6-109

Bai, Y. -C., Chang, Y. -Y., Hussain, M., Lu, B., Zhang, J. -P., Song, X. -B., et al. (2020). Soil chemical and microbiological properties are changed by long-term chemical fertilizers that limit ecosystem functioning. *Microorganisms* 8:694. doi: 10.3390/microorganisms8050694

Bakker, P. A. H. M., Pieterse, C. M. J., de Jonge, R., and Berendsen, R. L. (2018). The soil-borne legacy. *Cell* 172, 1178–1180. doi: 10.1016/j.cell.2018.02.024

Bartsev, A. V., Deakin, W. J., Boukli, N. M., McAlvin, C. B., Stacey, G., Malnoë, P., et al. (2004). NopL, an effector protein of rhizobium sp. NGR234, thwarts activation of plant defense reactions. *Plant Physiol.* 134, 871–879. doi: 10.1104/pp.103.031740

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

Bernal, P., Allsopp, L. P., Filloux, A., and Llamas, M. A. (2017). The *Pseudomonas putida* T6SS is a plant warden against phytopathogens. *ISME J.* 11, 972–987. doi: 10.1038/ismej.2016.169

Bernal, P., LlamBernal, P., Llamas, M. A., and Filloux, A. (2018). Type VI secretion systems in plant-associated bacteria. *Environ. Microbiol.* 20, 1–15. doi: 10.1111/1462-2920.13956

Bertani, I., Abbruscato, P., Piffanelli, P., Subramoni, S., and Venturi, V. (2016). Rice bacterial endophytes: isolation of a collection, identification of beneficial strains and microbiome analysis. *Environ. Microbiol. Rep.* 8, 388–398. doi: 10.1111/1758-2229.12403

Bladergroen, M. R., Badelt, K., and Spaink, H. P. (2003). Infection-blocking genes of a symbiotic rhizobium leguminosarum strain that are involved in temperature-dependent protein secretion. *Mol. Plant-Microbe Interact.* 16, 53–64. doi: 10.1094/MPMI.2003.16.1.53

Bogino, P. C., Oliva, M. D. L. M., Sorroche, F. G., and Giordano, W. (2013). The role of bacterial biofilms and surface components in plant-bacterial associations. *Int. J. Mol. Sci.* 14, 15838–15859. doi: 10.3390/ijms140815838

Brazelton, J. N., Pfeufer, E. E., Sweat, T. A., McSpadden Gardener, B. B., and Coenen, C. (2008). 2,4-Diacetylphloroglucinol alters plant root development. *Mol. Plant-Microbe Interact.* 21, 1349–1358. doi: 10.1094/MPMI-21-10-1349

Burkinshaw, B. J., and Strynadka, N. C. (2014). Assembly and structure of the T3SS. *Biochim. Biophys. Acta* 1843, 1649–1663. doi: 10.1016/j.bbamcr.2014.01.035

Cao, Y., Pi, H., Chandrangsu, P., Li, Y., Wang, Y., Zhou, H., et al. (2018). Antagonism of two plant-growth promoting bacillus velezensis isolates against Ralstonia solanacearum and Fusarium oxysporum. *Sci. Rep.* 8, 1–14. doi: 10.1038/s41598-018-22782-z

Carrión, V. J., Cordovez, V., Tyc, O., Etalo, D. W., de Bruijn, I., de Jager, V. C. L., et al. (2018). Involvement of Burkholderiaceae and sulfurous volatiles in disease-suppressive soils. *ISME J.* 12, 2307–2321. doi: 10.1038/s41396-018-0186-x

Carrión, V. J., Perez-Jaramillo, J., Cordovez, V., Tracanna, V., De Hollander, M., Ruiz-Buck, D., et al. (2019). Pathogen-induced activation of disease-suppressive functions in the endophytic root microbiome. *Science* 366, 606–612. doi: 10.1126/science.aaw9285

Chandler, S., Van Hese, N., Coutte, F., Jacques, P., Höfte, M., and De Vleesschauwer, D. (2015). Role of cyclic lipopeptides produced by Bacillus subtilis in mounting induced immunity in rice (*Oryza sativa* L.). *Physiol. Mol. Plant Pathol.* 91, 20–30. doi: 10.1016/j.pmpp.2015.05.010

Chapelle, E., Mendes, R., Bakker, P. A. H. M., and Raaijmakers, J. M. (2016). Fungal invasion of the rhizosphere microbiome. *ISME J.* 10, 265–268. doi: 10.1038/ismej.2015.82

Cheng, Z., Park, E., and Glick, B. R. (2007). 1-Aminocyclopropane-1-carboxylate deaminase from pseudomonas putida UW4 facilitates the growth of canola in the presence of salt. *Can. J. Microbiol.* 53, 912–918. doi: 10.1139/W07-050

Christie, P. J., and Cascales, E. (2005). Structural and dynamic properties of bacterial type IV secretion systems (review). *Mol. Membr. Biol.* 22, 51–61. doi: 10.1080/09687860500063316

Christie, P. J., Whitaker, N., and González-Rivera, C. (2014). Mechanism and structure of the bacterial type IV secretion systems. *Biochim. Biophys. Acta-Mol. Cell Res.* 1843, 1578–1591. doi: 10.1016/j.bbamcr.2013.12.019

Cianciotto, N. P. (2005). Type II secretion: A protein secretion system for all seasons. *Trends Microbiol.* 13, 581–588. doi: 10.1016/j.tim.2005.09.005

Clark, F. E. (1949). Soil microorganisms and plant roots. *Adv. Agron.* 1, 241–288. doi: 10.1016/S0065-2113(08)60750-6

Cohen, A. C., Bottini, R., and Piccoli, P. N. (2008). Azospirillum brasilense Sp 245 produces ABA in chemically-defined culture medium and increases ABA content in arabidopsis plants. *Plant Growth Regul.* 54, 97–103. doi: 10.1007/s10725-007-9232-9

Cosme, A. M., Becker, A., Santos, M. R., Sharypova, L. A., Santos, P. M., and Moreira, L. M. (2008). The outer membrane protein TolC from Sinorhizobium meliloti affects protein secretion, polysaccharide biosynthesis, antimicrobial resistance, and symbiosis. *Mol. Plant-Microbe Interact.* 21, 947–957. doi: 10.1094/MPMI-21-7-0947

Dai, W. J., Zeng, Y., Xie, Z. P., and Staehelin, C. (2008). Symbiosis-promoting and deleterious effects of NopT, a novel type 3 effector of rhizobium sp. strain NGR234. *J. Bacteriol.* 190, 5101–5110. doi: 10.1128/JB.00306-08

Daval, S., Lebreton, L., Gazengel, K., Boutin, M., Guillerm-Erckelboudt, A. Y., and Sarniguet, A. (2011). The biocontrol bacterium *Pseudomonas fluorescens* Pf29Arp strain affects the pathogenesis-related gene expression of the take-all fungus Gaeumannomyces graminis var. tritici on wheat roots. *Mol. Plant Pathol.* 12, 839–854. doi: 10.1111/j.1364-3703.2011.00715.x

Deakin, W. J., and Broughton, W. J. (2009). Symbiotic use of pathogenic strategies: Rhizobial protein secretion systems. *Nat. Rev. Microbiol.* 7, 312–320. doi: 10.1038/nrmicro2091

Decoin, V., Barbey, C., Bergeau, D., Latour, X., Feuilloley, M. G. J., Orange, N., et al. (2014). A type VI secretion system is involved in *Pseudomonas fluorescens* bacterial competition. *PLoS One* 9:e89411. doi: 10.1371/journal.pone.0089411

Díaz-Zorita, M., and Fernández-Canigia, M. V. (2009). Field performance of a liquid formulation of Azospirillum brasilense on dryland wheat productivity. *Eur. J. Soil Biol.* 45, 3–11. doi: 10.1016/j.ejsobi.2008.07.001

Dobbelaere, S., Croonenborghs, A., Thys, A., Vande Broek, A., and Vanderleyden, J. (1999). Phytostimulatory effect of Azospirillum brasilense wild type and mutant strains altered in IAA production on wheat. *Plant Soil* 212, 155–164. doi: 10.1023/A:1004658000815

Downie, J. A. (2010). The roles of extracellular proteins, polysaccharides and signals in the interactions of rhizobia with legume roots. *FEMS Microbiol. Rev.* 34, 150–170. doi: 10.1111/j.1574-6976.2009.00205.x

Duran, P., Thiergart, T., Garrido-Oter, R., Agler, M., Kemen, E., and Schulze-Lefert, P. (2018). Microbial Interkingdom interactions in roots promote Arabidopsis survival. *Cell* 175, 973–983.e14. doi: 10.1016/j.cell.2018.10.020

Farace, G., Fernandez, O., Jacquens, L., Coutte, F., Krier, F., Jacques, P., et al. (2015). Cyclic lipopeptides from Bacillus subtilis activate distinct patterns of defence responses in grapevine. *Mol. Plant Pathol.* 16, 177–187. doi: 10.1111/mpp.12170

Finkel, O. M., Castrillo, G., Herrera Paredes, S., Salas González, I., and Dangl, J. L. (2017). Understanding and exploiting plant beneficial microbes. *Curr. Opin. Plant Biol.* 38, 155–163. doi: 10.1016/j.pbi.2017.04.018

Finkel, O. M., Salas-González, I., Castrillo, G., Conway, J. M., Law, T. F., Teixeira, P. J. P. L., et al. (2020). A single bacterial genus maintains root development in a complex microbiome. bioRxiv [Preprint] doi:10.1101/645655.

Finnie, C., Hartley, N. M., Findlay, K. C., and Downie, J. A. (1997). The *Rhizobium leguminosarum prsDE* genes are required for secretion of several proteins, some of which influence nodulation, symbiotic nitrogen fixation and exopolysaccharide modification. *Mol. Microbiol.* 25, 135–146. doi: 10.1046/j.1365-2958.1997.4471803.x

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

Frederix, M., Edwards, A., Swiderska, A., Stanger, A., Karunakaran, R., Williams, A., et al. (2014). Mutation of praR in *Rhizobium leguminosarum* enhances root biofilms, improving nodulation competitiveness by increased expression of attachment proteins. *Mol. Microbiol.* 93, 464–478. doi: 10.1111/mmi.12670

García-Gutiérrez, L., Zeriouh, H., Romero, D., Cubero, J., de Vicente, A., and Pérez-García, A. (2013). The antagonistic strain *Bacillus subtilis* UMAF6639 also confers protection to melon plants against cucurbit powdery mildew by activation of jasmonate- and salicylic acid-dependent defence responses. *Microb. Biotechnol.* 6, 264–274. doi: 10.1111/1751-7915.12028

García 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

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

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

Gourion, B., Berrabah, F., Ratet, P., and Stacey, G. (2015). Rhizobium-legume symbioses: the crucial role of plant immunity. *Trends Plant Sci.* 20, 186–194. doi: 10.1016/j.tplants.2014.11.008

Großkinsky, D. K., Tafner, R., Moreno, M. V., Stenglein, S. A., De Salamone, I. E. G., Nelson, L. M., et al. (2016). Cytokinin production by *Pseudomonas fluorescens* G20-18 determines biocontrol activity against pseudomonas syringae in Arabidopsis. *Sci. Rep.* 6:23310. doi: 10.1038/srep23310

Guilleroux, M., and Osbourn, A. (2004). Gene expression during infection of wheat roots by the "take-all" fungus Gaeumannomyces graminis. *Mol. Plant Pathol.* 5, 203–216. doi: 10.1111/j.1364-3703.2004.00219.x

Hacquard, S., Garrido-Oter, R., González, A., Spaepen, S., Ackermann, G., Lebeis, S., et al. (2015). Microbiota and host nutrition across plant and animal kingdoms. *Cell Host Microbe* 17, 603–616. doi: 10.1016/j.chom.2015.04.009

Hansen, B. L., Pessotti, R.d. C., Fischer, M. S., Collins, A., El-Hifnawi, L., Liu, M. D., et al. (2020). Cooperation, competition, and specialized metabolism in a simplified root nodule microbiome. *MBio* 11, 1–20. doi: 10.1128/mBio.01917-20

Helfrich, E. J. N., Vogel, C. M., Ueoka, R., Schäfer, M., Ryffel, F., Müller, D. B., et al. (2018). Bipartite interactions, antibiotic production and biosynthetic potential of the arabidopsis leaf microbiome. *Nat. Microbiol.* 3, 909–919. doi: 10.1038/s41564-018-0200-0

Hiltner, L. (1904). Uber nevere Erfahrungen und Probleme auf dem Gebiet der Boden Bakteriologie und unter besonderer Beurchsichtigung der Grundungung und Broche. *Arbeit. Deut. Landw. Ges. Berlin* 98, 59–78.

Ho, B. T., Dong, T. G., and Mekalanos, J. J. (2014). A view to a kill: the bacterial type VI secretion system. *Cell Host Microbe* 15, 9–21. doi: 10.1016/j.chom.2013.11.008

Hood, R. D., Singh, P., Hsu, F. S., Güvener, T., Carl, M. A., Trinidad, R. R. S., et al. (2010). A type VI secretion system of *Pseudomonas aeruginosa* targets a toxin to bacteria. *Cell Host Microbe* 7, 25–37. doi: 10.1016/j.chom.2009.12.007

Hotson, A., Chosed, R., Shu, H., Orth, K., and Mudgett, M. B. (2003). Xanthomonas type III effector XopD targets SUMO-conjugated proteins in planta. *Mol. Microbiol.* 50, 377–389. doi: 10.1046/j.1365-2958.2003.03730.x

Hubber, A. M., Sullivan, J. T., and Ronson, C. W. (2007). Symbiosis-induced cascade regulation of the Mesorhizobium loti R7A VirB/D4 type IV secretion system. *Mol. Plant-Microbe Interact.* 20, 255–261. doi: 10.1094/MPMI-20-3-0255

Hubber, A., Vergunst, A. C., Sullivan, J. T., Hooykaas, P. J. J., and Ronson, C. W. (2004). Symbiotic phenotypes and translocated effector proteins of the Mesorhizobium loti strain R7A VirB/D4 type IV secretion system. *Mol. Microbiol.* 54, 561–574. doi: 10.1111/j.1365-2958.2004.04292.x

İpek, M., Aras, S., Arıkan, Ş., Eşitken, A., Pırlak, L., Dönmez, M. F., et al. (2017). Root plant growth promoting rhizobacteria inoculations increase ferric chelate reductase (FC-R) activity and Fe nutrition in pear under calcareous soil conditions. *Sci. Hortic.* 219, 144–151. doi: 10.1016/j.scienta.2017.02.043

Ivanova, R. P., Bojinova, D. Y., Gruncharov, I. N., and Damgaliev, D. L. (2006). The Solubilization of rock phosphate by organic acids. *Phosphorus Sulfur Silicon Relat. Elem.* 181, 2541–2554. doi: 10.1080/10426500600758399

Jamali, H., Sharma, A., Roohi., and Srivastava, A. K. (2020). Biocontrol potential of Bacillus subtilis RH5 against sheath blight of rice caused by Rhizoctonia solani. *J. Basic Microbiol.* 60, 268–280. doi: 10.1002/jobm.201900347

Jha, C. K., Patel, B., and Saraf, M. (2012). Stimulation of the growth of *Jatropha curcas* by the plant growth promoting bacterium Enterobacter cancerogenus MSA2. *World J. Microbiol. Biotechnol.* 28, 891–899. doi: 10.1007/s11274-011-0886-0

Jiang, X., Beust, A., Sappa, P. K., Völker, U., Dinse, T., Herglotz, J., et al. (2019). Two functionally deviating type 6 secretion systems occur in the nitrogen-fixing Endophyte Azoarcus olearius BH72. *Front. Microbiol.* 10:459. doi: 10.3389/fmicb.2019.00459

Jiménez-Guerrero, I., Pérez-Montaño, F., Medina, C., Ollero, F. J., and López-Baena, F. J. (2015). NopC is a rhizobium-specific type 3 secretion system effector secreted by sinorhizobium (ensifer) fredii HH103. *PLoS One* 10:e0142866. doi: 10.1371/journal.pone.0142866

Jinal, H. N., and Amaresan, N. (2020). Characterization of medicinal plant-associated biocontrol *Bacillus subtilis* (SSL2) by liquid chromatography-mass spectrometry and evaluation of compounds by *in silico* and *in vitro* methods. *J. Biomol. Struct. Dyn.* 38, 500–510. doi: 10.1080/07391102.2019.1581091

Kambara, K., Ardissone, S., Kobayashi, H., Saad, M. M., Schumpp, O., Broughton, W. J., et al. (2009). Rhizobia utilize pathogen-like effector proteins during symbiosis. *Mol. Microbiol.* 71, 92–106. doi: 10.1111/j.1365-2958.2008.06507.x

Kang, S. -M., Latif Khan, A., You, Y. -H., Kim, J. -G., Kamran, M., and Lee, I. -J. (2014). Gibberellin production by newly isolated strain Leifsonia soli SE134 and its potential to promote plant growth. *J. Microbiol. Biotechnol.* 24, 106–112. doi: 10.4014/jmb.1304.04015

Kang, S. M., Asaf, S., Kim, S. J., Yun, B. W., and Lee, I. J. (2016). Complete genome sequence of plant growth-promoting bacterium Leifsonia xyli SE134, a possible gibberellin and auxin producer. *J. Biotechnol.* 239, 34–38. doi: 10.1016/j.jbiotec.2016.10.004

Kim, M. J., Radhakrishnan, R., Kang, S. M., You, Y. H., Jeong, E. J., Kim, J. G., et al. (2017). Plant growth promoting effect of *Bacillus amyloliquefaciens* H-2-5 on crop plants and influence on physiological changes in soybean under soil salinity. *Physiol. Mol. Biol. Plants* 23, 571–580. doi: 10.1007/s12298-017-0449-4

Kloepper, J. W., Lifshitz, R., and Zablotowicz, R. M. (1989). Free-living bacterial inocula for enhancing crop productivity. *Trends Biotechnol.* 7, 39–44. doi: 10.1016/0167-7799(89)90057-7

Kobayashi, H., Naciri-Graven, Y., Broughton, W. J., and Perret, X. (2004). Flavonoids induce temporal shifts in gene-expression of nod-box controlled loci in rhizobium sp. NGR234. *Mol. Microbiol.* 51, 335–347. doi: 10.1046/j.1365-2958.2003.03841.x

Korenblum, E., Dong, Y., Szymanski, J., Panda, S., Jozwiak, A., Massalha, H., et al. (2020). Rhizosphere microbiome mediates systemic root metabolite exudation by root-to-root signaling. *Proc. Natl. Acad. Sci. U. S. A.* 117, 3874–3883. doi: 10.1073/pnas.1912130117

Krehenbrink, M., and Allan, J. A. (2008). Identification of protein secretion systems and novel secreted proteins in rhizobium leguminosarum bv. Viciae. *BMC Genomics* 9:55. doi: 10.1186/1471-2164-9-55

Kuc, J. A., and Tuzun, S. (1992). Proposed definitions related to induced disease resistance. *Biocontrol Sci. Tech.* 2, 349–351. doi: 10.1080/09583159209355251

Kuldau, G. A., De Vos, G., Owen, J., McCaffrey, G., and Zambryski, P. (1990). The virB operon of agrobacterium tumefaciens pTiC58 encodes 11 open reading frames. *MGG Mol. Gen. Genet.* 221, 256–266. doi: 10.1007/BF00261729

Kumar, A., and Dubey, A. (2020). Rhizosphere microbiome: engineering bacterial competitiveness for enhancing crop production. *J. Adv. Res.* 24, 337–352. doi: 10.1016/j.jare.2020.04.014

Kwak, M. J., Kong, H. G., Choi, K., Kwon, S. K., Song, J. Y., Lee, J., et al. (2018). Rhizosphere microbiome structure alters to enable wilt resistance in tomato. *Nat. Biotechnol.* 36, 1100–1116. doi: 10.1038/nbt.4232

Kwak, Y. S., Bonsall, R. F., Okubara, P. A., Paulitz, T. C., Thomashow, L. S., and Weller, D. M. (2012). Factors impacting the activity of 2,4-diacetylphloroglucinol-producing *Pseudomonas fluorescens* against take-all of wheat. *Soil Biol. Biochem.* 54, 48–56. doi: 10.1016/j.soilbio.2012.05.012

Lacroix, B., and Citovsky, V. (2016). A functional bacterium-to-plant DNA transfer machinery of rhizobium etli. *PLoS Pathog.* 12:e1005502. doi: 10.1371/journal.ppat.1005502

Lastochkina, O., Seifikalhor, M., Aliniaeifard, S., Baymiev, A., Pusenkova, L., Garipova, S., et al. (2019). Bacillus spp.: efficient biotic strategy to control postharvest diseases of fruits and vegetables. *Plan. Theory* 8:97. doi: 10.3390/plants8040097

Levy, A., Conway, J. M., Dangl, J. L., and Woyke, T. (2018a). Elucidating bacterial gene functions in the plant microbiome. *Cell Host Microbe* 24, 475–485. doi: 10.1016/j.chom.2018.09.005

Levy, A., Salas Gonzalez, I., Mittelviefhaus, M., Clingenpeel, S., Herrera Paredes, S., Miao, J., et al. (2018b). Genomic features of bacterial adaptation to plants. *Nat. Genet.* 50, 138–150. doi: 10.1038/s41588-017-0012-9

Lin, H. -H., Huang, H. -M., Yu, M., Lai, E. -M., Chien, H. -L., and Liu, C. -T. (2018). Functional exploration of the bacterial type VI secretion system in mutualism: *Azorhizobium caulinodans*ORS571– *Sesbania rostrata* as a research model. *Mol. Plant-Microbe Interact.* 31, 856–867. doi: 10.1094/MPMI-01-18-0026-R

Liu, K., Newman, M., McInroy, J. A., Hu, C. H., and Kloepper, J. W. (2017). Selection and assessment of plant growth-promoting rhizobacteria for biological control of multiple plant diseases. *Phytopathology* 107, 928–936. doi: 10.1094/PHYTO-02-17-0051-R

López-Baena, F. J., Ruiz-Sainz, J. E., Rodríguez-Carvajal, M. A., and Vinardell, J. M. (2016). Bacterial molecular signals in the Sinorhizobium fredii-soybean symbiosis. *Int. J. Mol. Sci.* 17:755. doi: 10.3390/ijms17050755

Lugtenberg, B., and Kamilova, F. (2009). Plant-growth-promoting Rhizobacteria. *Annu. Rev. Microbiol.* 63, 541–556. doi: 10.1146/annurev.micro.62.081307.162918

Marie, C., Deakin, W. J., Ojanen-Reuhs, T., Diallo, E., Reuhs, B., Broughton, W. J., et al. (2004). TtsI, a key regulator of rhizobium species NGR234 is required for type III-dependent protein secrection and synthesis of rhamnose-rich polysaccharides. *Mol. Plant-Microbe Interact.* 17, 958–966. doi: 10.1094/MPMI.2004.17.9.958

Mavrodi, D. V., Joe, A., Mavrodi, O. V., Hassan, K. A., Weller, D. M., Paulsen, I. T., et al. (2011). Structural and functional analysis of the type III secretion system from *Pseudomonas fluorescens* Q8r1-96. *J. Bacteriol.* 193, 177–189. doi: 10.1128/JB.00895-10

Mergaert, P. (2018). Role of antimicrobial peptides in controlling symbiotic bacterial populations. *Nat. Prod. Rep.* 35, 336–356. doi: 10.1039/c7np00056a

Miwa, H., and Okazaki, S. (2017). How effectors promote beneficial interactions. *Curr. Opin. Plant Biol.* 38, 148–154. doi: 10.1016/j.pbi.2017.05.011

Molina, R., Rivera, D., Mora, V., López, G., Rosas, S., Spaepen, S., et al. (2018). Regulation of IAA biosynthesis in *Azospirillum brasilense* under environmental stress conditions. *Curr. Microbiol.* 75, 1408–1418. doi: 10.1007/s00284-018-1537-6

Mongiardini, E. J., Pérez-Giménez, J., Althabegoiti, M. J., Covelli, J., Quelas, J. I., López-García, S. L., et al. (2009). Overproduction of the rhizobial adhesin RapA1 increases competitiveness for nodulation. *Soil Biol. Biochem.* 41, 2017–2020. doi: 10.1016/j.soilbio.2009.07.016

Mosquito, S., Bertani, I., Licastro, D., Compant, S., Myers, M. P., Hinarejos, E., et al. (2019). In planta colonization and role of T6SS in two rice Kosakonia endophytes. *Mol. Plant-Microbe Interact.* 33, 349–363. doi: 10.1094/MPMI-09-19-0256-R

Mukherjee, S., Keitany, G., Li, Y., Wang, Y., Ball, H. L., Goldsmith, E. J., et al. (2006). Yersinia YopJ acetylates and inhibits kinase activation by blocking phosphorylation. *Science* 312, 1211–1214. doi: 10.1126/science.1126867

Mullins, A. J., Murray, J. A. H., Bull, M. J., Jenner, M., Jones, C., Webster, G., et al. (2019). Genome mining identifies cepacin as a plant-protective metabolite

of the biopesticidal bacterium *Burkholderia ambifaria*. *Nat. Microbiol.* 4, 996–1005. doi: 10.1038/s41564-019-0383-z

Nascimento, F. X., Tavares, M. J., Franck, J., Ali, S., Glick, B. R., and Rossi, M. J. (2019). ACC deaminase plays a major role in *Pseudomonas fluorescens* YsS6 ability to promote the nodulation of alpha- and Betaproteobacteria rhizobial strains. *Arch. Microbiol.* 201, 817–822. doi: 10.1007/s00203-019-01649-5

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

Ongena, M., Jacques, P., Touré, Y., Destain, J., Jabrane, A., and Thonart, P. (2005). Involvement of fengycin-type lipopeptides in the multifaceted biocontrol potential of Bacillus subtilis. *Appl. Microbiol. Biotechnol.* 69, 29–38. doi: 10.1007/s00253-005-1940-3

Orozco-Mosqueda, M.del. C., Duan, J., DiBernardo, M., Zetter, E., Campos-García, J., Glick, B. R., et al. (2019). The production of ACC deaminase and trehalose by the plant growth promoting bacterium pseudomonas sp. UW4 synergistically protect tomato plants against salt stress. *Front. Microbiol.* 10:1392. doi: 10.3389/fmicb.2019.01392

Orozco-Mosqueda, M.d. C., Glick, B. R., and Santoyo, G. (2020). ACC deaminase in plant growth-promoting bacteria (PGPB): an efficient mechanism to counter salt stress in crops. *Microbiol. Res.* 235:126439. doi: 10.1016/j.micres.2020.126439

Pallai, R., Hynes, R. K., Verma, B., and Nelson, L. M. (2012). Phytohormone production and colonization of canola (*Brassica napus* l.) roots by *Pseudomonas fluorescens* 6-8 under gnotobiotic conditions. *Can. J. Microbiol.* 58, 170–178. doi: 10.1139/W11-120

Pedley, K. F., and Martin, G. B. (2005). Role of mitogen-activated protein kinases in plant immunity. *Curr. Opin. Plant Biol.* 8, 541–547. doi: 10.1016/j.pbi.2005.07.006

Perret, X., Freiberg, C., Rosenthal, A., Broughton, W. J., and Fellay, R. M. (1999). High-resolution transcriptional analysis of the symbiotic plasmid of rhizobium sp. NGR234. *Mol. Microbiol.* 32, 415–425. doi: 10.1046/j.1365-2958.1999.01361.x

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

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

Poole, P., Ramachandran, V., and Terpolilli, J. (2018). Rhizobia: from saprophytes to endosymbionts. *Nat. Rev. Microbiol.* 16, 291–303. doi: 10.1038/nrmicro.2017.171

Puente, M. E., Li, C. Y., and Bashan, Y. (2004). Microbial populations and activities in the rhizoplane of rock-weathering desert plants II. Growth promotion of cactus seedlings. *Plant Biol.* 6, 643–650. doi: 10.1055/s-2004-821101

Raaijmakers, J. M., Vlami, M., and De Souza, J. T. (2002). Antibiotic production by bacterial biocontrol agents. *Antonie Van Leeuwenhoek* 81, 537–547. doi: 10.1023/a:1020501420831

Rivera, D., Mora, V., Lopez, G., Rosas, S., Spaepen, S., Vanderleyden, J., et al. (2018). New insights into indole-3-acetic acid metabolism in *Azospirillum brasilense*. *J. Appl. Microbiol.* 125, 1774–1785. doi: 10.1111/jam.14080

Rodrigues, J. A., López-Baena, F. J., Ollero, F. J., Vinardell, J. M., Espuny, M. D. R., Bellogín, R. A., et al. (2007). NopM and NopD are rhizobial nodulation outer proteins: identification using LC-MALDI and LC-ESI with a monolithic capillary column. *J. Proteome Res.* 6, 1029–1037. doi: 10.1021/pr060519f

Rodríguez, 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

Rohde, J. R., Breitkreutz, A., Chenal, A., Sansonetti, P. J., and Parsot, C. (2007). Type III secretion effectors of the IpaH family are E3 ubiquitin ligases. *Cell Host Microbe* 1, 77–83. doi: 10.1016/j.chom.2007.02.002

Russo, D. M., Williams, A., Edwards, A., Posadas, D. M., Finnie, C., Dankert, M., et al. (2006). Proteins exported via the PrsD-PrsE type I secretion system and the acidic exopolysaccharide are involved in biofilm formation by *Rhizobium leguminosarum*. *J. Bacteriol.* 188, 4474–4486. doi: 10.1128/JB.00246-06

Saad, M. M., Staehelin, C., Broughton, W. J., and Deakin, W. J. (2008). Protein-protein interactions within type III secretion system-dependent pili of *Rhizobium* sp. strain NGR234. *J. Bacteriol.* 190, 750–754. doi: 10.1128/JB.01116-07

Sachs, J. L., Quides, K. W., and Wendlandt, C. E. (2018). Legumes versus rhizobia: a model for ongoing conflict in symbiosis. *New Phytol.* 219, 1199–1206. doi: 10.1111/nph.15222

Salinero-Lanzarote, A., Pacheco-Moreno, A., Domingo-Serrano, L., Durán, D., Ormeño-Orrillo, E., Martínez-Romero, E., et al. (2019). Type VI secretion system of rhizobium etli Mim1 has a positive effect in symbiosis. *FEMS Microbiol. Ecol.* 95:fiz054. doi: 10.1093/femsec/fiz054

Scagliola, M., Pii, Y., Mimmo, T., Cesco, S., Ricciuti, P., and Crecchio, C. (2016). Characterization of plant growth promoting traits of bacterial isolates from the rhizosphere of barley (*Hordeum vulgare* L.) and tomato (Solanum lycopersicon L.) grown under Fe sufficiency and deficiency. *Plant Physiol. Biochem.* 107, 187–196. doi: 10.1016/j.plaphy.2016.06.002

Schmeisser, C., Liesegang, H., Krysciak, D., Bakkou, N., Le Quéré, A., Wollherr, A., et al. (2009). Rhizobium sp. strain NGR234 possesses a remarkable number of secretion systems. *Appl. Environ. Microbiol.* 75, 4035–4045. doi: 10.1128/AEM.00515-09

Sgro, G. G., Oka, G. U., Souza, D. P., Cenens, W., Bayer-Santos, E., Matsuyama, B. Y., et al. (2019). Bacteria-killing type IV secretion systems. *Front. Microbiol.* 10:1078. doi: 10.3389/fmicb.2019.01078

Shahid, I., Rizwan, M., Baig, D. N., Saleem, R. S., Malik, K. A., and Mehnaz, S. (2017). Secondary metabolites production and plant growth promotion by *Pseudomonas chlororaphis* and *P. aurantiaca* strains isolated from cactus, cotton, and Para grass. *J. Microbiol. Biotechnol. J. Microbiol. Biotechnol.* 27, 480–491. doi: 10.4014/jmb.1601.01021

Sharma, A., and Johri, B. N. (2003). Growth promoting influence of siderophore-producing pseudomonas strains GRP3A and PRS9 in maize (*Zea mays* L.) under iron limiting conditions. *Microbiol. Res.* 158, 243–248. doi: 10.1078/0944-5013-00197

Skorpil, P., Saad, M. M., Boukli, N. M., Kobayashi, H., Ares-Orpel, F., Broughton, W. J., et al. (2005). NopP, a phosphorylated effector of *Rhizobium* sp. strain NGR234, is a major determinant of nodulation of the tropical legumes *Flemingia congesta* and *Tephrosia vogelii*. *Mol. Microbiol.* 57, 1304–1317. doi: 10.1111/j.1365-2958.2005.04768.x

Smit, G., Kijne, J. W., and Lugtenberg, B. J. (1987). Involvement of both cellulose fibrils and a Ca2+−dependent adhesin in the attachment of *Rhizobium leguminosarum* to pea root hair tips. *J. Bacteriol.* 169, 4294–4301. doi: 10.1128/jb.169.9.4294-4301.1987

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., Dobbelaere, S., Croonenborghs, A., and Vanderleyden, J. (2008). Effects of Azospirillum brasilense indole-3-acetic acid production on inoculated wheat plants. *Plant Soil* 312, 15–23. doi: 10.1007/s11104-008-9560-1

Srinivasan, V. B., Vaidyanathan, V., and Rajamohan, G. (2015). AbuO, a tolc-like outer membrane protein of Acinetobacter baumannii, is involved in antimicrobial and oxidative stress resistance. *Antimicrob. Agents Chemother.* 59, 1236–1245. doi: 10.1128/AAC.03626-14

Sullivan, J. T., Trzebiatowski, J. R., Cruickshank, R. W., Gouzy, J., Brown, S. D., Elliot, R. M., et al. (2002). Comparative sequence analysis of the symbiosis island of *Mesorhizobium loti* strain R7A. *J. Bacteriol.* 184, 3086–3095. doi: 10.1128/JB.184.11.3086-3095.2002

Tampakaki, A. P. (2014). Commonalities and differences of T3SSs in rhizobia and plant pathogenic bacteria. *Front. Plant Sci.* 5:114. doi: 10.3389/fpls.2014.00114

Teulet, A., Busset, N., Fardoux, J., Gully, D., Chaintreuil, C., Cartieaux, F., et al. (2019). The rhizobial type III effector ErnA confers the ability to form nodules in legumes. *Proc. Natl. Acad. Sci. U. S. A.* 116, 21758–21768. doi: 10.1073/pnas.1904456116

Trapet, P., Avoscan, L., Klinguer, A., Pateyron, S., Citerne, S., Chervin, C., et al. (2016). The *Pseudomonas fluorescens* siderophore pyoverdine weakens *Arabidopsis thaliana* defense in favor of growth in iron-deficient conditions. *Plant Physiol.* 171, 675–693. doi: 10.1104/pp.15.01537

Tzfira, T., Vaidya, M., and Citovsky, V. (2004). Involvement of targeted proteolysis in plant genetic transformation by agrobacterium. *Nature* 431, 87–92. doi: 10.1038/nature02857

Van Wees, S. C. M., Pieterse, C. M. J., Trijssenaar, A., Van't Westende, Y. A. M., Hartog, F., and Van Loon, L. C. (1997). Differential induction of systemic resistance in arabidopsis by biocontrol bacteria. *Mol. Plant-Microbe Interact.* 10, 716–724. doi: 10.1094/MPMI.1997.10.6.716

Vande Broek, A., Lambrecht, M., Eggermont, K., and Vanderleyden, J. (1999). Auxins upregulate expression of the indole-3-pyruvate decarboxylase gene in *Azospirillum brasilense*. *J. Bacteriol.* 181, 1338–1342. doi: 10.1128/jb.181.4.1338-1342.1999

Vesga, P., Flury, P., Vacheron, J., Keel, C., Croll, D., and Maurhofer, M. (2020). Transcriptome plasticity underlying plant root colonization and insect invasion by Pseudomonas protegens. *ISME J.* 14, 2766–2782. doi: 10.1038/s41396-020-0729-9

Viprey, V., Del Greco, A., Golinowski, W., Broughton, W. J., and Perret, X. (1998). Symbiotic implications of type III protein secretion machinery in rhizobium. *Mol. Microbiol.* 28, 1381–1389. doi: 10.1046/j.1365-2958.1998.00920.x

Wang, D., Yang, S., Tang, F., and Zhu, H. (2012). Symbiosis specificity in the legume - rhizobial mutualism. *Cell. Microbiol.* 14, 334–342. doi: 10.1111/j.1462-5822.2011.01736.x

Weller, D. M. (2007). Pseudomonas biocontrol agents of soilborne pathogens: looking back over 30 years. *Phytopathology* 97, 250–256. doi: 10.1094/PHYTO-97-2-0250

Weselowski, B., Nathoo, N., Eastman, A. W., MacDonald, J., and Yuan, Z. C. (2016). Isolation, identification and characterization of *Paenibacillus polymyxa* CR1 with potentials for biopesticide, biofertilization, biomass degradation and biofuel production. *BMC Microbiol.* 16:244. doi: 10.1186/s12866-016-0860-y

Wu, G., Liu, Y., Xu, Y., Zhang, G., Shen, Q., and Zhang, R. (2018). Exploring elicitors of the beneficial Rhizobacterium *Bacillus amyloliquefaciens* SQR9 to induce plant systemic resistance and their interactions with plant signaling pathways. *Mol. Plant-Microbe Interact.* 31, 560–567. doi: 10.1094/MPMI-11-17-0273-R

Xin, D. W., Liao, S., Xie, Z. P., Hann, D. R., Steinle, L., Boller, T., et al. (2012). Functional analysis of NopM, a novel E3 ubiquitin ligase (NEL) domain effector of *Rhizobium* sp. strain NGR234. *PLoS Pathog.* 8:e1002707. doi: 10.1371/journal.ppat.1002707

Yang, M., Mavrodi, D. V., Mavrodi, O. V., Thomashow, L. S., and Weller, D. M. (2020). Exploring the pathogenicity of pseudomonas brassicacearum Q8r1-96 and other strains of the *Pseudomonas fluorescens* complex on tomato. *Plant Dis.* 104, 1026–1031. doi: 10.1094/pdis-09-19-1989-re

Yasmin, F., Othman, R., Sijam, K., and Saad, M. S. (2009). Characterization of beneficial properties of plant growth-promoting rhizobacteria isolated from sweet potato rhizosphere. *Afr. J. Microbiol. Res.* 3, 815–821.

Yu, K., Pieterse, C. M. J., Bakker, P. A. H. M., and Berendsen, R. L. (2019a). Beneficial microbes going underground of root immunity. *Plant Cell Environ.* 42, 2860–2870. doi: 10.1111/pce.13632

Yu, K., Tichelaar, R., Liu, Y., Savant, N., Lagendijk, E., Van Kuijk, S., et al. (2019b). Plant-beneficial *Pseudomonas* spp. Suppress local root immune responses by Gluconic acid-mediated lowering of environmental pH. *SSRN Electron. J.* 42, 2860–2870. doi: 10.2139/ssrn.3396501

Yu, X., Ai, C., Xin, L., and Zhou, G. (2011). The siderophore-producing bacterium, *Bacillus subtilis* CAS15, has a biocontrol effect on Fusarium wilt and promotes the growth of pepper. *Eur. J. Soil Biol.* 47, 138–145. doi: 10.1016/j.ejsobi.2010.11.001

Zamioudis, C., and Pieterse, C. M. J. (2012). Modulation of host immunity by beneficial microbes. *Mol. Plant-Microbe Interact.* 25, 139–150. doi: 10.1094/MPMI-06-11-0179

Zehner, S., Schober, G., Wenzel, M., Lang, K., and Göttfert, M. (2008). Expression of the Bradyrhizobium japonicum type III secretion system in legume nodules and analysis of the associated tts box promoter. *Mol. Plant-Microbe Interact.* 21, 1087–1093. doi: 10.1094/MPMI-21-8-1087

Zhalnina, K., Louie, K. B., Hao, Z., Mansoori, N., da Rocha, U. N., Shi, S., et al. (2018). Dynamic root exudate chemistry and microbial substrate preferences drive patterns in rhizosphere microbial community assembly. *Nat. Microbiol.* 3, 470–480. doi: 10.1038/s41564-018-0129-3

Zhang, L., Chen, X. J., Lu, H. B., Xie, Z. P., and Staehelin, C. (2011). Functional analysis of the type 3 effector nodulation outer protein L (NopL) from *Rhizobium* sp. NGR234: symbiotic effects, phosphorylation, and interference with mitogen-activated protein kinase signaling. *J. Biol. Chem.* 286, 32178–32187. doi: 10.1074/jbc.M111.265942

Zheng, N., and Shabek, N. (2017). Ubiquitin ligases: structure, function, and regulation. *Annu. Rev. Biochem.* 86, 129–157. doi: 10.1146/annurev-biochem-060815-014922

Zhou, L., Wang, C., Wang, G. -H., Wei, Z. -W., Fu, Q. -X., Hang, X. -H., et al. (2020). Chemical targeting and manipulation of type III secretion in the Phytopathogen Xanthomonas campestris for control of disease. *Appl. Environ. Microbiol.* 86, e02349–e02419. doi: 10.1128/AEM.02349-19

Zupan, J. R., and Zambryski, P. (1995). Transfer of t-DNA from agrobacterium to the plant cell. *Plant Physiol.* 107, 1041–1047. doi: 10.1104/pp.107.4.1041

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

MINI REVIEW
published: 09 November 2020
doi: 10.3389/fpls.2020.594530

Elicitors of Plant Immunity Triggered by Beneficial Bacteria

Jelena Pršić and Marc Ongena**

Microbial Processes and Interactions Laboratory, Terra Teaching and Research Center, Gembloux Agro-Bio Tech, University of Liège, Gembloux, Belgium

The molecular basis of plant immunity triggered by microbial pathogens is being well-characterized as a complex sequential process leading to the activation of defense responses at the infection site, but which may also be systemically expressed in all organs, a phenomenon also known as systemic acquired resistance (SAR). Some plant-associated and beneficial bacteria are also able to stimulate their host to mount defenses against pathogen ingress via the phenotypically similar, induced systemic resistance phenomenon. Induced systemic resistance resembles SAR considering its mechanistic principle as it successively involves recognition at the plant cell surface, stimulation of early cellular immune-related events, systemic signaling via a fine-tuned hormonal cross-talk and activation of defense mechanisms. It thus represents an indirect but efficient mechanism by which beneficial bacteria with biocontrol potential improve the capacity of plants to restrict pathogen invasion. However, according to our current vision, induced systemic resistance is specific considering some molecular aspects underpinning these different steps. Here we overview the chemical diversity of compounds that have been identified as induced systemic resistance elicitors and thereby illustrating the diversity of plants species that are responsive as well as the range of pathogens that can be controlled via this phenomenon. We also point out the need for further investigations allowing better understanding how these elicitors are sensed by the host and the diversity and nature of the stimulated defense mechanisms.

Keywords: plant immunity, systemic resistance, defense mechanism, molecular patterns, plant growth promoting rhizobacteria, biocontrol

OPEN ACCESS

Edited by:
Roeland Lucas Berendsen, Utrecht University, Netherlands

Reviewed by:
Pierre Pétriacq, Université de Bordeaux, France
Zhonglin Mou, University of Florida, United States

****Correspondence:***
Jelena Pršić
jelena.prsic@student.uliege.be
Marc Ongena
marc.ongena@uliege.be

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

Received: *13 August 2020*
Accepted: *07 October 2020*
Published: *09 November 2020*

Citation:
Pršić J and Ongena M (2020) Elicitors of Plant Immunity Triggered by Beneficial Bacteria. Front. Plant Sci. 11:594530. doi: 10.3389/fpls.2020.594530

INTRODUCTION

Some bacteria isolated from the root microbiome have been selected for their remarkable beneficial effect provided to their host plant and are referred to as plant growth-promoting rhizobacteria (PGPR) (Backer et al., 2018; Singh et al., 2019). These PGPR favor plant growth notably by facilitating nutrient availability and modulating the host's hormonal balance but also display plant protective activity toward pathogen ingress. This biocontrol potential relies on several traits including the ability to efficiently compete for space and nutrients with pathogens, a strong direct antagonistic activity based on secretion of low-size antimicrobials or hydrolytic enzymes and the capacity to stimulate induced systemic resistance (ISR) (Pieterse et al., 2014; Köhl et al., 2019). ISR is a systemically expressed resistance state that renders the host less susceptible to subsequent infection, and it is of great interest from an agronomical perspective because effective against a broad spectrum of microbial pathogens, nematodes, and insects (Pieterse et al., 2014;

Grady et al., 2016; Rashid and Chung, 2017; Mhatre et al., 2018). Phenotypically, ISR resembles the systemic acquired resistance (SAR) mounted upon pathogen perception, which culminates from a complex immune-related process. At the front line, molecular features of invading microbes are detected with high specificity and sensitivity in the apoplast by a range of plasma membrane-anchored immune sensors referred to as pattern recognition receptors (PRR). These PRRs bind precise structural motifs (epitopes) of larger (macro)molecules retaining important functions for microbial fitness and thus widely conserved across species and termed microbe-associated molecular patterns (MAMPs). Some of the best described MAMPs from bacterial pathogens are flg22, a 22 amino acids portion of the flagellin protein, elf18 which is the epitope of the elongation factor EF-Tu, surface-exposed lipopolysaccharides (LipidA), and peptidoglycan (sugar backbone) (Schellenberger et al., 2019). The binding of MAMP by PRR initiates within minutes early immune-related events in responsive cells such as ion fluxes, oxidative burst, and phosphorylation cascade. Furthermore, defense mechanisms *sensu stricto* such as cell wall reinforcement and production of antimicrobial enzymes and secondary metabolites, referred to as phytoalexins, are stimulated (Piasecka et al., 2015). This PRR-mediated immune response [pattern triggered immunity (PTI)] is robust as it can detect MAMPs at nanomolar concentrations leading to fast and strong defensive responses (high transcriptional activation) but associated with growth-defense tradeoffs that reduce plant fitness (Huot et al., 2014). However, adapted pathogens use protein effectors injected into host cells to dampen PTI and subvert this first line of immune reaction. In turn, plants have evolved intracellular sensors called R (resistance) proteins, which interfere with these effectors leading to the effector-triggered type of immunity (ETI) which may, like PTI, lead to SAR (Kanyuka and Rudd, 2019; Schellenberger et al., 2019).

Due to the conserved nature of MAMPs, the plant's receptor equipment would enable detection not only of pathogenic bacteria, but also of beneficials such as rhizobial symbiots and root-associated epiphytic PGPR (Zipfel and Oldroyd, 2017). These beneficial microbes have thus to evade (by hiding or changing their MAMPs) or suppress (by secreting effectors that interfere with immune responses and signaling) PTI in order to establish a mutualistic relationship with their host (Stringlis et al., 2018; Yu et al., 2019). This has been recently illustrated with the *Pseudomonas simiae* strain WCS417 first detected as a pathogen via its flg22 epitope, but which then strongly attenuates the host immune response presumably via T3SS-mediated delivery of effectors (Berendsen et al., 2015; Stringlis et al., 2019). Subversion of host immune reaction allows PGPR to sustainably colonize the rhizoplane and establish threshold populations necessary for providing beneficial functions including ISR triggering via the secretion of other elicitors not related to MAMPs.

Here we provide an updated overview of those PGPR determinants responsible for ISR elicitation that are in most cases small-size compounds secreted by the colonizing bacteria even if some proteins isolated from *Brevibacillus laterosporus*, *B. amyloliquefaciens*, and *Saccharothrix yanglingensis* have also been recently proposed as bacterial triggers (**Table 1**) (Wang et al., 2015, 2016; Zhang et al., 2018). We highlight their chemical diversity and structure- and dose-dependent activity but only refer to compounds that have been tested in pure (proven) form at biologically relevant concentrations and/or via specifically suppressed mutants and for which the ability to stimulate systemic resistance was clearly demonstrated to be independent from direct antimicrobial effect.

PGPR METABOLITES IDENTIFIED AS ELICITORS OF PLANT SYSTEMIC RESISTANCE

Acyl-Homoserine Lactones

Many Gram-negative bacteria produce *N*-acyl-homoserine lactones (AHLs) as quorum-sensing molecules involved in cell-to-cell communication in order to monitor their behavior according to population density. Some AHLs are not only the mean of communication between bacterial cells, but also may be used as signal in inter-kingdom interaction and act as plant growth promoting compounds and/or as immunity elicitors (reviewed in Schikora et al., 2016). A first study showed, by using an AHL-suppressed mutant of *Serratia liquefaciens* MG1, that induced resistance in tomato toward *Alternaria alternata* by this strain is AHL-dependent (Schuhegger et al., 2006). Several works performed with purified molecules further revealed that AHL bioactivity is structure-dependent. AHLs with short length acyl chains have been mostly demonstrated to promote plant growth, whereas AHLs with longer fatty acid (C12, C14) are better described as elicitors of resistance (Schenk et al., 2012; Zarkani et al., 2013; Schikora et al., 2016). The long-chain *N*-3-oxo-tetradecanoyl-L-homoserine lactone (oxo-C14-HSL; **Figure 1-1**) has been amply demonstrated for immunity elicitation in many pathosystems. Upon treatment at 6 μM, oxo-C14-HSL successfully induced systemic resistance against biotrophs (*Blumeria graminis* f. sp. *hordei*, *Puccinia hordei*, *Golovinomyces orontii*) and hemibiotroph (*P. syringae* DC3000) in barley and Arabidopsis, but not against the necrotrophs *Botrytis cinerea* and *Plectosphaerella cucumerina* BMM (Schikora et al., 2011; Schenk et al., 2012; Wehner et al., 2019). ISR-eliciting activity of oxo-C14-HSL was also observed in wheat and tomato against *Puccinia graminis* f. sp. *tritici* and *Phytophthora infestans*, respectively (Hernández-Reyes et al., 2014). Collectively, these data indicate that the plant defensive response triggered by the same oxo-C14-HSL molecule may not be efficient in enhancing resistance against phytopathogens with necrotrophic lifestyle.

Cyclic Lipopeptides

Rhizobacterial cyclic lipopeptides (CLPs) are multifunctional secondary metabolites involved in developmental processes such as motility and biofilm formation and in biocontrol primarily based on their antimicrobial activity (Ongena and Jacques, 2008; Geudens and Martins, 2018). However, some CLPs secreted by beneficial bacilli and pseudomonads have emerged as an important category of plant immunity elicitors as well.

TABLE 1 | PGPR produced elicitors of systemic resistance.

Elicitor	Strain	Plant	Pathogen	Method	References
Acyl-homoserine lactones					
Precise structure not defined	*Serratia liquefaciens* MG1	Tomato	*Alternaria alternata*	Mutant	Schuhegger et al., 2006
oxo-C14-HSL_		Arabidopsis Barley	*P. syringae* pv. *tomato* DC3000 *Golovinomyces orontii Blumeria graminis* f. sp. *hordei*	Pure 6 μM	Schikora et al., 2011
		Arabidopsis	*P. syringae* pv *tomato* DC3000	Pure 6 μM	Schenk et al., 2012
	Sinorhizobium meliloti Rm2011	Arabidopsis	*P. syringae* pv *tomato*	Mutant	Zarkani et al., 2013
	Sinorhizobium meliloti	Barley Wheat Tomato	*Blumeria graminis* f. sp. *hordei* *Puccinia graminis* f. sp. *tritici* *Phytophthora infestans*	Mutant	Hernández-Reyes et al., 2014
Cyclic lipopeptides					
Surfactin	*Bacillus subtilis* 168	Bean Tomato	*Botrytis cinerea*	Mutant, pure 5 μM	Ongena et al., 2007
	B. subtilis UMAF6639	Melon	*Podosphaera fusca*	Mutant, Mutant + pure, pure 10 μM	García-Gutiérrez et al., 2013
	B. amyloliquefaciens S499/FZB42/QST713, *B. subtilis* 98S/BNO1, *Paenibacillus polymyxa* 56	Tomato	*B. cinerea*	SRF-producing strains, pure 10 μM	Cawoy et al., 2014
	B. amyloliquefaciens S13-3	Strawberry	*Colletotrichum gloeosporioides*	Pure 50 μM	Yamamoto et al., 2015
	B. amyloliquefaciens FZB42-AK3	*Lolium perenne* L.	*Magnaporthe oryzae*	Semi-purified ~250, ~500 μM	Rahman et al., 2015
	B. subtilis	Peanut	*Sclerotium rolfsii*	Pure 5, 10 μM	Rodríguez et al., 2018
	B. amyloliquefaciens S499	Wheat	*Zymoseptoria tritici*	Pure 1, 10, 100 μM	Le Mire et al., 2018
	B. subtilis BBG131/BBG125/ Bs2504	*idem*	*idem*	Pure surfactin + mycosubtilin 100 μM, 50/50	Mejri et al., 2018
Mycosubtilin	*idem*	*idem*	*idem*	Pure ~100 μM	*same study*
Fengycin	*B. amyloliquefaciens* FZB42	Tomato	*S. sclerotiorum*	Pure ~60 μM	Farzand et al., 2019
Iturin	*B. amyloliquefaciens* S13-3	Strawberry	*Colletotrichum gloeosporioides*	Pure 10 μM	Yamamoto et al., 2015
	B. amyloliquefaciens 41B-1	Cotton	*Verticillium dahliae*	Pure 50 μM	Han et al., 2015
		Arabidopsis	*C. gloeosporioides*	Pure 10 μM	Kawagoe et al., 2015
	B. vallismortis EXTN-1	Chili pepper	*Phytophthora capsici*	Pure 1, 10 μM	Park et al., 2016
Massetolide A	*Pseudomonas fluorescens* SS101	Tomato	*P. infestans*	Mutant, pure ~ 45, 90 μM	Tran et al., 2007
Orfamide	*Pseudomonas* sp. CMR12a	*Brassica chinensis* Bean	*R. solani* AG 2-1 *R. solani* AG 4-HGI	Mutant	Olorunleke et al., 2015
	Pseudomonas sp. CMR12a	Bean	*R. solani* AG2-2	Mutant, pure 1–100 nM	Ma et al., 2016
	Pseudomonas sp. CMR12a	Rice	*Cochliobolus miyabeanus*	Mutant, pure 25 μM	Ma et al., 2017
Sessilin	*Pseudomonas* sp. CMR12a	Bean	*R. solani* AG 2-2 *R. solani* AG 4-HGI	Mutant	D'aes et al., 2011
	Pseudomonas sp. CMR12a	*Brassica chinensis* Bean	*R. solani* AG 2-1 *R. solani* AG 4-HGI	Mutant	Olorunleke et al., 2015
	Pseudomonas sp. CMR12a	Bean	*R. solani AG2-2*	Mutant, crude extract 1, 10 μg/L	Ma et al., 2016
Lokisin	*Pseudomonas* sp. COR10	Rice	*M. oryzae*	Crude extract 25 μg/ml, one CLP-producing strains	Omoboye et al., 2019
WLIP	*Pseudomonas* sp. COW10	*idem*	*idem*	*Idem*	*same study*
Entolysin	*Pseudomonas* sp. COR5	*idem*	*idem*	*Idem*	*same study*
Rhamnolipids					
	P. aeruginosa	Grapevine	*B. cinerea*	Pure ~150 μM	Varnier et al., 2009
	P. aeruginosa	Arabidopsis	*P. syringae* pv *tomato, Hyaloperonospora arabidopsidis, B. cinerea*	Pure ~300 μM	Sanchez et al., 2012
	P. aeruginosa	*Brassica napus*	*B. cinerea*	Pure 10, 100 μM	Monnier et al., 2018

(Continued)

TABLE 1 | Continued

Elicitor	Strain	Plant	Pathogen	Method	References
***N*-alkylated benzylamine derivative**					
NABD	*P. putida* BTP1	Bean	*B. cinerea*	Mutant, pure 0.2 μM	Ongena et al., 2005
	P. putida BTP1	Bean Cucumber	*B. cinerea* *Colletotrichum lagenarium*	Pure 1 μM	Ongena et al., 2008
Siderophores					
Pyoverdine	*P. fluorescens* CHA0	Tobacco	*Tobacco necrosis virus* (TNV)	Mutant	Maurhofer et al., 1994
	P. fluorescens WCS374	Radish	*Fusarium oxysporum* f. sp. *raphani*	Mutant, pure 70 μg/root	Leeman et al., 1996
	P. putida WCS358	Tomato Bean Arabidopsis	*Colletotrichum lindemuthianum* *B. cinerea* *P. syringae* pv. *tomato*	Mutant, Pure 3.02 μg pyoverdine/g soil	Meziane et al., 2005
	P. putida WCS358r	*Eucalyptus urophylla*	*Ralstonia solanacearum*	Mutant, pure 10–100 μg/ml	Ran et al., 2005
	P. fluorescens WCS374r	Rice	*M. oryzae*	Mutant, pure 70 μg per root	De Vleesschauwer et al., 2008
Salicylic acid	*P. aeruginosa 7NSK2*	Bean	*B. cinerea*	Pure 100 nM	De Meyer et al., 1999
	P. aeruginosa 7NSK2	Grapevine	*B. cinerea*	Mutant	Verhagen et al., 2010
Pyochelin + SA/pyoverdine	*P. aeruginosa* 7NSK2	Tomato	*B. cinerea*	Mutant	Audenaert et al., 2002
Elicitors with antibiotic function					
DAPG	*P. fluorescens* CHA0	Tomato	*Meloidogyne javanica*	Mutant	Siddiqui and Shaukat, 2003
	P. fluorescens CHA0	Arabidopsis	*Peronospora parasitica*	Mutant, pure 10, 100 μM	Iavicoli et al., 2003
	P. fluorescens Q2-87	Arabidopsis	*P. syringae* pv. *tomato*	Mutant, pure 10–250 μM	Weller et al., 2012
		Arabidopsis	*P. syringae* pv. *tomato* *B. cinerea*	Pure 10, 100, 200 μM	Chae et al., 2020
Phenazine	*Pseudomonas* sp. CMR12a	Bean	*R. solani* AG 2-2 *R. solani* AG 4- HGI	Mutant	D'aes et al., 2011
	Pseudomonas sp. CMR12a	*Brassica chinensis* Bean	*R. solani* AG 2-1 *R. solani* AG 4-HGI.	Mutant	Olorunleke et al., 2015
	Pseudomonas sp. CMR12a	Rice Bean	*R. solani AG2-2*	Mutant pure 0.1, 1 μM	Ma et al., 2016
Volatile organic compounds					
2,3-butanediol	*B. subtilis* GB03, *B. amyloliquefaciens* IN937a	Arabidopsis	*Erwinia carotovora* subsp. *carotovora*	Mutant	Ryu et al., 2004
	P. chlororaphis O6	Tobacco	*E. carotovora*	Pure 100 μg/root	Han et al., 2006
		Pepper	*Cucumber mosaic virus*, *Tobacco mosaic virus*, *Pepper mottle virus*, *Tomato yellow leaf curl virus*, *Tomato spotted wilt virus*	Pure 1, 5, 10 mM	Kong et al., 2018
	Enterobacter aerogenes	Maize	*Setosphaeria turcica*	Pure 22 mM	D'Alessandro et al., 2014
3-pentanol	*B. amyloliquefaciens* IN937a	Pepper	*Xanthomonas axonopodis* pv. *vesicatoria*	Pure 10 μM, 1 mM	Choi et al., 2014
	B. subtilis GB03	Arabidopsis	*P. syringae* pv. *tomato* DC3000	Pure 100 nM, 10 μM	Song et al., 2015
Tridecane	*Paenibacillus polymyxa* E681	Arabidopsis	*Pseudomonas syringae* pv. *maculicola* ES4326	Pure 100 μM	Lee et al., 2012
Hexadecane	*Paenibacillus polymyxa* E681	Arabidopsis	*P. syringae* pv *maculicola*, *Pectobacterium carotovorum* subsp. *carotovorum*	Pure 1, 100 μM	Park et al., 2013

Abbreviations: oxo-C14-HSL, N,N-dimethyl-N- tetradecyl-N-benzylammonium; NABD, N,N-dimethyl-N- tetradecyl-N-benzylammonium; DAPG, 2,4-diacetyl phloroglucinol.

Surfactin is a heptapeptide occurring as a mix of naturally co-produced homologs varying in the length of the fatty acid chain (**Figure 1-2**). This CLP is among the *Bacillus* compounds best described as trigger of systemic resistance. When applied as pure compound on roots, in micromolar amounts (5–10 μM), surfactin demonstrated to induce ISR in bean, tomato, tobacco, against *B. cinerea,* in melon against *Podosphaera fusca*, and peanut, against *Sclerotium rolfsii* (Ongena et al., 2007; García-Gutiérrez et al., 2013; Cawoy et al., 2014; Rodríguez et al., 2018). The structure of this CLP may strongly impact its

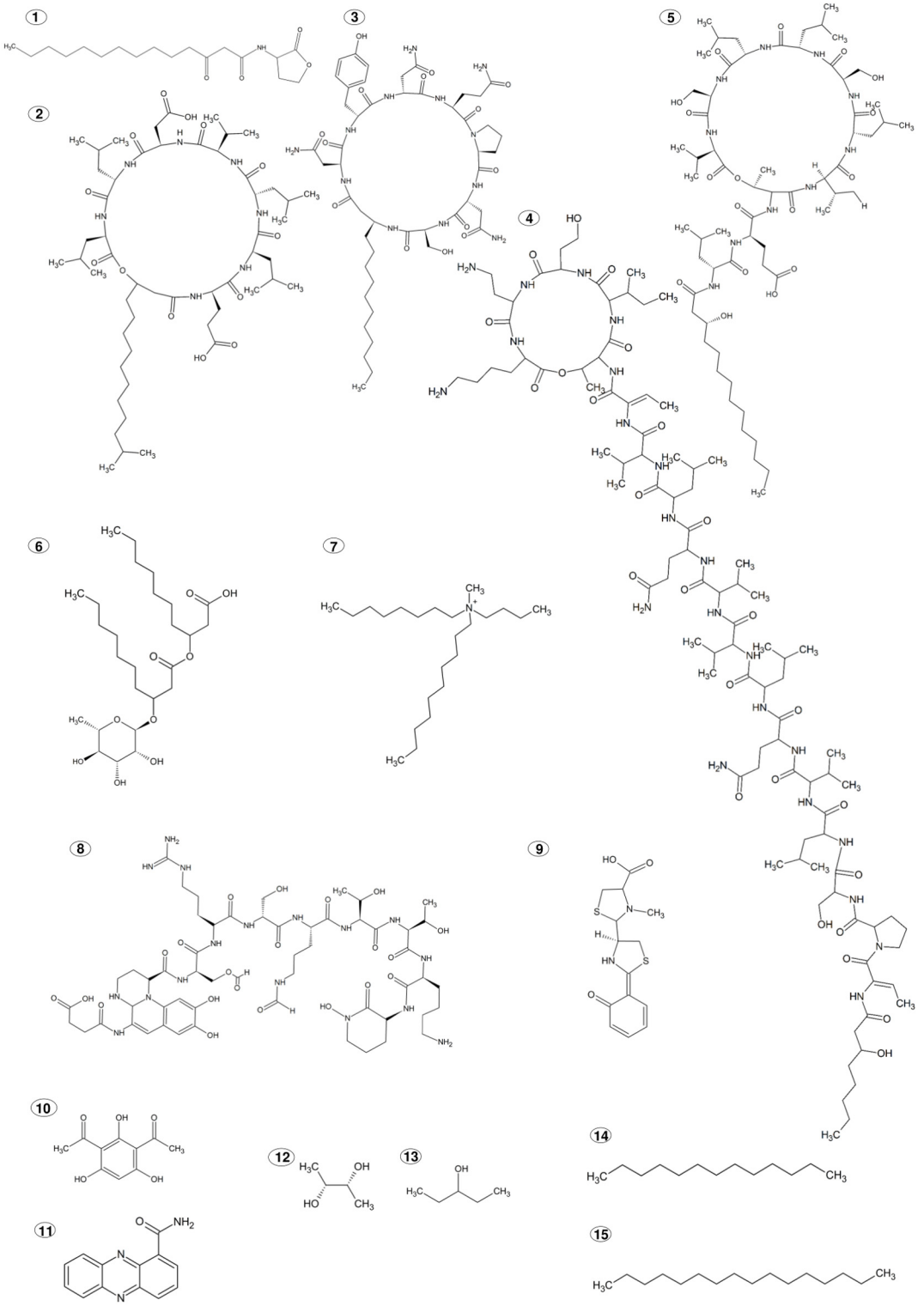

FIGURE 1 | Chemical structures of PGPR produced ISR elicitors. **(1)** *N*-3-oxo-tetradecanoyl-L-homoserine lactone; **(2)** Surfactin; **(3)** Iturin; **(4)** Sessilin; **(5)** Orfamide; **(6)** Rhamnolipid, L-rhamnosyl-3-hydroxydecanoyl-3-hydroxydecanoic acid; **(7)** *N,N*-dimethyl-*N*- tetradecyl-*N*-benzylammonium; **(8)** Pyoverdine; **(9)** Pyochelin; **(10)** 2,4-diacetyl phloroglucinol; **(11)** phenazine-1-carboxamide; **(12)** 2,3-butanediol; **(13)** 3-pentanol; **(14)** Tridecane; **(15)** Hexadecane.

elicitor activity as observed on tobacco cells. Linear and/or methylated derivatives are much less efficient and only homologs with long C14 and C15 acyl chains are active at inducing early immune-related events, unlike C12 and C13 (Jourdan et al., 2009; Henry et al., 2011). Despite its high bioactivity on dicot plant roots, surfactin shows low competence in mounting immunity when applied on dicots leaves or monocots roots (Rahman et al., 2015; Yamamoto et al., 2015; Mejri et al., 2018). Induction of systemic resistance and/or plant defenses by other CLPs produced by biocontrol bacilli has also been occasionally reported. In tomato and grapevine, fengycin triggered ISR against *B. cinerea* and *Plasmopara viticola* (Farzand et al., 2019; Li et al., 2019), while CLPs from the iturin group (**Figure 1-3**) were shown to have a similar role in strawberry (Yamamoto et al., 2015), cotton (Han et al., 2015), grapevine (Farace et al., 2015) and Arabidopsis (Wu et al., 2018). Additionally illustrating the dependence of CLP-mediated ISR on the plant organ, iturin showed bioactivity on roots at concentration of 50 μM, whereas it was active already at 1–10 μM when applied on leaves (Han et al., 2015; Kawagoe et al., 2015; Yamamoto et al., 2015; Park et al., 2016). The activation of defense genes in Arabidopsis by iturin foliar treatment was also dependent on the molecule's structure, i.e., cyclization and/or length of the b-hydroxy fatty acid chain (Kawagoe et al., 2015).

Several studies have also reported the involvement of *Pseudomonas* sp. CLPs in plant resistance stimulation on various pathosystems. Massetolide A was the first reported for its resistance-inducing activity in tomato against *P. infestans* (Tran et al., 2007). Recent works conducted with *Pseudomonas* sp. strain CMR12a revealed that two other types of CLPs, sessilin and orfamide (**Figures 1-4,5**, respectively) are involved in ISR-triggering albeit differently according to the pathosystem tested. Works combining the use of biosynthesis mutants and CLP extracts showed that both sessilin and orfamide are determinants of ISR in bean against *Rhizoctonia solani*, but that a balanced production is needed for optimal effect. These two compounds are indeed active at precise range of concentrations from 0.001 to 0.1 μM for pure orfamide, and 0.001 and 0.01 mg/L for sessilin as crude extract (Olorunleke et al., 2015; Ma et al., 2016). In monocots, such as rice, *Pseudomonas* CLP structure is crucial in determining elicitor effect on a given pathosystem. For example, orfamide applied at 25 μM was identified as elicitor inducing resistance to *Cochliobolus miyabeanus*, but it is not active against *Magnaporthe oryzae* (Ma et al., 2017). Nevertheless, CLPs such as WLIP, lokisin, and entolysin were recently described to successfully induce resistance toward this last pathogen, confirming elicitor specificity for certain pathosystems (Omoboye et al., 2019).

Rhamnolipids

Rhamnolipids (RLs) are amphiphilic glycolipids produced by various species including pathogenic isolates, but also some plant beneficial *Pseudomonas* and *Burkholderia* species (Perneel et al., 2008; Abdel-Mawgoud et al., 2010). They are essential for bacterial surface motility and biofilm development (Vatsa et al., 2010; Chrzanowski et al., 2012). Mono- and di-RLs (**Figure 1-6**) tested as pure compounds have been shown to elicit plant defense responses and to induce resistance against various pathogens in grapevine, Arabidopsis, and *Brassica napus* using a wide range of concentrations from approximately 10 up to 300 μM (Varnier et al., 2009; Sanchez et al., 2012; Monnier et al., 2018, 2020).

N-Alkylated Benzylamine Derivative

Although its function for bacterial life is not clear, an *N*-alkylated benzylamine derivative (NABD; **Figure 1-7**) produced by *P. putida* BTP1, was identified as elicitor (Ongena et al., 2005). Treatment of bean and cucumber roots with pure NABD at low micromolar concentration elicited similar protective effect compared to living cells. On the other hand, in tomato, the pure elicitor induced a lower protective effect than observed with the producing strain, suggesting the synthesis of an additional ISR determinant in that case (Ongena et al., 2008).

Siderophores

To ensure their growth in iron-limited environments, microorganisms have evolved powerful Fe^{3+}-acquisition systems based on the secretion of high-affinity iron-chelating molecules termed siderophores. PGPR siderophores are also known to antagonize pathogen populations by decreasing iron amounts in soil, resulting in competition for this essential element (Kramer et al., 2020). However, some siderophores also act as plant immunity elicitors and pyoverdines (also referred to as pseudobactines; **Figure 1-8**) produced by various fluorescent pseudomonads were, in the 90's, among the first PGPR metabolites proposed as ISR elicitors. Their key role in systemic resistance induction was notably shown for *P. fluorescens* CHA0 on tobacco infected by *Tobacco necrosis virus*, and in the protection provided by *P. fluorescens* WCS374 to radish against Fusarium wilt (Maurhofer et al., 1994; Leeman et al., 1996). These chromopeptides were further described as inducers of resistance in various plant species such as bean, tomato, Arabidopsis, tobacco, eucalyptus and rice against a range of microbial pathogens (Meziane et al., 2005; Ran et al., 2005; De Vleesschauwer et al., 2008). In addition to pyoverdine, *P. aeruginosa* 7NSK2 also forms the chelating agent pyochelin (**Figure 1-9**) and its precursor salicylic acid (SA). When tested on pathosystem grapevine/*B. cinerea*, SA produced by *P. aeruginosa* 7NSK2 was crucial for mounting the plant immunity (De Meyer et al., 1999; Verhagen et al., 2010). However, for ISR stimulated in tomato by the same strain, an important role for SA could not be excluded, but probably combined with the action of other metabolites including pyochelin which may somehow also retain some eliciting activity (Audenaert et al., 2002).

Elicitors With Antibiotic Function

To ensure fitness in the competitive rhizosphere niche, PGPR produce a wide range of secondary metabolites best identified for their antimicrobial function such as non-ribosomal peptides, polyketides, bacteriocins, terpenes, phenazines, quinolones, or rhamnolipids (Raaijmakers and Mazzola, 2012; Zhao and Kuipers, 2016; Tracanna et al., 2017). Interestingly, some of these antibiotics were also shown to act as signal for ISR stimulation at similar concentrations, making them promising tools for biocontrol with dual action on pathogen populations (Kenawy

et al., 2019). One such antibiotic 2,4-diacetyl phloroglucinol (**Figure 1-10**), formed by *P. fluorescens*, is triggering resistance in Arabidopsis against various pathogens, such as *Peronospora parasitica*, *P. syringae* pv. *tomato*, and *B. cinerea,* upon application at relatively high 10–100 μM concentrations (Iavicoli et al., 2003; Weller et al., 2012; Chae et al., 2020). 2,4-diacetyl phloroglucinol can also induce resistance against nematodes as shown with *P. protegens* CHA0 for the reduction of infection caused by *Meloidogyne javanica* on tomato roots (Siddiqui and Shaukat, 2003). Besides, other *Pseudomonas* antibiotics of the phenazine-type were also reported to induce resistance. Notably phenazine-1-carboxamide (**Figure 1-11**) in rice toward *M. oryzae* at 0.1–1 μM, in bean toward *R. solani*, and pyocyanin produced by *P. aeruginosa* 7NSK2 in the pathosystem tomato/*B. cinerea* (Audenaert et al., 2002; D'aes et al., 2011; Ma et al., 2016).

Volatile Organic Compounds

Most of the well-characterized PGPR elicitors are soluble compounds, but some volatile organic compounds (VOCs) were as well-reported to induce systemic resistance in the host plant, showing that these metabolites can also act as infochemicals involved in inter-kingdom communication (Kai et al., 2016). The most studied VOC immunity elicitor is 2,3-butanediol (2,3-BD; **Figure 1-12**) produced from glucose in the central metabolism (Yang et al., 2013). Its bioactivity was first assessed on Arabidopsis by application on roots, where *B. subtilis* GB03 induced resistance against *Erwinia carotovora* subsp. *carotovora*, while mutants deprived in 2,3-BD or its precursor acetoin production were inactive (Ryu et al., 2004). Bioactivity, when treated on roots in relatively high mM concentrations, was demonstrated as well in the pathosystems maize/*Setosphaeria turcica*, tobacco/*Erwinia carotovora*, and in pepper against multiple viruses (**Table 1**), where the immunity eliciting ability was structure-dependent. Namely, among the three forms, 2R,3R-BD (R), 2S,3S-BD (S) and 2R,3S-BD (meso), the S form is the least active (Han et al., 2006; D'Alessandro et al., 2014; Kong et al., 2018). However, on pepper against *Xanthomonas axonopodis* pv. *vesicatoria*, another VOC 3-pentanol (10 μM, 1 mM; **Figure 1-13**) showed higher activity than 2,3-BD (Choi et al., 2014). Beside 3-pentanol, long-chain VOCs tridecane and hexadecane (**Figures 1-14,15**, respectively) showed bioactivity as well at μM (100) concentration (Lee et al., 2012; Park et al., 2013).

MOLECULAR BASIS OF PLANT IMMUNIZATION: PGPR vs. MAMP ELICITORS

The potential of PGPR to induce plant immunity mainly relies on the secretion of a range of structurally diverse low-molecular weight metabolites. However, the molecular mechanisms driving recognition of these elicitors at the plant cell surface are poorly understood. By contrast with MAMPs from pathogens, there is no indication so far for specific PRRs involved in the perception of PGPR elicitors. They activate immune responses only at relatively high μM concentrations compared to MAMPs, suggesting that they are not sensed via high-affinity receptors. CLPs are known to promptly insert into biological membranes causing defects, pore formation and cell lysis in a range of (micro)organisms (Balleza et al., 2019). Furthermore, immune response triggered by the *Bacillus* CLP surfactin was fully conserved in protease-treated tobacco cells, and successive applications do not lead to some refractory state due to the saturation of high-affinity binding sites (as receptors). Based on these and the fact that surfactin readily interact with sphingolipid-enriched microdomains in the plasma membrane, it was suggested that this CLP is perceived by plant cells via a lipid-mediated process. This is supported by the strong structure-dependent activity showing that only long fatty acid chain homologs are active at triggering early immune-related events in tobacco cells because they should display stronger interaction with lipid bilayers (Jourdan et al., 2009; Henry et al., 2011). Rhamnolipids also readily fit into plant lipid-based bilayer models. Therefore, it was proposed that by inserting into plasma membranes, these compounds provoke subtle changes in lipid dynamics that could be related to plant defense induction (Davis et al., 2010; Monnier et al., 2018; Schellenberger et al., 2019). As other alkyl-chain containing elicitors, AHLs could also be perceived by plant cells via a receptor-independent but lipid-mediated process, by analogy with data recently obtained on mammalian cells (Schikora et al., 2016; Song et al., 2018). It may explain why a given compound does not act at the same level on different targets, considering that the lipid composition in the plasma membrane differs according to the plant species/organs.

The way PGPR elicitors are perceived at the plasma membrane level is not clear, but there is still a clear convergence between PGPR-triggered immunity and pathogen-induced PTI in the early steps of downstream signaling. The best studied PGPR elicitors induce similar early immune events as observed upon pathogen perception, such as oxidative burst, ion fluxes, and phosphorylation cascade (Jourdan et al., 2009; Schikora et al., 2011; Cho et al., 2013; Cawoy et al., 2014; Farace et al., 2015; Rahman et al., 2015). Detection of MAMPs from pathogens leads to a fast and strong defensive response, but also to a costly reduction of the plant growth and fitness (Huot et al., 2014). On the other hand, PGPR and/or their elicitors usually prime the host to stimulate defense mechanisms but only after pathogen challenge as observed upon treatment with surfactin, AHLs, or with bacteria producing NADB or pyoverdines (De Vleesschauwer et al., 2008; Mariutto et al., 2011; Cawoy et al., 2014; Debois et al., 2015; Schikora et al., 2016). Again by contrast with PTI, PGPR elicitor-priming is not associated with major transcriptional reprogramming until the pathogen is detected and does not involve fitness costs but still prepares the plant for mounting a robust defense (Martinez-Medina et al., 2016; Mauch-Mani et al., 2017). Globally, the defense mechanisms restricting pathogen ingress that are stimulated by beneficial bacteria or their elicitors resemble those observed upon pathogen MAMP perception. It notably means up-regulation of genes involved in the synthesis of antimicrobial enzymes/proteins or metabolites and in hormone signaling. Higher expression of genes such are pathogenesis- related, lipoxygenase, plant defensin factor, and phenylalanine ammonia lyase, is often reported in elicitor pretreated plants after pathogen infection

(Ongena et al., 2007; García-Gutiérrez et al., 2013; Zarkani et al., 2013; Song et al., 2015; Park et al., 2016; Yi et al., 2016; Kong et al., 2018; Song et al., 2019). Besides, PGPR elicitors also trigger stomatal closure and enhance cell wall reinforcement which serves as a structural barrier to pathogen invasion via callose deposition or accumulation of phenolic compounds and lignin (De Vleesschauwer et al., 2006; García-Gutiérrez et al., 2013; Schenk et al., 2014; Rodríguez et al., 2018).

DISCUSSION

From an agronomic perspective, ISR triggered by PGPR is interesting since the phenomenon is considered to provide long-lasting and broad-spectrum protection without causing growth cost and is not *a priori* conducive for development of resistance in pathogens (Köhl et al., 2019). However, the success of PGPR as stimulators of plant defenses is so far rather limited due to a range of factors. These include our global lack of knowledge about the nature and mode of action of their elicitors. Indeed, ISR determinants were identified mainly from a limited number of species (*Pseudomonas* spp. and *Bacillus* spp.), leaving elicitors from many other PGPR to be discovered. Moreover, in the last decades, tremendous advances have been done on understanding the basics of MAMP perception during PTI, but the mechanistic of PGPR elicitor recognition at the plant plasma membrane level and the molecular events underlying PGPR-induced priming remain largely obscure. As the lipid phase is suspected to act as docking platform for some of these elicitors, experimental biophysics and *in silico* dynamic modeling using appropriate biomimetic vesicles represent interesting approaches to get further insights into the physico-chemical basis of the interactions (Deleu et al., 2014; Balleza et al., 2019; Nishimura and Matsumori, 2020). It would help to explain why some compounds are only efficient on specific plants/tissues according to the nature and proportions of lipids in their domain-structured plasma membranes (Gronnier et al., 2018). Also, the variety of pathosystems tested so far is still limited and additional research on agriculturally important crops and pathogens is needed in order to better appreciate their potential at a larger market scale.

Furthermore, PGPR elicitors in most instances are active at micromolar doses but only a few studies suggest that quantities produced by bacteria *in planta* are sufficient to locally reach such threshold around the roots (D'Alessandro et al., 2014; Debois et al., 2015). Determining the amounts of elicitors produced by PGPR under natural conditions is still important, but not an easy task. It would require optimal extraction from rhizosphere samples and the most-advanced MS-based metabolomics allowing high sensitivity for their detection and quantification. Also, environmental factors may affect ISR efficiency (Williams et al., 2018), but their impact on the production of elicitors by PGPR remains poorly appreciated. Low temperature, acidic pH or poor oxygen availability are among the rhizosphere specific abiotic parameters that affect bacterial physiology and which may also modulate the production of secondary metabolites including elicitors as reported for *Bacillus* lipopeptides (Fahim et al., 2012; Pertot et al., 2013; Zhao and Kuipers, 2016). Biotic factors such as interactions with other microbial species of the soil microbiome or chemical cross-talk with the host plant, may also influence elicitor production under natural conditions (Debois et al., 2015; Wu et al., 2015; Venturi and Keel, 2016; Andrić et al., 2020). A better evaluation of the impact of all these factors deserve further investigation and is necessary to better anticipate inconsistencies in PGPR efficacy observed upon application under field conditions.

Integrating all this missing information should also lead to more rational determine the range of pathosystems, and environmental conditions in which PGPR-based bioproducts would be the most efficient. An alternative is to make from these bacterial immunogenic compounds microbial-derived products for the phytosanitary market provided that they can be produced and formulated in cost-effective industrial processes. This is feasible for some metabolites with high production rate in bioreactors, such as *Bacillus* lipopeptides (Zanotto et al., 2019; Brück et al., 2020), but the dose and structure dependent activity should guide a rational selection of the strain, optimization of culture conditions and extensive testing in field experiments in comparison with chemical products.

AUTHOR CONTRIBUTIONS

Both authors conceived the idea, designed the outlines of the review, and wrote the manuscript.

FUNDING

Research in the laboratory is supported by the EU Interreg FWVL V portfolio SmartBiocontrol and by the EOS project ID30650620 from the FWO/F.R.S.-FNRS (Fonds National de la Recherche Scientifique). MO is senior research associate at the F.R.S.-F.N.R.S.

ACKNOWLEDGMENTS

We gratefully acknowledge S. Andrić, F. Boubsi, A. Anckaert, G. Gilliard, and T. Mayer for critically reading the manuscript and their valuable suggestions and discussions.

REFERENCES

Abdel-Mawgoud, A. M., Lepine, F., and Deziel, E. (2010). Rhamnolipids: diversity of structures, microbial origins and roles. *Appl. Microbiol. Biotechnol.* 86, 1323–1336. doi: 10.1007/s00253-010-2498-2

Andrić, S., Meyer, T., and Ongena, M. (2020). *Bacillus* responses to plant-associated fungal and bacterial communities. *Front. Microbiol.* 11:1350. doi: 10.3389/fmicb.2020.01350

Audenaert, K., Pattery, T., Cornelis, P., and Höfte, M. (2002). Induction of Systemic Resistance to *Botrytis cinerea* in Tomato by *Pseudomonas aeruginosa* 7NSK2:

role of salicylic acid, pyochelin, and pyocyanin. *Mol. Plant Microbe Interact.* 15, 1147-1156. doi: 10.1094/MPMI.2002.15.11.1147

Backer, R., Rokem, J. S., Ilangumaran, G., Lamont, J., Praslickova, D., Ricci, E., et al. (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

Balleza, D., Alessandrini, A., and Beltrán García, M. J. (2019). Role of lipid composition, physicochemical interactions, and membrane mechanics in the molecular actions of microbial cyclic lipopeptides. *J. Memb. Biol.* 252, 131–157. doi: 10.1007/s00232-019-00067-4

Berendsen, R. L., van Verk, M. C., Stringlis, I. A., Zamioudis, C., Tommassen, J., Pieterse, C. M. J., et al. (2015). Unearthing the genomes of plant-beneficial *Pseudomonas* model strains WCS358. WCS374 and WCS417. *BMC Genom.* 16:539. doi: 10.1186/s12864-015-1632-z

Brück, H. L., Coutte, F., Dhulster, P., Gofflot, S., Jacques, P., and Delvigne, F. (2020). Growth dynamics of bacterial populations in a two-compartment biofilm bioreactor designed for continuous surfactin Biosynthesis. *Microorganisms* 8:679. doi: 10.3390/microorganisms8050679

Cawoy, H., Mariutto, M., Henry, G., Fisher, C., Vasilyeva, N., Thonart, P., et al. (2014). Plant defense stimulation by natural isolates of *Bacillus* depends on efficient surfactin production. *Mol. Plant Microbe Interact.* 27, 87–100. doi: 10.1094/MPMI-09-13-0262-R

Chae, D. H., Kim, D. R., Cheong, M. S., Lee, Y. B., and Kwak, Y. S. (2020). Investigating the induced systemic resistance mechanism of 2,4-diacetylphloroglucinol (Dapg) using dapg hydrolase-transgenic arabidopsis. *Plant Pathol. J.* 36, 255–266. doi: 10.5423/PPJ.OA.02.2020.0031

Cho, S. M., Kim, Y. H., Anderson, A. J., and Kim, Y. C. (2013). Nitric oxide and hydrogen peroxide production are involved in systemic drought tolerance induced by 2R,3R-butanediol in *Arabidopsis thaliana*. *Plant Pathol. J.* 29, 427–434. doi: 10.5423/PPJ.OA.07.2013.0069

Choi, H. K., Song, G. C., Yi, H. S., and Ryu, C. M. (2014). Field evaluation of the bacterial volatile derivative 3-pentanol in priming for induced resistance in pepper. *J. Chem. Ecol.* 40, 882–892. doi: 10.1007/s10886-014-0488-z

Chrzanowski, Ł, Ławniczak, Ł, and Czaczyk, K. (2012). Why do microorganisms produce rhamnolipids? *World J. Microbiol. Biotechnol.* 28, 401–419. doi: 10.1007/s11274-011-0854-8

D'aes, J., Hua, G. K. H., De Maeyer, K., Pannecoucque, J., Forrez, I., Ongena, M., et al. (2011). Biological control of rhizoctonia root rot on bean by phenazine and cyclic lipopeptide-producing *Pseudomonas* CMR12a. *Phytopathology* 101, 996–1004. doi: 10.1094/PHYTO-11-10-0315

D'Alessandro, M., Erb, M., Ton, J., Brandenburg, A., Karlen, D., Zopfi, J., et al. (2014). Volatiles produced by soil-borne endophytic bacteria increase plant pathogen resistance and affect tritrophic interactions. *Plant Cell and Environment* 37, 813–826. doi: 10.1111/pce.12220

Davis, B. M., Jensen, R., Williams, P., and O'Shea, P. (2010). The interaction of N-acylhomoserine lactone quorum sensing signaling molecules with biological membranes: implications for inter-kingdom signaling. *PLoS One* 5:e0013522. doi: 10.1371/journal.pone.0013522

De Meyer, G., Capieau, K., Audenaert, K., Buchala, A., Métraux, J. P., and Höfte, M. (1999). Nanogram amounts of salicylic acid produced by the rhizobacterium *Pseudomonas aeruginosa* 7NSK2 activate the systemic acquired resistance pathway in bean. *Mol. Plant Microbe Interact.* 12, 450–458. doi: 10.1094/MPMI.1999.12.5.450

De Vleesschauwer, D., Cornelis, P., and Höfte, M. (2006). Redox-active pyocyanin secreted by *Pseudomonas aeruginosa* 7NSK2 triggers systemic resistance to *Magnaporthe grisea* but enhances *Rhizoctonia solani* susceptibility in rice. *Mol. Plant Microbe Interact.* 19, 1406–1419. doi: 10.1094/MPMI-19-1406

De Vleesschauwer, D., Djavaheri, M., Bakker, P. A. H. M., and Höfte, M. (2008). *Pseudomonas fluorescens* WCS374r-induced systemic resistance in rice against *Magnaporthe oryzae* is based on pseudobactin-mediated priming for a salicylic acid-repressible multifaceted defense response. *Plant Physiol.* 148, 1996–2012. doi: 10.1104/pp.108.127878

Debois, D., Fernandez, O., Franzil, L., Jourdan, E., de Brogniez, A., Willems, L., et al. (2015). Plant polysaccharides initiate underground crosstalk with bacilli by inducing synthesis of the immunogenic lipopeptide surfactin. *Environ. Microbiol. Rep.* 7, 570–582. doi: 10.1111/1758-2229.12286

Deleu, M., Crowet, J., Nasir, M. N., and Lins, L. (2014). Complementary biophysical tools to investigate lipid speci fi city in the interaction between bioactive molecules and the plasma membrane: a review. *Biochim. Biophys. Acta Biomemb.* 1838, 3171–3190. doi: 10.1016/j.bbamem.2014.08.023

Fahim, S., Dimitrov, K., Gancel, F., Vauchel, P., Jacques, P., and Nikov, I. (2012). Bioresource Technology Impact of energy supply and oxygen transfer on selective lipopeptide production by *Bacillus subtilis* BBG21. *Bioresour. Technol.* 126, 1–6. doi: 10.1016/j.biortech.2012.09.019

Farace, G., Fernandez, O., Jacquens, L., Coutte, F., Krier, F., Jacques, P., et al. (2015). Cyclic lipopeptides from *Bacillus subtilis* activate distinct patterns of defence responses in grapevine. *Mol. Plant Pathol.* 16, 177–187. doi: 10.1111/mpp.12170

Farzand, A., Moosa, A., Zubair, M., Khan, A. R., Massawe, V. C., Tahir, H. A. S., et al. (2019). Suppression of *Sclerotinia sclerotiorum* by the induction of systemic resistance and regulation of antioxidant pathways in tomato using fengycin produced by *Bacillus amyloliquefaciens* FZB42. *Biomolecules* 9:613. doi: 10.3390/biom9100613

García-Gutiérrez, L., Zeriouh, H., Romero, D., Cubero, J., de Vicente, A., and Pérez-García, A. (2013). The antagonistic strain *Bacillus subtilis* UMAF6639 also confers protection to melon plants against cucurbit powdery mildew by activation of jasmonate- and salicylic acid-dependent defence responses. *Microb. Biotechnol.* 6, 264–274. doi: 10.1111/1751-7915.12028

Geudens, N., and Martins, J. C. (2018). Cyclic lipodepsipeptides from *Pseudomonas* spp. - biological swiss-army knives. *Front. Microbiol.* 9:1867. doi: 10.3389/fmicb.2018.01867

Grady, E. N., MacDonald, J., Liu, L., Richman, A., and Yuan, Z. C. (2016). Current knowledge and perspectives of *Paenibacillus*: a review. *Microb. Cell Factories* 15:203. doi: 10.1186/s12934-016-0603-7

Gronnier, J., Gerbeau-Pissot, P., Germain, V., Mongrand, S., and Simon-Plas, F. (2018). Divide and rule: plant plasma membrane organization. *Trends Plant Sci.* 23, 899–917. doi: 10.1016/j.tplants.2018.07.007

Han, Q., Wu, F., Wang, X., Qi, H., Shi, L., Ren, A., et al. (2015). The bacterial lipopeptide iturins induce *Verticillium dahliae* cell death by affecting fungal signalling pathways and mediate plant defence responses involved in pathogen-associated molecular pattern-triggered immunity. *Environ. Microbiol.* 17, 1166–1188. doi: 10.1111/1462-2920.12538

Han, S. H., Lee, S. J., Moon, J. H., Park, K. H., Yang, K. Y., Cho, B. H., et al. (2006). GacS-dependent production of 2R, 3R-butanediol by *Pseudomonas chlororaphis* O6 is a major determinant for eliciting systemic resistance against *Erwinia carotovora* but not against *Pseudomonas syringae* pv. *tabaci in tobacco*. *Mol. Plant Microbe Interact.* 2006 19, 924–930. doi: 10.1094/MPMI-19-0924

Henry, G., Deleu, M., Jourdan, E., Thonart, P., and Ongena, M. (2011). The bacterial lipopeptide surfactin targets the lipid fraction of the plant plasma membrane to trigger immune-related defence responses. *Cell. Microbiol.* 13, 1824–1837. doi: 10.1111/j.1462-5822.2011.01664.x

Hernández-Reyes, C., Schenk, S. T., Neumann, C., Kogel, K. H., and Schikora, A. (2014). N-acyl-homoserine lactones-producing bacteria protect plants against plant and human pathogens. *Microb. Biotechnol.* 7, 580–588. doi: 10.1111/1751-7915.12177

Huot, B., Yao, J., Montgomery, B. L., and He, S. Y. (2014). Growth-defense tradeoffs in plants: a balancing act to optimize fitness. *Mol. Plant* 7, 1267–1287. doi: 10.1093/mp/ssu049

Iavicoli, A., Boutet, E., Buchala, A., and Métraux, J.-P. (2003). Induced systemic resistance in *Arabidopsis thaliana* in response to root inoculation with *Pseudomonas fluorescens* CHA0. *Mol. Plant Microbe Interact.* 16, 851–858. doi: 10.1094/MPMI.2003.16.10.851

Jourdan, E., Henry, G., Duby, F., Dommes, J., Barthélemy, J. P., Thonart, P., et al. (2009). Insights into the defense-related events occurring in plant cells following perception of surfactin-type lipopeptide from *Bacillus subtilis*. *Mol. Plant Microbe Interact.* 22, 456–468. doi: 10.1094/MPMI-22-4-0456

Kai, M., Effmert, U., and Piechulla, B. (2016). Bacterial-plant-interactions: approaches to unravel the biological function of bacterial volatiles in the rhizosphere. *Front. Microbiol.* 7:108. doi: 10.3389/fmicb.2016.00108

Kanyuka, K., and Rudd, J. J. (2019). Cell surface immune receptors: the guardians of the plant's extracellular spaces. *Curr. Opin. Plant Biol.* 50, 1–8. doi: 10.1016/j.pbi.2019.02.005

Kawagoe, Y., Shiraishi, S., Kondo, H., Yamamoto, S., Aoki, Y., and Suzuki, S. (2015). Cyclic lipopeptide iturin A structure-dependently induces defense response

in Arabidopsis plants by activating SA and JA signaling pathways. *Biochem. Biophys. Res. Commun.* 460, 1015–1020. doi: 10.1016/j.bbrc.2015.03.143

Kenawy, A., Dailin, D. J., Abo-Zaid, G. A., Abd Malek, R., Ambehabati, K. K., Zakaria, K. H., et al. (2019). "Biosynthesis of antibiotics by PGPR and their roles in biocontrol of plant diseases," in *Plant Growth Promoting Rhizobacteria for Sustainable Stress Management*, ed. R. Z. Sayyed (Singapore: Springer), 1–36. doi: 10.1007/978-981-13-6986-5_1

Köhl, J., Kolnaar, R., and Ravensberg, W. J. (2019). Mode of action of microbial biological control agents against plant diseases: relevance beyond efficacy. *Front. Plant Sci.* 10:845. doi: 10.3389/fpls.2019.00845

Kong, H. G., Shin, T. S., Kim, T. H., and Ryu, C. M. (2018). Stereoisomers of the bacterial volatile compound 2,3-butanediol differently elicit systemic defense responses of pepper against multiple viruses in the field. *Front. Plant Sci.* 9:90. doi: 10.3389/fpls.2018.00090

Kramer, J., Özkaya, Ö, and Kümmerli, R. (2020). Bacterial siderophores in community and host interactions. *Nat. Rev. Microbiol.* 18, 152–163. doi: 10.1038/s41579-019-0284-4

Le Mire, G., Siah, A., Brisset, M.-N., Gaucher, M., Deleu, M., and Jijakli, M. (2018). Surfactin protects wheat against Zymoseptoria tritici and activates both salicylic acid- and jasmonic acid-dependent defense responses. *Agriculture* 8:11. doi: 10.3390/agriculture8010011

Lee, B., Farag, M. A., Park, H. B., Kloepper, J. W., Lee, S. H., and Ryu, C. M. (2012). Induced resistance by a long-chain bacterial volatile: elicitation of plant systemic defense by a C13 volatile produced by *Paenibacillus polymyxa*. *PLoS One* 7:e48744. doi: 10.1371/journal.pone.0048744

Leeman, M., Den Ouden, F. M., Van Pelt, J. A., Dirkx, F. P. M., Steijl, H., Bakker, P. A. H. M., et al. (1996). Iron availability affects induction of systemic resistance to *Fusarium* wilt of radish by *Pseudomonas fluorescens*. *Phytopathology* 86, 149–155. doi: 10.1094/Phyto-86-149

Li, Y., Héloir, M. C., Zhang, X., Geissler, M., Trouvelot, S., Jacquens, L., et al. (2019). Surfactin and fengycin contribute to the protection of a *Bacillus subtilis* strain against grape downy mildew by both direct effect and defence stimulation. *Mol. Plant Pathol.* 20, 1037–1050. doi: 10.1111/mpp.12809

Ma, Z., Hoang Hua, G. K. H., Ongena, M., and Höfte, M. (2016). Role of phenazines and cyclic lipopeptides produced by *Pseudomonas* sp. *CMR*12a in induced systemic resistance on rice and bean. *Environ. Microbiol. Rep.* 8, 896–904. doi: 10.1111/1758-2229.12454

Ma, Z., Ongena, M., and Höfte, M. (2017). The cyclic lipopeptide orfamide induces systemic resistance in rice to *Cochliobolus miyabeanus* but not to *Magnaporthe oryzae*. *Plant Cell Rep.* 36, 1731–1746. doi: 10.1007/s00299-017-2187-z

Mariutto, M., Duby, F., Adam, A., Bureau, C., Fauconnier, M. L., Ongena, M., et al. (2011). The elicitation of a systemic resistance by *Pseudomonas putida* BTP1 in tomato involves the stimulation of two lipoxygenase isoforms. *BMC Plant Biol.* 11:29. doi: 10.1186/1471-2229-11-29

Martinez-Medina, A., Flors, V., Heil, M., Mauch-Mani, B., Pieterse, C. M. J., Pozo, M. J., et al. (2016). Recognizing plant defense priming. *Trends Plant Sci.* 21, 818–822. doi: 10.1016/j.tplants.2016.08.005

Mauch-Mani, B., Baccelli, I., Luna, E., and Flors, V. (2017). Defense priming: an adaptive part of induced resistance. *Annu. Rev. Plant Biol.* 2017, 485–512. doi: 10.1146/annurev-arplant-042916

Maurhofer, M., Hase, C., Meuwly, P., Metraux, J. P., and Defago, G. (1994). Induction of systemic resistance of tobacco to tobacco necrosis virus by the root-colonizing *Pseudomonas fluorescens* strain CHA0: influence of the gacA gene and of pyoverdine production. *Phytopathology* 84, 139–146. doi: 10.1094/Phyto-84-139

Mejri, S., Siah, A., Coutte, F., Magnin-Robert, M., Randoux, B., Tisserant, B., et al. (2018). Biocontrol of the wheat pathogen *Zymoseptoria tritici* using cyclic lipopeptides from *Bacillus subtilis*. *Environ. Sci. Pollut. Res.* 25, 29822–29833. doi: 10.1007/s11356-017-9241-9

Meziane, H., Van Der Sluis, I., Va Loon, L. C., Höfte, M., and Bakker, P. A. (2005). Determinants of *Pseudomonas putida* WCS358 involved in inducing systemic resistance in plants. *Mol. Plant Pathol.* 6, 177–185. doi: 10.1111/j.1364-3703.2005.00276

Mhatre, P. H., Karthik, C., Kadirvelu, K., Divya, K. L., Venkatasalam, E. P., Srinivasan, S., et al. (2018). Plant growth promoting rhizobacteria (PGPR): a potential alternative tool for nematodes bio-control. *Biocatal. Agricult. Biotechnol.* 17, 119–128. doi: 10.1016/j.bcab.2018.11.009

Monnier, N., Cordier, M., Dahi, A., Santoni, V., Guenin, S., Clement, C., et al. (2020). Semi-purified rhamnolipid mixes protect *Brassica napus* against *Leptosphaeria maculans* early infections. *Phytopathology* 110, 834–842. doi: 10.1094/phyto-07-19-0275-r

Monnier, N., Furlan, A., Botcazon, C., Dahi, A., Mongelard, G., Cordelier, S., et al. (2018). Rhamnolipids from *Pseudomonas aeruginosa* are elicitors triggering *Brassica napus* protection against *Botrytis cinerea* without physiological disorders. *Front. Plant Sci.* 9:1170. doi: 10.3389/fpls.2018.01170

Nishimura, S., and Matsumori, N. (2020). Chemical diversity and mode of action of natural products targeting lipids in the eukaryotic cell membrane. *Nat. Prod. Rep.* 37, 677–702. doi: 10.1039/c9np00059c

Olorunleke, F. E., Hua, G. K. H., Kieu, N. P., Ma, Z., and Höfte, M. (2015). Interplay between orfamides, sessilins and phenazines in the control of Rhizoctonia diseases by *Pseudomonas* sp. CMR12a. *Environ. Microbiol. Rep.* 7, 774–781. doi: 10.1111/1758-2229.12310

Omoboye, O. O., Oni, F. E., Batool, H., Yimer, H. Z., De Mot, R., and Höfte, M. (2019). *Pseudomonas* cyclic lipopeptides suppress the rice blast fungus *Magnaporthe oryzae* by induced resistance and direct antagonism. *Front. Plant Sci.* 10:901. doi: 10.3389/fpls.2019.00901

Ongena, M., and Jacques, P. (2008). *Bacillus* lipopeptides: versatile weapons for plant disease biocontrol. *Trends in Microbiol.* 16, 115–125. doi: 10.1016/j.tim.2007.12.009

Ongena, M., Jourdan, E., Adam, A., Paquot, M., Brans, A., Joris, B., et al. (2007). Surfactin and fengycin lipopeptides of *Bacillus subtilis* as elicitors of induced systemic resistance in plants. *Environ. Microbiol.* 9, 1084–1090. doi: 10.1111/j.1462-2920.2006.01202.x

Ongena, M., Jourdan, E., Adam, A., Schäfer, M., Budzikiewicz, H., and Thonart, P. (2008). Amino acids, iron, and growth rate as key factors influencing production of the *Pseudomonas putida* BTP1 benzylamine derivative involved in systemic resistance induction in different plants. *Microb. Ecol.* 55, 280–292. doi: 10.1007/s00248-007-9275-5

Ongena, M., Jourdan, E., Schäfer, M., Kech, C., Budzikiewicz, H., Luxen, A., et al. (2005). Isolation of an N-alkylated benzylamine derivative from *Pseudomonas putida* BTP1 as elicitor of induced systemic resistance in bean. *Mol. Plant Microbe Interact.* 18, 562–569. doi: 10.1094/MPMI-18-0562

Park, H. B., Lee, B., Kloepper, J. W., and Ryu, C. M. (2013). One shot-two pathogens blocked: exposure of Arabidopsis to hexadecane, a long chain volatile organic compound, confers induced resistance against both *Pectobacterium carotovorum* and *Pseudomonas syringae*. *Plant Signal. Behav.* 8:e24619. doi: 10.4161/psb.24619

Park, K., Park, Y. S., Ahamed, J., Dutta, S., Ryu, H., Lee, S. H., et al. (2016). Elicitation of induced systemic resistance of chili pepper by iturin a analogs derived from *Bacillus vallismortis* EXTN-1. *Can. J. Plant Sci.* 96, 564–570. doi: 10.1139/cjps-2015-0199

Perneel, M., D'Hondt, L., De Maeyer, K., Adiobo, A., Rabaey, K., and Hofte, M. (2008). Phenazines and biosurfactants interact in the biological control of soil-borne diseases caused by *Pythium* spp. *Environ. Microbiol.* 10, 778–788. doi: 10.1111/j.1462-2920.2007.01501.x

Pertot, I., Puopolo, G., Hosni, T., Pedrotti, L., Jourdan, E., and Ongena, M. (2013). Limited impact of abiotic stress on surfactin production in planta and on disease resistance induced by Bacillus amyloliquefaciens S499 in tomato and bean. *FEMS Microbiol. Ecol.* 86, 505–519. doi: 10.1111/1574-6941.12177

Piasecka, A., Jedrzejczak-Rey, N., and Bednarek, P. (2015). Secondary metabolites in plant innate immunity: conserved function of divergent chemicals. *New Phytol.* 206, 948–964. doi: 10.1111/nph.13325

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

Raaijmakers, J. M., and Mazzola, M. (2012). Diversity and natural functions of antibiotics produced by beneficial and plant pathogenic bacteria. *Annu. Rev. Phytopathol.* 50, 403–424. doi: 10.1146/annurev-phyto-081211-172908

Rahman, A., Uddin, W., and Wenner, N. G. (2015). Induced systemic resistance responses in perennial ryegrass against *Magnaporthe oryzae* elicited by semi-purified surfactin lipopeptides and live cells of *Bacillus amyloliquefaciens*. *Mol. Plant Pathol.* 16, 546–558. doi: 10.1111/mpp.12209

Ran, L. X., Li, Z. N., Wu, G. J., Van Loon, L. C., and Bakker, P. A. H. M. (2005). Induction of systemic resistance against bacterial wilt in *Eucalyptus urophylla*

by fluorescent *Pseudomonas* spp. *Eur. J. Plant Pathol.* 113, 59–70. doi: 10.1007/s10658-005-0623-3

Rashid, M. H. O., and Chung, Y. R. (2017). Induction of systemic resistance against insect herbivores in plants by beneficial soil microbes. *Front. Plant Sci.* 8:1816. doi: 10.3389/fpls.2017.01816

Rodríguez, J., Tonelli, M. L., Figueredo, M. S., Ibáñez, F., and Fabra, A. (2018). The lipopeptide surfactin triggers induced systemic resistance and priming state responses in *Arachis hypogaea* L. *Eur. J. Plant Pathol.* 152, 845–851. doi: 10.1007/s10658-018-1524-6

Ryu, C. M., Farag, M. A., Hu, C. H., Reddy, M. S., Kloepper, J. W., and Paré, P. W. (2004). Bacterial volatiles induce systemic resistance in Arabidopsis. *Plant Physiol.* 134, 1017–1026. doi: 10.1104/pp.103.026583

Sanchez, L., Courteaux, B., Hubert, J., Kauffmann, S., Renault, J. H., Clément, C., et al. (2012). Rhamnolipids elicit defense responses and induce disease resistance against biotrophic, hemibiotrophic, and necrotrophic pathogens that require different signaling pathways in Arabidopsis and highlight a central role for salicylic acid. *Plant Physiology* 160, 1630–1641. doi: 10.1104/pp.112.201913

Schellenberger, R., Touchard, M., Clément, C., Baillieul, F., Cordelier, S., Crouzet, J., et al. (2019). Apoplastic invasion patterns triggering plant immunity: plasma membrane sensing at the frontline. *Mol. Plant Pathol.* 20, 1602–1616. doi: 10.1111/mpp.12857

Schenk, S. T., Hernández-Reyes, C., Samans, B., Stein, E., Neumann, C., Schikora, M., et al. (2014). N-acyl-homoserine lactone primes plants for cell wall reinforcement and induces resistance to bacterial pathogens via the salicylic acid/oxylipin pathway. *Plant Cell* 26, 2708–2723. doi: 10.1105/tpc.114.126763

Schenk, S. T., Stein, E., Kogel, K. H., and Schikora, A. (2012). Arabidopsis growth and defense are modulated by bacterial quorum sensing molecules. *Plant Signal. Behav* 7, 178-181. doi: 10.4161/psb.18789

Schikora, A., Schenk, S. T., and Hartmann, A. (2016). Beneficial effects of bacteria-plant communication based on quorum sensing molecules of the N-acyl homoserine lactone group. *Plant Mol. Biol.* 90, 605–612. doi: 10.1007/s11103-016-0457-8

Schikora, A., Schenk, S. T., Stein, E., Molitor, A., Zuccaro, A., and Kogel, K. H. (2011). N-acyl-homoserine lactone confers resistance toward biotrophic and hemibiotrophic pathogens via altered activation of AtMPK6. *Plant Physiol.* 157, 1407–1418. doi: 10.1104/pp.111.180604

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

Siddiqui, I. A., and Shaukat, S. S. (2003). Suppression of root-knot disease by *Pseudomonas fluorescens* CHA0 in tomato: importance of bacterial secondary metabolite, 2,4-diacetylpholoroglucinol. *Soil Biol Biochem.* 35, 1615–1623. doi: 10.1016/j.soilbio.2003.08.006

Singh, M., Singh, D., Gupta, A., Pandey, K. D., Singh, P. K., and Kumar, A. (2019). "Plant growth promoting rhizobacteria: application in biofertilizers and bio-control of phytopathogens," in *PGPR Amelioration in Sustainable Agriculture*, eds A. K. Singh, A. Kumar, and P. K. Singh (Cambridge: Woodhead Publishing, Inc), 2019.

Song, D., Meng, J., Cheng, J., Fan, Z., Chen, P., Ruan, H., et al. (2018). *Pseudomonas aeruginosa* quorum-sensing metabolite induces host immune cell death through cell surface lipid domain dissolution. *Nat. Microbiol.* 4, 97–111. doi: 10.1038/s41564-018-0290-8

Song, G. C., Choi, H. K., and Ryu, C. M. (2015). Gaseous 3-pentanol primes plant immunity against a bacterial speck pathogen, *Pseudomonas syringae* pv. *tomato via salicylic acid and jasmonic acid-dependent signaling pathways in arabidopsis*. *Front. Plant Sci.* 6:821. doi: 10.3389/fpls.2015.00821

Song, G. C., Riu, M., and Ryu, C. M. (2019). Beyond the two compartments Petri-dish: Optimising growth promotion and induced resistance in cucumber exposed to gaseous bacterial volatiles in a miniature greenhouse system. *Plant Methods* 15:9. doi: 10.1186/s13007-019-0395-y

Stringlis, I. A., De Jonge, R., and Pieterse, C. M. J. (2019). The age of coumarins in plant-microbe interactions. *Plant Cell Physiol.* 60, 1405–1419. doi: 10.1093/pcp/pcz076

Stringlis, I. A., Proietti, S., Hickman, R., Van Verk, M. C., Zamioudis, C., and Pieterse, C. M. J. (2018). Root transcriptional dynamics induced by beneficial rhizobacteria and microbial immune elicitors reveal signatures of adaptation to mutualists. *Plant J.* 93, 166–180. doi: 10.1111/tpj.13741

Tracanna, V., de Jong, A., Medema, M. H., and Kuipers, O. P. (2017). Mining prokaryotes for antimicrobial compounds: from diversity to function. *FEMS Microbiol. Rev.* 41, 417–429. doi: 10.1093/femsre/fux014

Tran, H., Ficke, A., Asiimwe, T., Höfte, M., and Raaijmakers, J. M. (2007). Role of the cyclic lipopeptide massetolide a in biological control of *Phytophthora infestans* and in colonization of tomato plants by *Pseudomonas fluorescens*. *New Phytol.* 175, 731–742. doi: 10.1111/j.1469-8137.2007.02138.x

Varnier, A. L., Sanchez, L., Vatsa, P., Boudesocque, L., Garcia-Brugger, A., Rabenoelina, F., et al. (2009). Bacterial rhamnolipids are novel MAMPs conferring resistance to *Botrytis cinerea* in grapevine. *Plant Cell Environ.* 32, 178–193. doi: 10.1111/j.1365-3040.2008.01911.x

Vatsa, P., Sanchez, L., Clement, C., Baillieul, F., and Dorey, S. (2010). Rhamnolipid biosurfactants as new players in animal and plant defense against microbes. *Int. J. Mol. Sci.* 11, 5095–5108. doi: 10.3390/ijms11125095

Venturi, V., and Keel, C. (2016). Signaling in the Rhizosphere. *Trends Plant Sci.* 21, 187–198. doi: 10.1016/j.tplants.2016.01.005

Verhagen, B. W. M., Trotel-Aziz, P., Couderchet, M., Höfte, M., and Aziz, A. (2010). Pseudomonas spp.-*induced systemic resistance to Botrytis cinerea* is associated with induction and priming of defence responses in grapevine. *J. Exp. Bot.* 61, 249–260. doi: 10.1093/jxb/erp295

Wang, H., Yang, X., Guo, L., Zeng, H., and Qiu, D. (2015). PeBL1, a novel protein elicitor from *Brevibacillus laterosporus* strain A60, activates defense responses and systemic resistance in *Nicotiana benthamiana*. *Appl. Environ. Microbiol.* 81, 2706–2716. doi: 10.1128/AEM.03586-14

Wang, N., Liu, M., Guo, L., Yang, X., and Qiu, D. (2016). A novel protein elicitor (PeBA1) from *Bacillus amyloliquefaciens* NC6 induces systemic resistance in tobacco. *Int. J. Biol. Sci.* 12, 757–767. doi: 10.7150/ijbs.14333

Wehner, G., Kopahnke, D., Richter, K., Kecke, S., Schikora, A., and Ordon, F. (2019). Priming is a suitable strategy to enhance resistance towards leaf rust in barley. *Phytob. J.* 3, 46–51. doi: 10.1094/PBIOMES-09-18-0041-R

Weller, D. M., Mavrodi, D. V., Van Pelt, J. A., Pieterse, C. M. J., Van Loon, L. C., and Bakker, P. A. H. M. (2012). Induced systemic resistance in *Arabidopsis thaliana* against *Pseudomonas syringae* pv. *tomato by* 2,4-diacetylphloroglucinol-producing *Pseudomonas fluorescens*. *Phytopathology* 102, 403–412. doi: 10.1094/PHYTO-08-11-0222

Williams, A., Pétriacq, P., Beerling, D. J., Cotton, T. E. A., and Ton, J. (2018). Impacts of atmospheric CO2 and soil nutritional value on plant responses to rhizosphere colonization by soil bacteria. *Front. Plant Sci.* 871:1493. doi: 10.3389/fpls.2018.01493

Wu, G., Liu, Y., Xu, Y., Zhang, G., Shen, Q., and Zhang, R. (2018). Exploring elicitors of the beneficial rhizobacterium *Bacillus amyloliquefaciens* SQR9 to induce plant systemic resistance and their interactions with plant signaling pathways. *Mol. Plant Microbe Interact.* 31, 560–567. doi: 10.1094/MPMI-11-17-0273-R

Wu, K., Fang, Z., Guo, R., Pan, B., Shi, W., Yuan, S., et al. (2015). Pectin enhances bio-control efficacy by inducing colonization and secretion of secondary metabolites by Bacillus amyloliquefaciens SQY 162 in the rhizosphere of tobacco. *PLoS One* 10:e127418. doi: 10.1371/journal.pone.0127418

Yamamoto, S., Shiraishi, S., and Suzuki, S. (2015). Are cyclic lipopeptides produced by *Bacillus amyloliquefaciens* S13-3 responsible for the plant defence response in strawberry against *Colletotrichum gloeosporioides*? *Lett. Appl. Microbiol.* 60, 379–386. doi: 10.1111/lam.12382

Yang, T., Rao, Z., Zhang, X., Xu, M., Xu, Z., and Yang, S. T. (2013). Improved Production of 2,3-Butanediol in *Bacillus amyloliquefaciens* by Over-Expression of Glyceraldehyde-3-Phosphate Dehydrogenase and 2,3-butanediol Dehydrogenase. *PLoS One* 8:e76149. doi: 10.1371/journal.pone.0076149

Yi, H. S., Ahn, Y. R., Song, G. C., Ghim, S. Y., Lee, S., Lee, G., et al. (2016). Impact of a bacterial volatile 2,3-butanediol on *Bacillus subtilis* rhizosphere robustness. *Front. Microbiol.* 7:993. doi: 10.3389/fmicb.2016.00993

Yu, K., Pieterse, C. M. J., Bakker, P. A. H. M., and Berendsen, R. L. (2019). Beneficial microbes going underground of root immunity. *Plant Cell Environ.* 42, 2860–2870. doi: 10.1111/pce.13632

Zanotto, A. W., Valério, A., de Andrade, C. J., and Pastore, G. M. (2019). New sustainable alternatives to reduce the production costs for surfactin 50 years

after the discovery. *Appl. Microbiol. Biotechnol.* 103, 8647–8656. doi: 10.1007/s00253-019-10123-7

Zarkani, A. A., Stein, E., Röhrich, C. R., Schikora, M., Evguenieva-Hackenberg, E., Degenkolb, T., et al. (2013). Homoserine lactones influence the reaction of plants to rhizobia. *Int. J. Mol. Sci.* 14, 17122–17146. doi: 10.3390/ijms140817122

Zhang, Y., Yan, X., Guo, H., Zhao, F., and Huang, L. (2018). A novel protein elicitor BAR11 from *Saccharothrix yanglingensis* Hhs.*015* improves plant resistance to pathogens and interacts with catalases as targets. *Front. Microbiol.* 9:700. doi: 10.3389/fmicb.2018.00700

Zhao, X., and Kuipers, O. P. O. P. (2016). Identification and classification of known and putative antimicrobial compounds produced by a wide variety of Bacillales species. *BMC Genomics* 17:882. doi: 10.1186/s12864-016-3224-y

Zipfel, C., and Oldroyd, G. E. D. (2017). Plant signalling in symbiosis and immunity. *Nature* 43, 328–336. doi: 10.1038/nature22009

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

ORIGINAL RESEARCH
published: 16 November 2020
doi: 10.3389/fpls.2020.599904

The Soil Nutrient Environment Determines the Strategy by Which *Bacillus velezensis* HN03 Suppresses *Fusarium* wilt in Banana Plants

Xiaoyan Wu[1,2†], Ying Shan[1,3†], Yi Li[1,3,4], Qinfen Li[1,3] and Chunyuan Wu[1,3,4]*

[1] Environment and Plant Protection Institute, Chinese Academy of Tropical Agricultural Sciences, Haikou, China, [2] Key Laboratory of Integrated Pest Management on Tropical Crops, Ministry of Agriculture and Rural Affairs, Danzhou, China, [3] Danzhou Scientific Observing and Experimental Station of Agro-Environment, Ministry of Agriculture and Rural Affairs, Danzhou, China, [4] Hainan Engineering Research Center for Non-point Source and Heavy Metal Pollution Control, Haikou, China

Biological control agents (BCAs) are considered as one of the most important strategies for controlling *Fusarium* wilt, and bioorganic fertilizer, in particular, has been extensively investigated. However, little is known regarding how a biocontrol microorganism affects the suppression mechanisms when combined with different amendments. In this study, a pot experiment was performed using banana plants to investigate the different mechanisms by which the biocontrol bacterium *Bacillus velezensis* HN03 (isolated from our laboratory) and amendments suppress *Fusarium* wilt. The incidence of banana wilt was decreased under HN03 and was reduced further when HN03 was combined with compost, particularly wormcast. In the suppression of *Fusarium* wilt, HN03 was found to influence the soil environment in various ways. HN03 increased the peroxidase level, which improves plant defense, and was highest when combined with wormcast, being 69 times higher than when combined with cow dung compost. The high accumulation of Mg and P in the "HN03 + wormcast" and Zn and Mn in the "HN03 + cow dung" treatments was negatively correlated with disease incidence. Furthermore, HN03 re-established the microbial community destroyed by the pathogen and further increased the level of suppression in the wormcast. HN03 also enhanced the functional traits of the soil, including defensive mechanism-related traits, and these traits were further enhanced by the combination of HN03 + wormcast.

Keywords: *Bacillus velezensis*, *Fusarium wilt*, soil nutrient environment, plant immunity, bacteria community

OPEN ACCESS

Edited by:
Paulo José Pereira Lima Teixeira, University of São Paulo, Brazil

Reviewed by:
Luciano Kayser Vargas, State Secretariat of Agriculture, Livestock and Irrigation, Brazil
Bruno Brito Lisboa, State Secretariat of Agriculture, Livestock and Irrigation, Brazil

***Correspondence:**
Chunyuan Wu
cywu@catas.cn

† These authors have contributed equally to this work

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

Received: *28 August 2020*
Accepted: *27 October 2020*
Published: *16 November 2020*

Citation:
Wu X, Shan Y, Li Y, Li Q and Wu C (2020) The Soil Nutrient Environment Determines the Strategy by Which Bacillus velezensis HN03 Suppresses Fusarium wilt in Banana Plants. Front. Plant Sci. 11:599904. doi: 10.3389/fpls.2020.599904

INTRODUCTION

Soil-borne pathogens are the causal agents of several plant diseases of global importance and cause substantial economic losses (Ruiz-Romero et al., 2018). *Fusarium* wilt disease has become a serious threat to Cavendish banana (*Musa acuminata* L. AAA group, cv. Cavendish), which is the most widely planted cash crop in South China, because this cultivar is susceptible to the soil-borne pathogen *Fusarium oxysporum* f. sp. *cubense* tropical race 4 (*FOC*4) (Shen et al., 2015).

The biological control of *Fusarium* wilt by antagonistic bacteria offers a promising strategy and has attracted major research attention (Fu et al., 2017; Xiong et al., 2017; Sun et al., 2018). Biological control agents (BCAs) reduce infections or disease through antibiosis, parasitism, or competition (for space and/or nutrients), induction of plant local/systemic resistance, plant growth promotion,

or changes in soil/plant microbiota (Bubici et al., 2019). Several antagonistic bacteria, such as *Bacillus*, *Trichoderma*, *Pseudomonas*, non-pathogenic *Fusarium*, and *Penicillium* strains, have been evaluated as possible means of controlling *Fusarium* wilt of banana (Raza et al., 2017); however, no single biological product can be recommended for widespread use to control this disease (Dita et al., 2018). It is thought that compost application can help reduce pathogen attack and improve soil health and nutrient levels (Mehta et al., 2014), but the application of compost alone often results in inconsistent levels of disease control (Lang et al., 2011). However, the manipulation of compost by inoculation or enrichment with specific antagonists to produce bio-organic fertilizer is believed to be a more efficient means of controlling soil-borne disease than the use of a single antagonistic microbe or compost type (Shen et al., 2013).

Recently, some studies have evaluated combinations of antagonistic microbes and compost in controlling *Fusarium* wilt (Fu et al., 2016, 2017; Huang et al., 2019). Changes to the soil microbial community are considered as the main mechanism through which bio-organic fertilizer promotes soil suppression of disease (Shen et al., 2013; Huang et al., 2017). The reported mechanisms include inhibiting soil-borne pathogen growth, reducing the population of pathogens, recovering the microbial populations damaged by pathogens, and altering the composition of the bacterial community (Lang et al., 2011; Qiu et al., 2012; Zhao et al., 2014). Previous studies have also showed that the manipulation of soil P level by organic fertilizer is one possible mechanism by which bio-organic fertilizer reduces the incidence of *Fusarium* disease (Yergeau et al., 2006). Some other studies demonstrated that bio-organic fertilizer could induce plant suppression of pathogens by activating the defense enzymes of the plant (Wang et al., 2015), such as the enhancement of peroxidase (POD) activity when controlling *Fusarium* wilt of pepper following bio-organic fertilizer application (Wu et al., 2015). Though previous studies suggest similar mechanisms of action for antagonistic bacteria, compost, and bioorganic fertilizer, such as the induction of plant resistance and regulation of the microbial community (Dita et al., 2018), few studies have focused on how these factors interact to suppress pathogens, and, particularly, how BCAs alter the functional and nutritional characteristics of microbial communities to enhance disease control.

Understanding the action modes of BCAs is essential for exploiting their potential for effective disease management (Bubici et al., 2019). Previous studies showed that improvements in seed growth, nutrient uptake, and soil microflora may be related to the strain (*Bacillus* spp.) inoculated into the compost (El-Hassan and Gowen, 2006). Additionally, BCAs combined with compost might demonstrate better biocontrol due to additive, or even synergistic, interactions between BCA and compost. For example, the combination of compatible supplementary sources with a biocontrol strain can improve phytopathogen suppression to a reliable level (Regassa et al., 2018). However, our understanding of the role of BCAs in mediating the control mechanisms of banana *Fusarium* wilt under different nutrient environments in the soil remains limited. Here, we focus on the specific interactions between a BCA (*Bacillus velezensis* HN03) and its soil nutrient environment, aiming to construct a more comprehensive understanding of the role of HN03 in mediating nutrients and structuring the functional characteristics of microbial communities, as well as its role in inducing defense enzymes to control banana *Fusarium* wilt in plants and soil.

The specific interactions between BCA and the highly complex soil environment are difficult to discern when investigating a soil-borne disease, as their understanding requires an assessment of the broader influences on plant and soil suppression. Recently, Illumina sequencing technology has been widely used in microbial communities associated with banana *Fusarium* wilt. Unique distributions of bacteria and fungi were observed in diseased and disease-free soil samples from banana fields (Zhou et al., 2019). The importance of microorganisms in soil nutrient cycling and their role in plant nutrition is well established (Kucey et al., 1989), and some nutrients are reported to be related to plant disease defense (Siddiqui et al., 2015). If BCA can mediate the nutrient profile to defend against banana *Fusarium* wilt in different environments, then different nutrients would be found to accumulate in the soil and plants. Moreover, if HN03 can mediate the structural functional characteristics of microbial communities to defend against banana *Fusarium* wilt under different environments, then different suppression levels and functions would be found in the soil. Finally, BCA mobilizes different composts to construct a new suppression system in the soil and plant, and in this new system, the defense abilities of HN03 or compost are strengthened. Furthermore, new defense mechanisms are formed in this complex system.

Here, we evaluated the comprehensive mechanism of suppression of *Fusarium* wilt by BCA in different environments. We hypothesized that the suppression mechanisms are involved in plant immunity, soil microbial community, trophic interactions, and specific functional traits. In order to test our hypothesis, a new biocontrol bacterium, *Bacillus velezensis* HN03, with strong adaptability to the environment and wide application to various plant soil-borne diseases, was isolated and identified. Furthermore, pot experiments with banana plants were designed to determine the capacity of HN03 to suppress *FOC*4 among different nutrient environments, following which HN03-mediated transformation of the main mechanism involved in improving suppression in compost was explored. Collectively our data showed that HN03 regulated the changes in the main suppression mechanisms to control banana *Fusarium* wilt in different environments, thus highlighting the significance of BCA and appropriate carriers in controlling soil-borne disease.

MATERIALS AND METHODS

Bacterial Strain

The bacterial strain designated HN03 used in this study was isolated from laterite soil in our laboratory, which was collected from rhizospheric soil of healthy bananas in Haikou City, Hainan Province, China, located at 19°56′34″N, 110°04′27.25″E. The isolation of HN03 using a method from Li et al. (2017).

Testing for Antifungal Activity Against Fungal Pathogens

Twelve strains of fungal pathogens (**Supplementary Table 1**) were selected from the Key Laboratory of Integrated Pest Management on Tropical Crops, including 10 strains of soil-borne pathogens and 2 strains of common pathogens. The antagonism of HN03 toward the pathogens was assessed by measuring the inhibition of the growth rate using a method from Rajaofera et al. (2017) with a slight modification. A 6 mm mycelial disk of a pathogenic fungus collected from the edge of an actively growing colony was placed into the center of a PDA (potato dextrose agar) plate. An inoculum of HN03 bacterial cells (0.2 μL) was delivered around the periphery of the target fungus within a radius of 2.5 cm.

Identification of the Bacterial Strain

The HN03 strain was physiologically and biochemically characterized using a 96-well plate test system (Kämpfer et al., 1991) by a GenIII Microplate (Biolog, Hayward, United States). Characterizations including carbon source utilization and antibiotic resistance are listed in **Supplementary Table 2**, and other characteristics are listed in **Supplementary Table 3** and were tested according to the instructions of Bergey's Manual of Systematic Bacteriology (Sneath, 1986; Wayne, 1986).

Molecular biological tests were performed to verify the identification based on the physiological and biochemical tests. To determine the phylogenetic affiliation of strain HN03 for molecular identification, genomic DNA was extracted using a Bacterial Genomic DNA Extraction Kit (Solarbio, Beijing, China) and purified using a Universal DNA Purification Kit (Tiangen, Beijing, China). The 16S ribosomal gene sequence of strain HN03 was amplified using primers 27F and M1492R as described by Ma et al. (2016). The PCR product was cloned into a pEasy-T1 cloning vector (TransGen, Beijing, China) for sequencing. The sequence of strain HN03 was submitted to GenBank to search for similar sequences using the BLAST algorithm[1] and was compared with sequences available on the EzTaxon-e server provided by EzTaxon[2] (Kim et al., 2012).

DNA–DNA hybridization was also performed with the strain with the closest similarities in biochemical and physiological characteristics and 16S rRNA gene sequences. The levels of DNA–DNA hybridization were determined using a modified optical renaturation method described by De et al. (1970) and Gillis et al. (1970).

Bio-Organic Fertilizer Preparation

A single colony of the HN03 strain was grown in 50 mL of nutrient broth at 30°C for 24 h (at 200 rpm), which was then inoculated in nutrient agar at a 1:100 (v/v) ratio and grown at 30°C for 2–3 d (at 200 rpm) before harvesting. Bacterial cells were collected by centrifugation at 8,000 (× g) for 10 min and were resuspended with the same volume of distilled water at a final concentration of 10^8 CFU/mL, which was used as the bacterial cell inoculum for antifungal activity tests and bio-organic fertilizer preparation.

A total of 600 earthworms, *Eisenia fetida*, were grown in a box with a 4,500 g mixture of cattle manure and sawdust for approximately 1.5 months. Then the earthworms were removed, and the processed mixture was air dried, crushed, and passed through a 2 mm mesh sieve to obtain the wormcast used in the current study. The cow dung was fermented and composted for about 1 month in a box while covered by a plastic film. The dung was air dried, crushed, and passed through a 2 mm mesh sieve.

Pot Experiment

Pot experiments were performed from July to October 2017 in the greenhouse of the Chinese Academy of Tropical Agricultural Sciences, located in Hainan, China. Banana seedlings (*Musa acuminata* L. AAA group, cv. Cavendish) with 3–4 true leaves, weighing 3.30 ± 0.16 g and 8.29 ± 0.31 g in mass in the above- and belowground parts, respectively, and approximately 5.16 ± 0.12 cm in height, were used for the experiment.

*FOC*4 was grown in PDA liquid culture at 28°C for 4–5 d (at 200 rpm). The culture was filtered through a sterile pledget to obtain a spore suspension, which was then diluted to a concentration greater than 5×10^5 spores/mL with distilled water.

The soil used in the pot experiment, classified as laterite (clay), was collected from Haikou City, Hainan Province, China, located at 19°56′38.3″N, 110°28′42.9″E, and had the following properties: pH 7.1; organic matter (OM) 7.4 g kg^{-1}; available nitrogen (AN) 18.9 mg kg^{-1}; available phosphorus (AP) 3.58 mg kg^{-1}; available potassium (AK) 78.9 mg kg^{-1}; total nitrogen (TN) 701.99 mg kg^{-1}; total phosphorus (TP) 278.38 mg kg^{-1}; and total potassium (TK) 4601.47 mg kg^{-1}. The properties were measured according to section "Resistance Activity and Mineral Nutrient Assays for Leaf and Soil."

Banana seedlings were grown in plastic pots (7 cm diameter, 16-m depth) with 2,000 g of culture medium. All plant roots were treated with the *FOC*4 spore suspension for 20 min after trimming the roots to 10 cm, except the healthy controls, which were treated with water for 20 min after root trimming. To explore the action modes of HN03 and its nutrient environments to soil-borne disease, a factorial design (2 × 3 + control) was set to two levels of inoculation (with HN03 and without it) and three levels of amendments (without amendment, wormcast and cow dung compost), plus the control S. The pot experiment included the following seven treatments: (1) healthy control (S): healthy plants were grown in untreated soil that was irrigated with 500 mL of water every 7 d; (2) disease control (S + F): *FOC*4-infected plants were grown in soil that was irrigated with 500 mL of water every 7 d; (3) HN03 treatment (S + F + B): *FOC*4-infected plants were grown in soil that was irrigated with 500 mL of 50-fold diluted HN03 bacterial cell inoculum every 7 days; (4) HN03 amended with wormcast treatment (S + F + B + EW): *FOC*4-infected plants were grown in soil containing 10% (w/w) of the wormcast bio-organic fertilizer that was irrigated with 500 mL of 50-fold diluted HN03 bacterial cell inoculum every 7 days; (5) wormcast treatment (S + F + EW): *FOC*4-infected plants

[1] https://www.ncbi.nlm.nih.gov

[2] https://www.ezbiocloud.net

were grown in soil containing 10% (w/w) of the wormcast bio-organic fertilizer that was irrigated with 500 mL of water every 7 days; (6) HN03 amended with cow dung compost treatment (S + F + B + CD): *FOC*4-infected plants were grown in soil containing 10% (w/w) of the cow dung compost bio-organic fertilizer that was irrigated with 500 mL of 50-fold diluted HN03 bacterial cell inoculum every 7 days; and (7) cow dung compost treatment (S + F + CD): *FOC*4-infected plants were grown in soil with 10% (w/w) of the cow dung compost bio-organic fertilizer that was irrigated with 500 mL of water every 7 days. Therefore, every 7 days, plants in treatments S, S + F, S + F + EW, and S + F + CD were irrigated with water, whereas plants in treatments S + F + B, S + F + B + EW, and S + F + B + CD were irrigated with the same amount of diluted HN03 bacterial cell inoculum. The seedlings were grown in a greenhouse without any pesticides or fertilizers for 90 d; the temperature ranged from 22 to 30°C, and relative humidity was from 75 to 85%. One plant was planted per pot, with three pots per replicate and three replicates per treatment, resulting in a total of 63 seedlings for the seven treatments.

Disease Incidence and Plant Growth Assessment

Seedling infection by *FOC*4 was recorded daily, and disease development investigated as disease incidence (DI) was recorded on a 5-grade scale from 0 to 4 as described by Huang et al. (2014): 0 = no wilting, 1 = 1–25% wilting, 2 = 26-50% wilting, 3 = 51-75% wilting, and 4 = 76-100% wilting or dead. The DI value of the different treatments was calculated according to the method described by Huang et al. (2014). The biocontrol efficacies (BE) were calculated as described by Tan et al. (2015).

Seedling pseudo-stem height (distance from the base of the plant to the point of the youngest emergent leaf) was measured. The fresh plants were dried in an oven at 70°C for 72 h until constant weight was reached, and the dry weight (weight of aboveground parts including the leaves and banana cauloid and weight of belowground parts including the banana corm and root) was measured on a scale (± 0.01 g).

Resistance Activity and Mineral Nutrient Assays for Leaf and Soil

After 90 d, leaf samples and soil samples were obtained from three biological replicate pots. The first and third leaves from the apex of the dominant stem were combined and used for plant tissue analysis.

The POD content in the banana seedlings was analyzed using their respective assay kits (Solarbio, Beijing, China).

The leaves detached from the stems were cleaned dried and ground using a ball mill. Tissue nitrogen (N) content was measured in a Carlo Erba NA 1,500 C/N analyzer (Milan, Italy) (Steiner et al., 2008). Tissue phosphorus (P) content was determined using Mo–Sb colorimetry (Kowalenko and Babuin, 2007). For mineral nutrients, potassium (K), calcium (Ca), magnesium (Mg), manganese (Mn), iron (Fe), and zinc (Zn) content were determined by an atomic absorption spectrophotometer (PerkinElmer PinAAcle 900T, Waltham, MA, United States) after extraction (Muhammad et al., 2010).

Soil samples were collected using a horticultural shovel and were air dried and crushed to pass through a 2-mm mesh sieve after removing the plant parts. Loss-on-ignition as a rough measure of soil OM was determined by igniting 2 g of soil in a muffle furnace at 600°C for 6 h followed by overnight cooling (Salehi et al., 2011). TP, AP, TN, AN, TK, and AK were measured according to Roiloa et al. (2015). Exchangeable Mg and Ca were determined by an atomic absorption spectrophotometer (PerkinElmer PinAAcle 900T, Waltham, MA United States) after extraction (Olorunfemi et al., 2018).

Soil DNA Extraction, PCR, and Sequencing

Total soil DNA was extracted from samples using a Power Soil DNA Isolation Kit (MO BIO Laboratories, Carlsbad, CA, United States) according to the manufacturer's protocol. DNA quality and quantity were assessed based on the ratios of 260/280 and 260/230 nm. The DNA was stored at −80°C until further processing. The V3–V4 region of the bacterial 16S rRNA gene was amplified (forward primer, 5′-ACTCCTACGGGAGGCAGCA-3′; reverse primer, 5′-GGACTACHVGGGTWTCTAAT-3′) (Mori et al., 2014) and combined with adapter sequences and bar code sequences. The ITS1 region of the fungi was amplified (forward primer, 5′-CTTGGTCATTTAGAGGAAGTAA-3′; reverse primer, 5′-GCTGCGTTCTTCATCGATGC-3′) (Fouquier et al., 2016) and combined with adapter sequences and bar code sequences. The PCR amplification was conducted in 50-μL reactions containing 10 μL of 5 × PCR buffer, 0.2 μL of Q5 High-Fidelity DNA Polymerase (NEB, Ipswich, United States), 10 μL of High GC Enhancer, 1 μL of dNTP, 10 μM each primer, and 60 ng of genomic DNA. The PCR amplification conditions were set as follows: an initial denaturation at 95°C for 5 min; followed by 15 cycles consisting of 95°, 50°C, and 72°C for 1 min; and a final extension step at 72°C for 7 min. The PCR products from the first round of PCR were purified through VAHTSTM DNA Clean Beads (Vazyme, Nanjing, China) and were used as the template in the second round of PCR. A second round of PCR was then performed in a 40-μL reaction containing 10 μL of PCR products from the first round of PCR, 20 μL of 2 × Phusion HF MM, 8 μL of ddH_2O, and 10 μM each primer. The PCR amplification conditions were set as follows: an initial denaturation at 98°C for 30 s; followed by 10 cycles consisting of 98°C for 10 s, 65°C for 30 s, and 72°C for 30 s; and a final extension step at 72°C for 5 min. Finally, all PCR products quantified by the Quant-iT^{TM} dsDNA HS Reagent (Invitrogen, Carlsbad, CA, United States) were pooled and used for high-throughput sequencing analysis of bacterial rRNA genes in an Illumina HiSeq 2,500 platform (Illumina, Santiago, United States) (2 × 250 paired ends) at Biomarker Technologies Corporation, Beijing, China.

Statistical Analyses

All data were analyzed using the statistical program IBM SPSS version 19 (2010 SPSS, Inc., Chicago, IL, United States). Data

are presented as the mean ± SD. Non-normally distributed variables were normalized using Bloom's Formula (Bo et al., 2009), and means were compared using Tukey's test at 5%. Differences were analyzed using one-way ANOVA and least significant difference (LSD) tests. Differences at $P < 0.05$ were considered statistically significant. PCA and RDA were conducted using Canoco version 5 (2012, Biometry, Plant Research International, the Netherlands). Sequence analysis was performed by the UPARSE version 10.0 software (2013, CA, United States) package using the UPARSE-OTU and UPARSE-OTUref algorithm (Edgar, 2013). In-house Perl scripts were used to analyze alpha (within samples) and beta (among samples) diversity. Sequences with ≥ 97% similarity were assigned to the same OTUs, and heatmaps based on the retained OTUs were constructed based on the Bray–Curtis distance and Binary–Jaccard distance (Winstanley et al., 2005) using BMKCloud[3]. In order to compute alpha diversity, we rarified the OTU table and calculated five metrics: Chao1 and ACE estimates for species abundance; Observed species estimates for the number of unique OTUs found in each sample; and Simpson and Shannon indexes using Mothur v1.30 (2013, MI, US) (Schloss et al., 2009). The smaller the Simpson index, the higher the community diversity. A greater Shannon index indicates a higher community diversity. Rarefaction curves were generated based on these three metrics (Wang et al., 2012; Zhao et al., 2016). Microorganism features distinguishing specific microbiota were identified using the LDA LEfSe method[4] for biomarker discovery, and an alpha significance level of 0.05 and an effect-size threshold of 2 were used for all biomarkers (Jiang et al., 2015). To determine the statistical differences of bacterial functions between the treatments, Statistical Analysis of Metagenomics Profile v2.1.3 (STAMP) software based on the Clusters of Orthologous Groups (COG) was used (Parks et al., 2014). Spearman's correlation analysis was performed to determine the links between the bacterial community and the environmental factors (AK, AP, AN, TK, TP, TN, OM, Ca, and Mg) using IBM SPSS version 19 (2010 SPSS, Inc., Chicago, IL, United States). The significance tests of Monte Carlo permutations were conducted to construct the appropriate models of the bacteria–environment relationships using Canoco version 5 (2012, Biometry, Plant Research International, Netherlands).

[3]www.biocloud.net

[4]http://huttenhower.sph.harvard.edu/lefse/

RESULTS

Identification of the Bacterial Strain

Strain HN03 was phenotypically characterized as a Gram-positive, rod-shaped, endospore-forming bacterium with positive oxidase activity. The strain produced proteases, cellulase, and amylase and was positive for the Voges–Proskauer test (**Supplementary Table 1**). HN03 grew well in the temperature range of 15–50°C, a NaCl content of 0–10% (w/v), and on medium amended with glucose, sucrose, glycerol, and D-mannitol. HN03 could assimilate carbon from a wide variety of sources and demonstrated good growth with various nitrogen source extracts (**Supplementary Table 2**).

BLAST analysis revealed a high level of similarity (99%) to the sequence of *B. velezensis*, as well as to those of other *Bacillus* species. Pairwise sequence similarities of the 16S rRNA genes of HN03 to the most closely related type strains revealed a high sequence similarity of 99.93% in both strains with that of *B. velezensis* and *B. siamensis*. The sequences of 16S rRNA of the HN03 strain were deposited in GenBank under accession No. MF155192. In the maximum-likelihood tree (**Figure 1**),

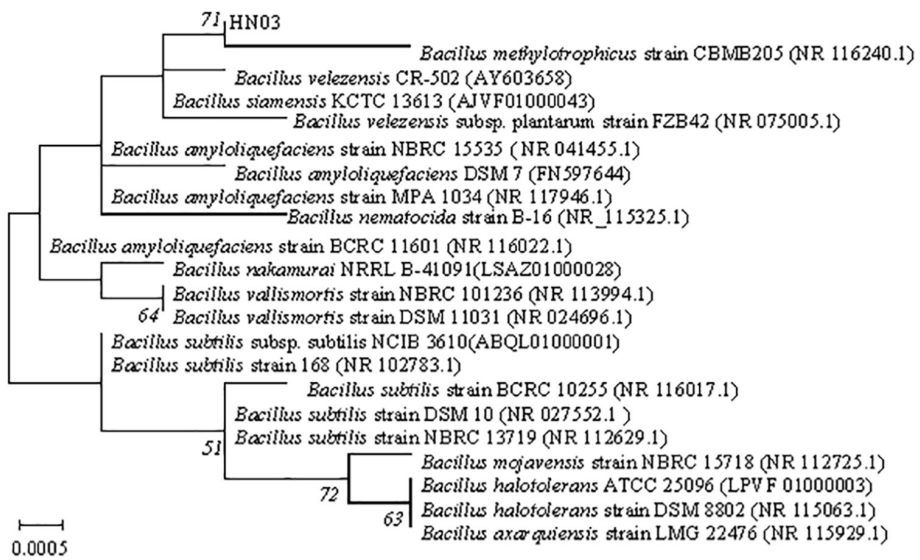

FIGURE 1 | Maximum-likelihood phylogenetic tree based on partial 16S rRNA gene sequences (1,424 bp) showing the position of strain HN03. Bootstrap values (>50%) based on 1,000 replications are shown at branch nodes. Bar = 0.0005 substitutions per nucleotide position.

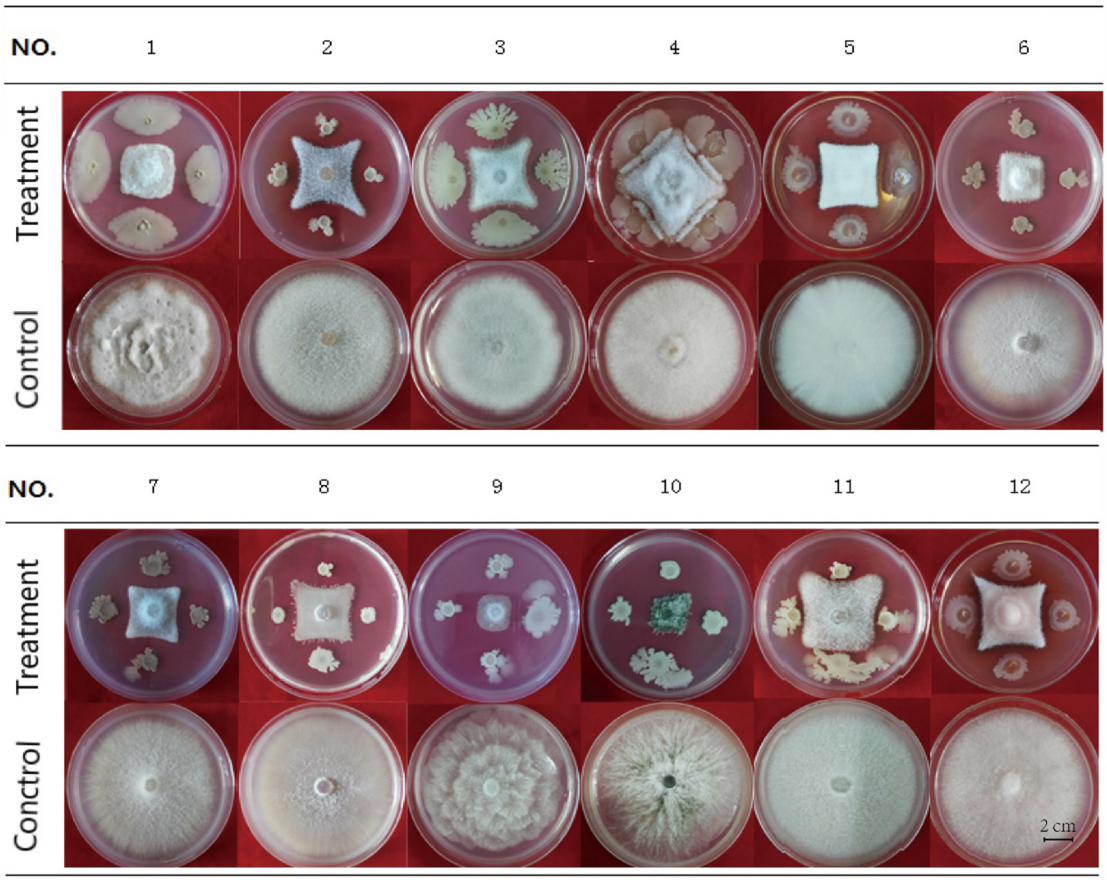

FIGURE 2 | Antifungal activity of HN03 against 12 pathogenic strains of fungi. A dual culture assay was used to determine the *in vitro* inhibition of mycelial growth. The fungal pathogens were co-cultured with the bacterial strain HN03 on potato dextrose agar (treatment, pathogen + HN03; control, pathogen only). No. 1, *FOC*4 (Hainan); No. 2, *FOC*4 (Vietnam); No. 3, *Fusarium oxysporum* f. sp. cubense 1 (Hainan); No. 4, *Fusarium solani* of Noni; No. 5, *Fusarium solani* of Annona squamosal; No. 6, *Fusarium oxysporum* f. sp. *radicis lycopersic*; No. 7, *Fusarium oxysporum* f. sp. *melonis*; No. 8, *Fusarium oxysporum* f. sp. *niveum*; No. 9, *Phytophthora nicotianae*; No. 10, *Colletotrichum gloeosporioides*; No. 11, *Fusarium solani* of Medicago; No. 12, *Fusarium solani* of Annona squamosal (Hainan).

HN03 was within a group containing *B. methylotrophicus* strain CBMB205, adjacent to *B. velezensis* CR-502, *B. siamensis* KCTC 13613, and *B. velezensis* subsp. *plantarum* strain FZB42, and was similar to the strain *Bacillus amyloliquefaciens* DSM 7. The biochemical and physiological data of those strains were reported in previous studies (Ruiz-Garcia et al., 2005; Madhaiyan et al., 2010; Sumpavapol et al., 2010; Borriss et al., 2011) and are compared with HN03 in **Supplementary Table 2**. The data showed that *B. velezensis* subsp. *plantarum* strain FZB42 had the highest similarity with HN03.

DNA–DNA hybridization showed that HN03 had 90.9% DNA–DNA relatedness to the closest reference isolate *B. velezensis* FZB42^T. According to these results, HN03 is closely related taxonomically to the plant-associated strains of *B. velezensis*.

Antifungal Activity of HN03

HN03 had inhibitory activity against a broad spectrum of fungal pathogens and suppressed the mycelial growth of the 12 tested strains (**Figure 2**), 10 of which were soil-borne pathogens and 2 of which were common pathogens. The activity of HN03 against *FOC4* (Vietnam) was 68.79%, followed by *FOC*4 at 68.47% (**Supplementary Table 3**).

Efficiency Against Banana *Fusarium* wilt of HN03 and Compost

Irrigation with HN03 rendered the dry weight of the underground parts significantly greater than that of the plants in the disease control (S + F) treatment and was highest in treatment "S + F + B + EW" (**Figure 3A**). The wormcast was a much better supplement than cow dung when measured based on the weight of the aboveground parts and the pseudo-stem height of the banana plants, while HN03 application weakened the significant differences observed in the aboveground parts between cow dung and wormcast.

In comparison to the disease control treatment (S + F), both the HN03 treatments (S + F + B) and compost treatments (S + F + EW and S + F + CD) significantly reduced the disease incidence (DI) of banana *Fusarium* wilt (**Figure 3B**). The

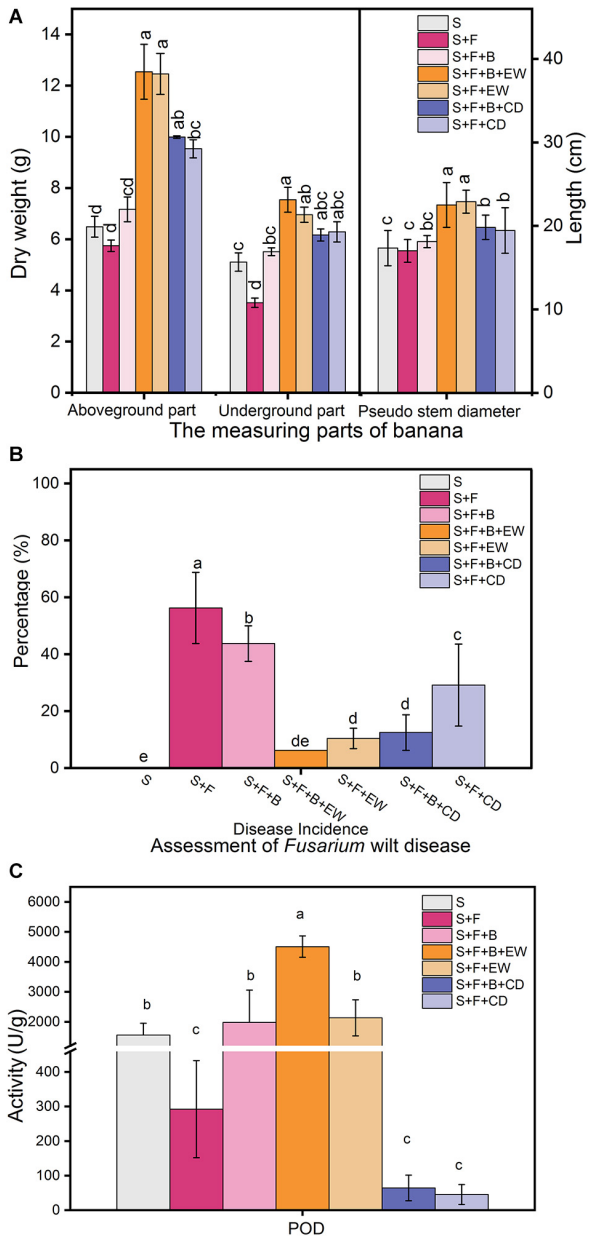

FIGURE 3 | Effects of the treatments on **(A)** dry weights of the aboveground and belowground parts and the height (cm) of the pseudo-stem of banana, **(B)** assessment of *Fusarium* wilt disease, and **(C)** contents of peroxidase (POD) in banana leaves in the pot experiment. Values are the mean ± SD of three replicates. S, healthy control (healthy soil); S + F, disease control (healthy soil + *FOC*4); S + F + B, healthy soil + *FOC*4 + HN03 treatment; S + F + B + EW, healthy soil + *FOC*4 + HN03 + wormcast treatment; S + F + EW, healthy soil + *FOC*4 + wormcast treatment; S + F + B + CD, healthy soil + *FOC*4 + HN03 + cow dung compost treatment; S + F + CD, healthy soil + *FOC*4 + cow dung compost treatment. Bars sharing the same letter are not significantly different based on Duncan's multiple range test at $P < 0.05$.

"S + F + B + EW" treatment showed the lowest DI among the *FOC*4-treated treatments, with a value of 6.25%. Interestingly, there was a significant difference in DI between the "S + F + CD" and "S + F + B + CD" treatments, but no significant difference in DI between "S + F + EW" and "S + F + B + EW."

Peroxidase in the Leaves

As shown in **Figure 3C**, the addition of HN03 increased the POD content in the banana seedlings, and the POD content of the plants in treatment "S + F + B" was approximately seven times higher than that in treatment "S + F." When HN03 was combined with wormcast in treatment "S + F + B + EW," the POD content of the plants increased significantly and was the highest among the seven treatments, and was more than two times that in the plants in treatment "S + F + EW." The wormcast was a much better supplement than cow dung, and the POD content of the plants in treatment "S + F + B + EW" was 69 times higher than that in treatment "S + F + B + CD".

Mineral Nutrients in the Leaf and Soil

The addition of HN03 increased the content of Mn in the banana leaves in treatments "S + F + B," "S + F + B + EW," and "S + F + B + CD," which had significantly higher contents than their comparable treatments without HN03 (S + F, S + F + EW, S + F + CD), and the highest contents were found in "S + F + B + CD." The content of Zn was high in the treatment "S + F" and highest in "S + F + B + CD." The contents of P and Mg in the banana leaves in the wormcast treatments ("S + F + B + EW" and ""S + F + EW") were significantly higher than those in the cow dung treatments ("S + F + B + CD" and "S + F + CD"), and the content of Mg in "S + F + B + EW" was significantly higher than in the other treatments (**Figure 4A**). In addition, the contents of N, K, and Ca of the leaves in all treatments were not significantly different from each other (**Figure 4B**).

The mineral nutrient concentrations of the three environments, initial soil (S_0), initial soil with wormcast $(S + EW)_0$, and natural soil with cow dung compost $(S + CD)_0$, were investigated before adding HN03. As shown in **Figure 5**, the TP, AP, AN, Ca, and Mg contents in $(S + EW)_0$ were significantly higher than those in CD and S_0, while the AK contents were highest in $(S + CD)_0$. After the 3-month pot experiments, the contents of TP, AP, Ca, and Mg remained at high levels in the wormcast treatments ("S + F + B + EW" and "S + F + EW"), and when combined with HN03 (S + F + B + EW), the TK and AN contents reached the highest level. In contrast, the contents of AK and OM remained at high levels in the cow dung treatments ("S + F + B + CD" and "S + F + CD"), and the contents of TN and OM reached the highest levels when combined with HN03 (S + F + B + CD).

Composition, Structure, and Functional Annotation of the Soil Microbial Community

The indices for community abundance, i.e., Chao 1 and ACE, and the indices for community diversity, i.e., Simpson and Shannon, were estimated (**Table 1**). The values of the Chao 1 and ACE indices for bacterial community abundance were significantly higher in the soil treated with HN03 than those in

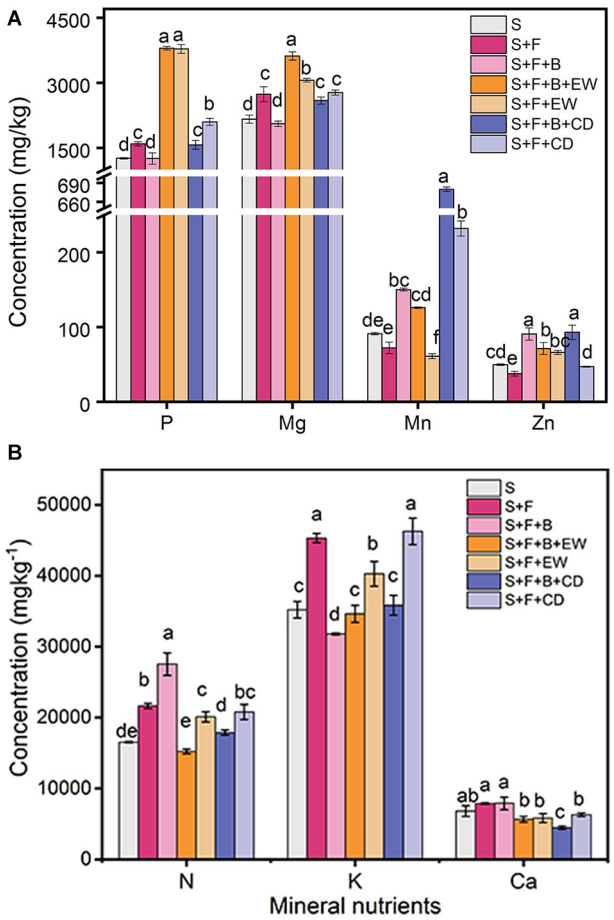

FIGURE 4 | Contents (mg/kg) of mineral nutrients in banana leaves in the pot experiment. **(A)** P, Mg, Mn, and Zn; **(B)** N, K, and Ca. Values are the mean ± SD of three replicates. S, healthy control; S, healthy control (healthy soil); S + F, disease control (healthy soil + *FOC*4); S + F + B, healthy soil + *FOC*4 + HN03 treatment; S + F + B + EW, healthy soil + *FOC*4 + HN03 + wormcast treatment; S + F + EW, healthy soil + *FOC*4 + wormcast treatment; S + F + B + CD, healthy soil + *FOC*4 + HN03 + cow dung compost treatment; S + F + CD, healthy soil + *FOC*4 + cow dung compost treatment. Bars sharing the same letter are not significantly different based on Tukey's test at $P < 0.05$.

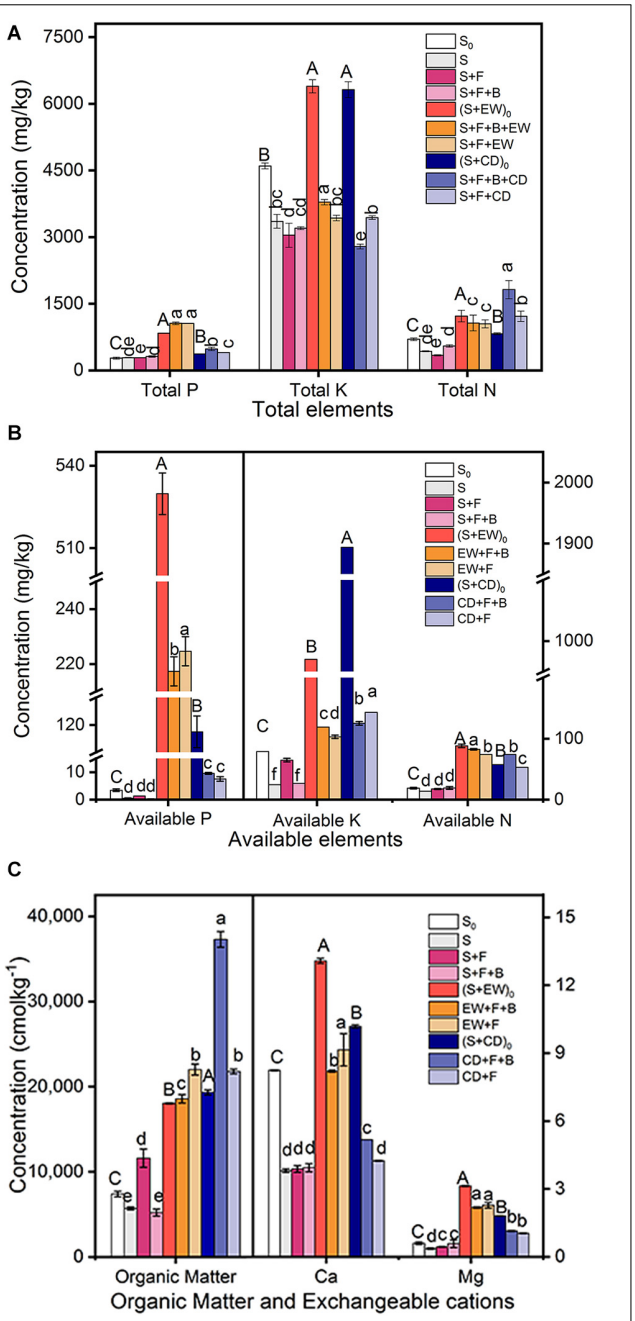

FIGURE 5 | Contents (mg/kg) of mineral nutrients in the soil in the pot experiment. **(A)** Total elements: TP, TK, and TN; **(B)** available elements: AP, AK, and AN; **(C)** organic matter (OM), Ca, and Mg. Values are the mean ± SD of three replicates. S_0, initial soil; $(S + EW)_0$, initial soil combined 10% (w/w) wormcast; $(S + CD)_0$, initial soil combined 10% (w/w) cow dung compost; S, healthy control (healthy soil); S + F, disease control (healthy soil + *FOC*4); S + F + B, healthy soil + *FOC*4 + HN03 treatment; S + F + B + EW, healthy soil + *FOC*4 + HN03 + wormcast treatment; S + F + EW, healthy soil + *FOC*4 + wormcast treatment; S + F + B + CD, healthy soil + *FOC*4 + HN03 + cow dung compost treatment; S + F + CD, healthy soil + *FOC*4 + cow dung compost treatment. In the graph, capital letters indicate significant differences in original soil properties, and lowercase letters indicate significant differences in the potting soil in the different treatments. Bars sharing the same letter are not significantly different based on Tukey's test at $P < 0.05$.

the "S + F" treatment, and the values were highest in the two treatments with wormcast. Additionally, the highest Shannon index and the lowest Simpson index were obtained in the "S + F + B + EW" treatment.

The heatmap analysis of the operational taxonomic units (OTUs) with hierarchical clustering based on the Bray–Curtis distance and Binary–Jaccard distance indicated that the community structural patterns differed significantly with respect to amendment types. In terms of bacteria (**Figure 6**), the treatments with cow dung compost or wormcast were clearly separated from the treatments without compost. For the groups in soil without compost, treatment "S + F" was clearly separated from "S and S + F + B." Groups with compost were divided into two groups: one with wormcast and one with cow dung compost. With the addition of HN03,

TABLE 1 | The means of OTU, Chao 1, ACE, Simpson, and Shannon indices for soil bacteria (97% similarity) challenged with *FOC*4.

Treatment	OTUs	ACE	Chao1	Simpson	Shannon
S	1268 ± 10cd	1350.21 ± 34.85cd	1373 ± 32bc	0.0068 ± 0.0006a	5.94 ± 0.01cd
S + F	1159 ± 27d	1238.24 ± 31.2d	1246 ± 39c	0.0064 ± 0.0003a	5.81 ± 0.04d
S + F + B	1351 ± 11bc	1431.72 ± 36.62abc	1460 ± 47ab	0.0056 ± 0.0001ab	6.00 ± 0.02c
S + F + B + EW	1405 ± 31a	1487.95 ± 52.28ab	1505 ± 67a	0.0041 ± 0.0004c	6.30 ± 0.03a
S + F + EW	1388 ± 15ab	1487.88 ± 7.84a	1509 ± 12a	0.0054 ± 0.0011ab	6.14 ± 0.05b
S + F + B + CD	1320 ± 32bc	1425.97 ± 21.96abc	1461 ± 18ab	0.0059 ± 0.0005a	5.99 ± 0.09c
S + F + CD	1305 ± 45c	1405.38 ± 31.75bc	1437 ± 31abc	0.0062 ± 0.0005a	6.01 ± 0.02c

Data are presented as the mean ± SD. Different letters in each column indicate significant differences on the basis of Tukey's test ($P < 0.05$). S, healthy control (healthy soil); S + F, disease control (healthy soil + FOC4); S + F + B, healthy soil + FOC4 + HN03 treatment; S + F + B + EW, healthy soil + FOC4 + HN03 + wormcast treatment; S + F + EW, healthy soil + FOC4 + wormcast treatment; S + F + B + CD, healthy soil + FOC4 + HN03 + cow dung compost treatment; S + F + CD, healthy soil + FOC4 + cow dung compost treatment.

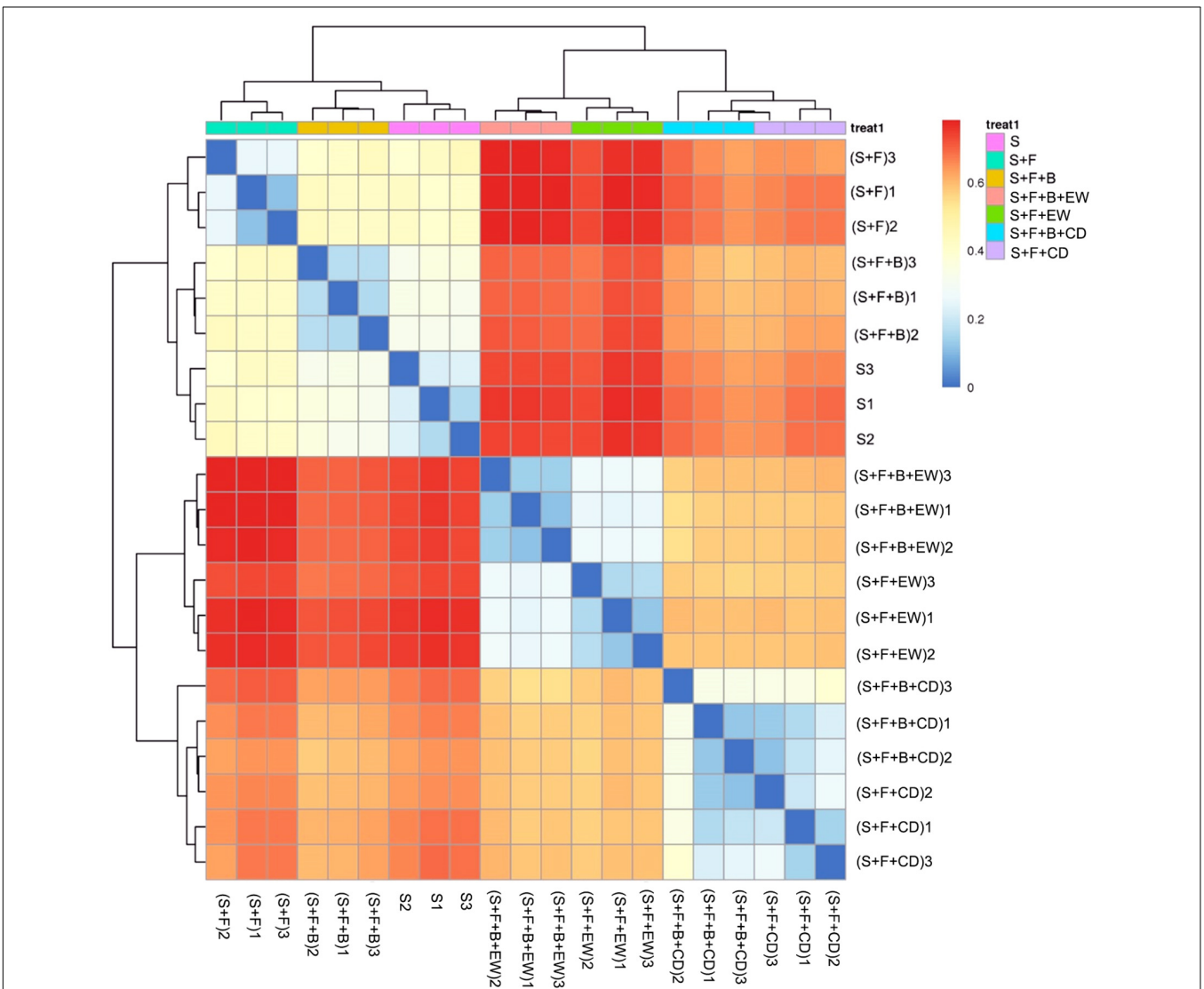

FIGURE 6 | Heatmap of similarities between the bacterial communities based on Bray–Curtis distance indices in the different treatments. S, healthy control (healthy soil); S + F, disease control (healthy soil + *FOC*4); S + F + B, healthy soil + *FOC*4 + HN03 treatment; S + F + B + EW, healthy soil + *FOC*4 + HN03 + wormcast treatment; S + F + EW, healthy soil + *FOC*4 + wormcast treatment; S + F + B + CD, healthy soil + *FOC*4 + HN03 + cow dung compost treatment; S + F + CD, healthy soil + *FOC*4 + cow dung compost treatment. Every treatment has three replicates and was named treatment or (treatment) + numbers (1, 2, 3). The color gradient from red to blue indicates increasing similarity. Panels within the figures indicate the similarity among soil samples.

the wormcast groups were well separated, whereas the cattle manure groups were not as well separated. In addition, treatment "S + F + B + EW" had the lowest bacterial community similarity with that of treatment "S + F." The grouping of fungal communities was similar to that of the bacterial communities (**Supplementary Figure 1**).

According to the comparative analysis of the microbiome in the soil samples of all treatments, differences were observed in the community structure and abundance of specific family groups. The 16S rRNA gene data using the linear discriminant analysis (LDA) effect size (LEfSe) approach was used to further reveal the abundance of the top 20 bacterial families and identify the key phylotypes among the treatments. The results are shown in **Figure 7**. Compared with the healthy soil without *FOC4*, the abundance of Comamonadaceae, Solibacteraceae, Methylophilaceae, Xanthomonadaceae_Incerae_Sedis, Cytophagaceae, and Xanthomonadaceae was decreased significantly in the soil of the "S + F" treatment, and the abundance of Cytophagaceae was the lowest among all treatments. In the soil inoculated with *FOC4*, the bacterial families Comamonadaceae, Methylophilaceae, and Xanthomonadaceae were recovered in the treatments with HN03, and the highest abundances of Xanthomonadaceae, Chitinophagaceae, Micrococcaceae, and Oxalobacteraceae were found in the "S + F" treatment. The highest abundance of Methylophilaceae was detected when HN03 was combined with wormcast. Cytophagaceae was significantly enriched in all amended treatments, especially in the treatment "S + F + B + EW." Additionally, the abundance of Solibacteraceae and Xanthomonadaceae_Incerae_Sedis was mainly recovered in the treatments amended with cow dung compost and wormcast, respectively. On the contrary, the abundance of Intrasporangiaceae, uncultured_bacterium_o_Acidmicrobiales, and uncultured_bacterium_o_Saccharibacteria was increased significantly in the soil of the "S + F" treatment compared with the healthy soil. The abundance of these bacterial families was significantly suppressed by HN03 or wormcast, especially HN03 combined with wormcast.

Several COG categories were further exploration to differ significantly between these groups (**Figure 8**) indicating that at a broad scale these groups are metabolically and functionally distinct from each other. Examining individual COG categories in detail indicates that the "S + F + B + EW" contains relatively more genes assigned to categories defense mechanisms compared with the other treatments. The genes assigned to categories cell motility and signal transduction mechanisms in the treatment "S + F" were relatively less compared with the healthy soil, and were increased significantly in "S + F + B + EW" (**Supplementary Figure 2**). HN03 enhanced significantly different COGs within categories such as cell wall/membrane/envelope biogenesis; cell motility; posttranslational modification, protein turnover, and chaperones; intracellular trafficking, secretion, and vesicular transport; inorganic ion transport and metabolism; defense mechanisms; signal transduction mechanisms; and function unknown, while the highest abundance of cell wall/membrane/envelope biogenesis; cell motility, posttranslational modification, protein turnover, and chaperones; intracellular trafficking, secretion, and vesicular transport; inorganic ion transport; and metabolism.

Associations of DI, Leaf and Soil Mineral Nutrition, and Microbes

According to the principal component analysis (PCA) (**Figures 9A,B**), the associations of DI, fresh weight, and BE with leaf mineral nutrition and soil mineral nutrition explained 97.23 and 96.62% of the variability, respectively, of all the data sets. The first component (PC1), which explained 90.67 and 90.02% of the total variation of DI associated with leaf and soil mineral nutrition, respectively, separated treatments "S," "S + F," and "S + F + B" from the treatments amended with cow dung compost or wormcast. The second component (PC2), which explained 6.56 and 6.6% of the total variation of DI associated with leaf and soil mineral nutrition, respectively, separated treatment "S + F" from treatments "S" and "S + F + B" and separated treatments "S + F + B + EW" and "S + F + EW" from treatments "S + F + B + CD" and "S + F + CD".

With respect to the leaf, the highest P and Mg contents were in treatment "S + F + B + EW," and the highest Mn and Zn contents were in treatment "S + F + B + CD." The higher Zn and Mn contents in treatment "S + F + B" were negatively correlated with DI (**Figure 9A**). The nutrient concentrations of TP, TK, TN, AP, AK, AN, Ca, Mg, and OM in the soil were negatively correlated with DI, particularly the TK and TN contents (**Figure 9B**).

Redundancy analysis (RDA) showed that the first and second RDA components were able to explain 73.71 and 72.51% of the total bacterial variation in leaf and soil nutrients, respectively (**Figures 9C,D**). The first component (RDA1), explaining 51.34 and 50.51% of the total variation of bacterial families with leaf and soil nutrients, respectively, separated the treatments "S," "S + F," and "S + F + B" from treatments amended with cow dung compost or wormcast. The second component (RDA2), explaining 22.37 and 22.01% of the total variation of bacterial families with leaf and soil nutrients, respectively, separated treatment "S + F" from treatments "S" and "S + F + B," and treatment "S + F + B + EW" from treatments "S + F + B + CD," "S + F + CD," and "S + F + EW."

The similarity in Euclidean distance between "S" and "S + F + B" was high, whereas the lowest similarity was detected between "S + F" and "S + F + B + EW" (**Figure 9**), which was consistent with the similarity of the bacterial groupings (**Figure 6**). When the plant and associated soil were threatened by *FOC*4, the addition of HN03 reshaped the elements and soil community structure into a healthy environment. In particular, the DI values were lower when HN03 was combined with wormcast.

The dominant bacterial communities were associated with soil nutrients in the different environments. Spearman's correlation analysis results based on the selected soil chemical properties and the top-20 bacterial families and Bacillaceae family abundance results revealed that among the candidate bacterial families,

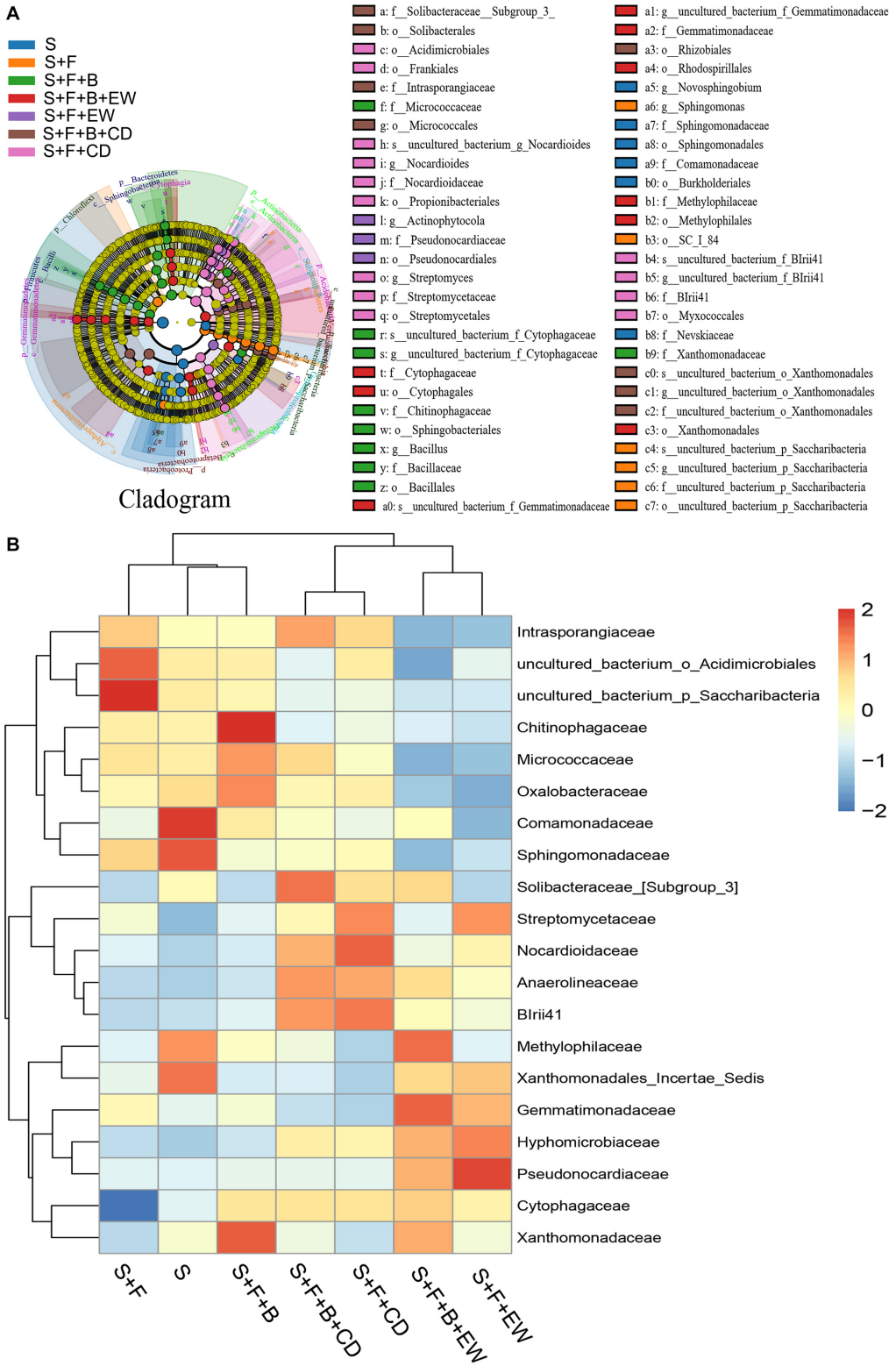

FIGURE 7 | Taxonomic cladogram obtained from LEfSe analysis of 16S sequences (relative abundance $P \geq 0.5\%$) **(A)**; top-20 heatmap similarities between the bacterial communities based on Euclidean distance indices of the different treatments **(B)**. S, healthy control (healthy soil); S + F, disease control (healthy soil + *FOC*4); S + F + B, healthy soil + *FOC*4 + HN03 treatment; S + F + B + EW, healthy soil + *FOC*4 + HN03 + wormcast treatment; S + F + EW, healthy soil + *FOC*4 + wormcast treatment; S + F + B + CD, healthy soil + *FOC*4 + HN03 + cow dung compost treatment; S + F + CD, healthy soil + *FOC*4 + cow dung compost treatment.

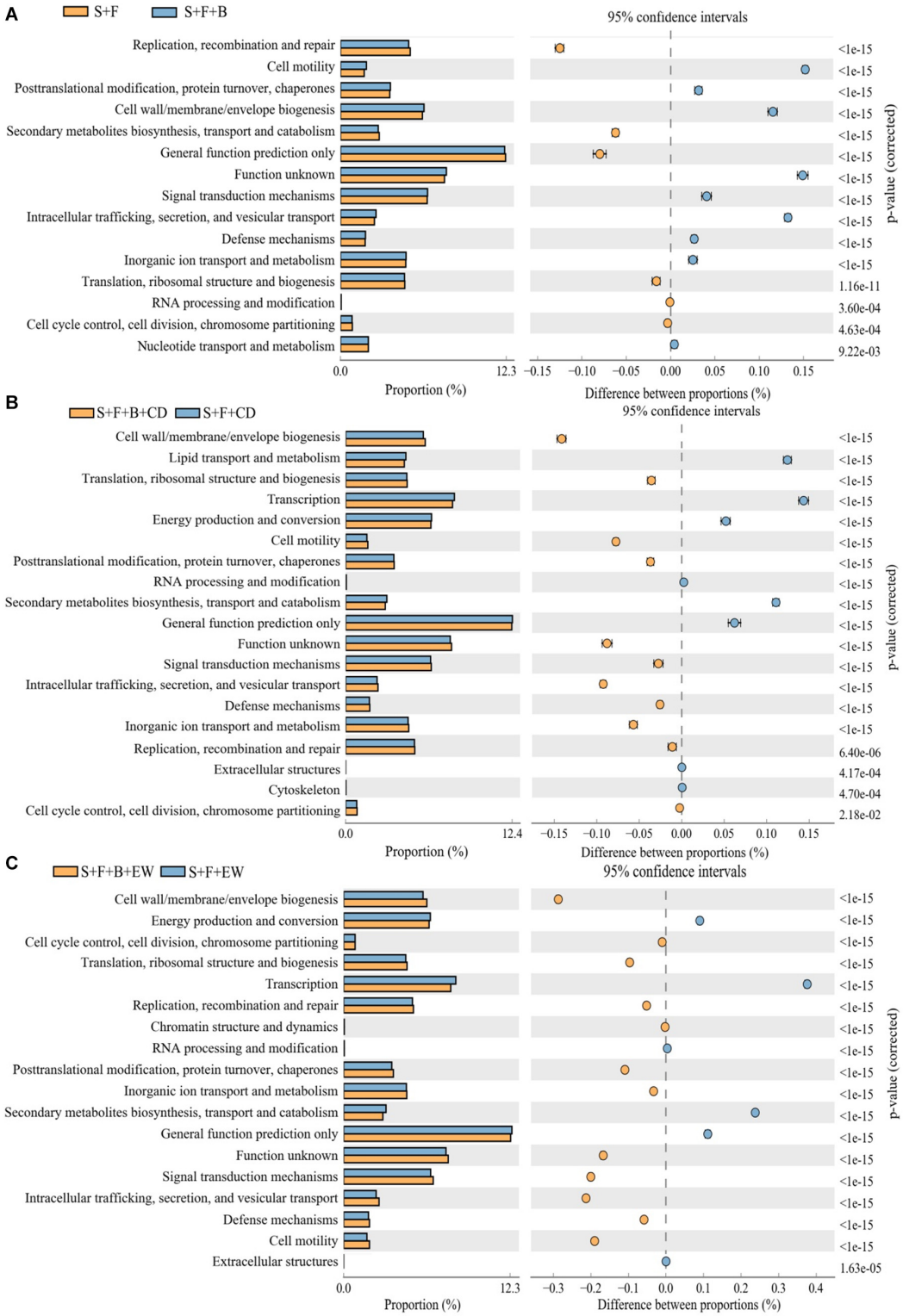

FIGURE 8 | The cluster of orthologous groups (COG) categories differed significantly between treatments. "S + B" and "S + B + C" treatments **(A)**, "S + F + B + CD" and "S + F + CD" **(B)**, and "S + F + B + EW" and "S + F + EW" **(C)**. Values are the mean ± SD of three replicates. S, healthy control (healthy soil); S + F, disease control (healthy soil + *FOC*4); S + F + B, healthy soil + *FOC*4 + HN03 treatment; S + F + B + EW, healthy soil + *FOC*4 + HN03 + wormcast treatment; S + F + EW, healthy soil + *FOC*4 + wormcast treatment; S + F + B + CD, healthy soil + *FOC*4 + HN03 + cow dung compost treatment; S + F + CD, healthy soil + *FOC*4 + cow dung compost treatment. Bars sharing the same letter are not significantly different on the basis of Tukey's test at $P < 0.05$.

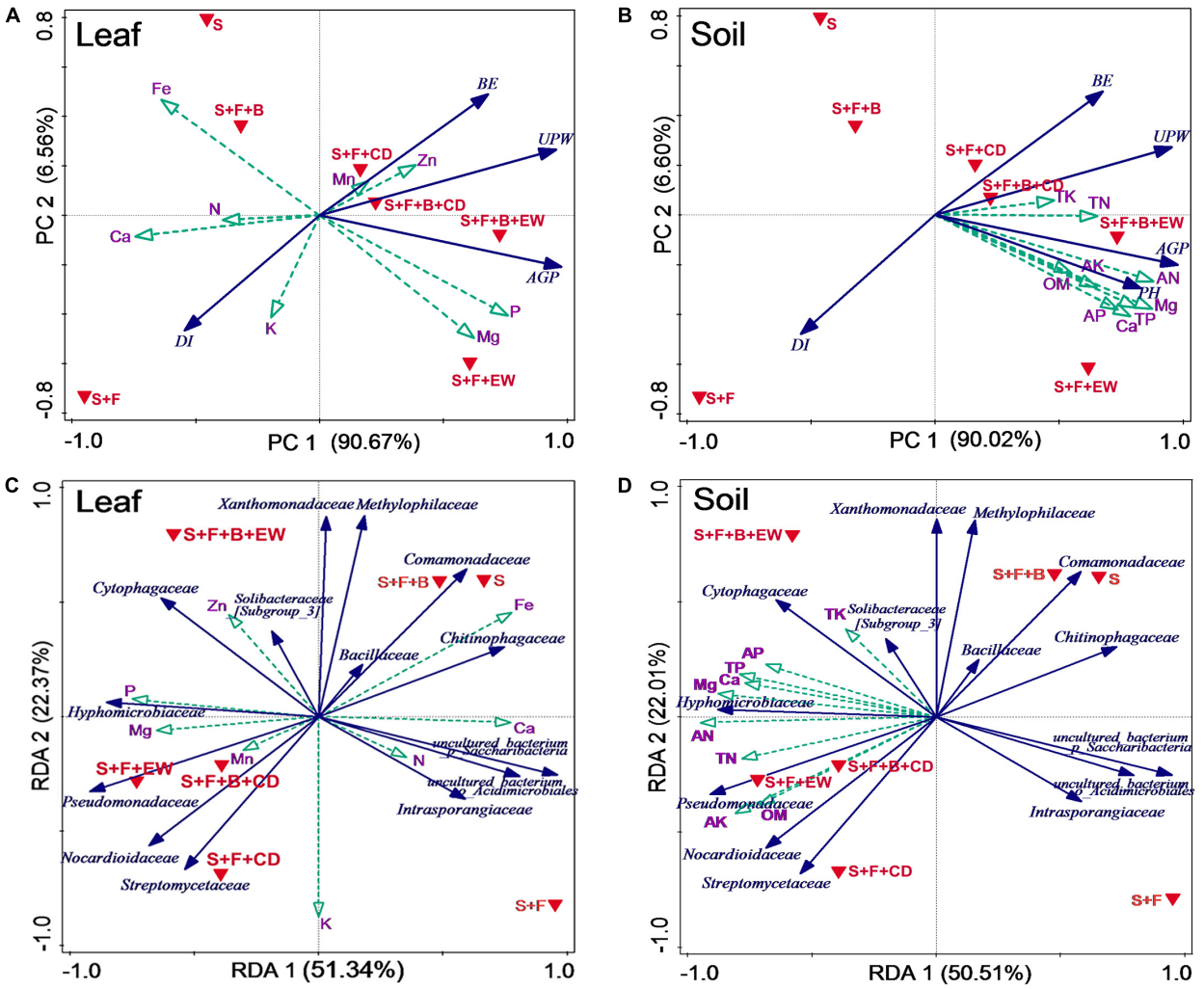

FIGURE 9 | Associations of DI (disease incidence), leaf and soil mineral nutrients, and microbes. Principal component analysis (PCA) based on biocontrol and selected leaves **(A)** and soil **(B)** mineral nutrients in the different treatments. Variable included DI (disease incidence), BE (biocontrol efficacy), UPW (belowground weight), AGP (aboveground weight), and PH (pseudo-stem height). Redundancy analysis (RDA) based on the relative abundance of bacterial families and selected leaves **(C)** and soil **(D)** mineral nutrients in the different treatments. S, healthy control (healthy soil); S + F, disease control (healthy soil + *FOC*4); S + F + B, healthy soil + *FOC*4 + HN03 treatment; S + F + B + EW, healthy soil + *FOC*4 + HN03 + wormcast treatment; S + F + EW, healthy soil + *FOC*4 + wormcast treatment; S + F + B + CD, healthy soil + *FOC*4 + HN03 + cow dung compost treatment; S + F + CD, healthy soil + *FOC*4 + cow dung compost treatment.

the abundance of Bacillaceae, of which the highest abundance occurred in treatment "S + F + B," was not correlated with nutrient elements in the soil. However, the abundances of Intrasporangiaceae, uncultured_bacterium_o_Acidimicrobiales, and uncultured_bacterium_o_Sphingobacteriales were negatively correlated with TP and Mg in the soil, and the highest abundance of these families occurred in the treatment "S + F," while the lowest occurred in the treatments of "S + F + EW" and "S + F + B + EW." Moreover, the contents of TP and Mg were higher in the treatments containing wormcast than the other treatments (**Figure 5**). The highest abundance of Gemmatimonadaceae was found in the treatments with wormcast and was positively correlated with TK (**Table 2**), while the concentration of TK was highest in the treatment "S + F + B + EW" (**Figure 5**).

DISCUSSION

Characteristics of HN03 and the Effects of Its Application on the Suppression of *Fusarium* wilt

The HN03 strain isolated from the soil was molecularly identified as *B. velezensis*. According to the results of the biochemical tests, the strain could utilize a wide range of carbon sources and physiological and biochemical characteristics that helped it adapt to the environment. *B. velezensis* strains have good potential for biocontrol and can promote plant growth (Cai et al., 2017; Liu et al., 2017), and they are also effective against *F. oxysporum* (Moreno-Velandia et al., 2018). In this study, we demonstrated that HN03 has antagonistic activity against a wide spectrum

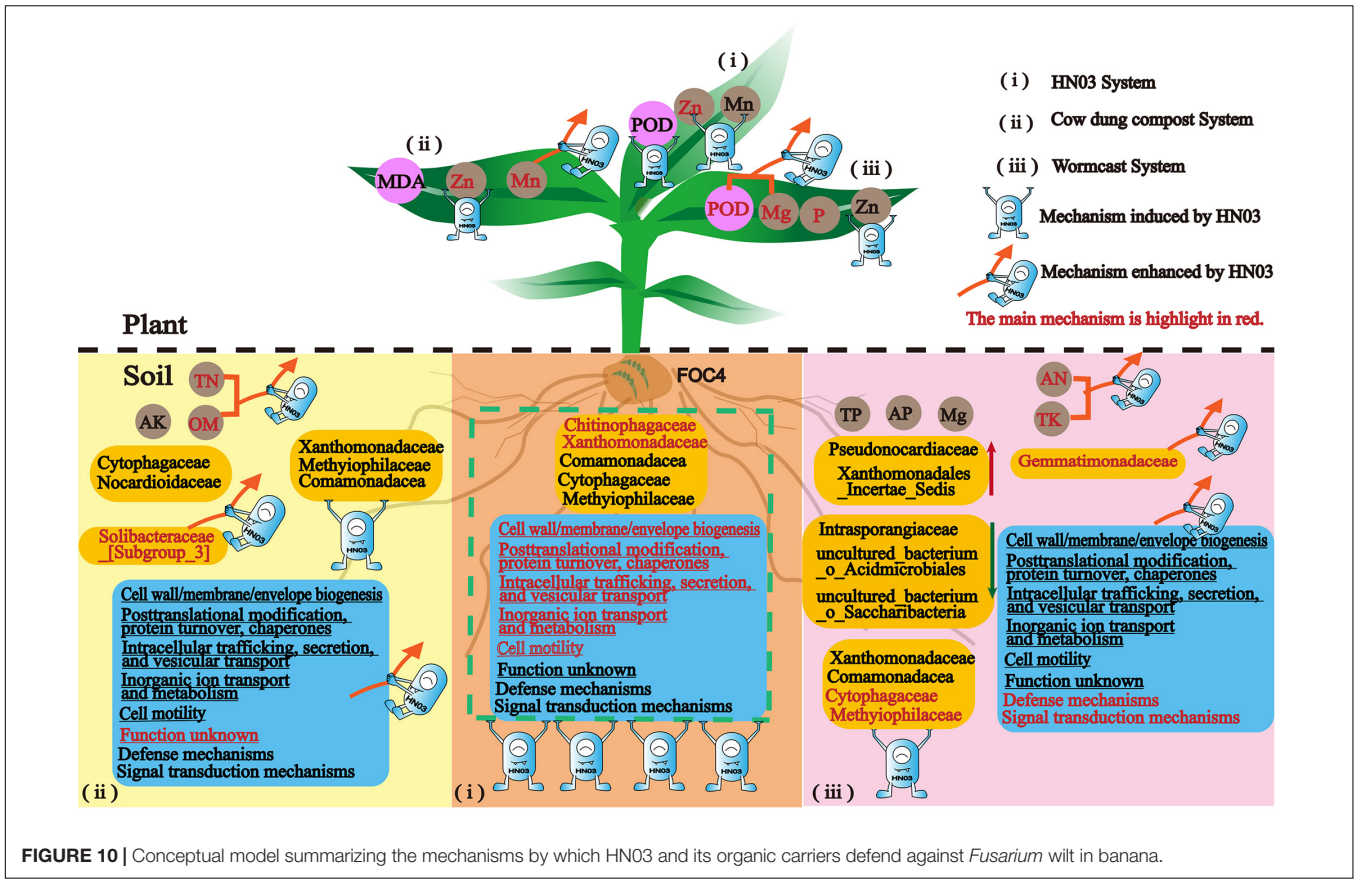

FIGURE 10 | Conceptual model summarizing the mechanisms by which HN03 and its organic carriers defend against *Fusarium* wilt in banana.

TABLE 2 | Spearman's coefficients of correlation (*r*) between 21 bacterial families (Bacillaceae and the top 20 families in abundance) and soil properties.

No.	Family	AK	AP	AN	TK	TP	TN	OM	Ca	Mg
1	Intrasporangiaceae	−0.02	−0.40	−0.47[b]	−0.78	−0.56[a]	−0.11	0.06	−0.52[b]	−0.50[b]
2	uncultured_bacterium_o_Acidimicrobiales	−0.60[a]	−0.69[a]	−0.77[a]	−0.41	−0.78[a]	−0.64[a]	−0.59[a]	−0.72[a]	−0.72[a]
3	uncultured_bacterium_o_Sphingobacteriales	−0.59[a]	−0.79[a]	−0.88[a]	−0.52[b]	−0.88[a]	−0.70[a]	−0.58[a]	−0.83[a]	−0.84[a]
4	Chitinophagaceae	−0.70[a]	−0.75[a]	−0.66[a]	−0.33	−0.66[a]	−0.61[a]	−0.77[a]	−0.68[a]	−0.66[a]
5	Micrococcaceae	−0.46[b]	−0.78[a]	−0.70[a]	−0.69[a]	−0.69[a]	−0.35	−0.45[b]	−0.69[a]	−0.67[a]
6	Oxalobacteraceae	−0.52[b]	−0.87[a]	−0.73[a]	−0.48[b]	−0.74[a]	−0.37	−0.62[a]	−0.79[a]	−0.76[a]
7	Comamonadaceae	−0.61[a]	−0.69[a]	−0.53[b]	−0.09	−0.61[a]	−0.43	−0.71[a]	−0.69[a]	−0.64[a]
8	Sphingomonadaceae	−0.55[b]	−0.69	−0.85	−0.45[b]	−0.86	−0.53[b]	−0.46[b]	−0.81	−0.80
9	Solibacteraceae_[Subgroup_3]	0.22	0.14	0.16	−0.08	0.04	0.35	0.25	−0.04	0.03
10	Streptomycetaceae	0.54[b]	0.41	0.31	−0.06	0.38	0.39	0.55[b]	0.48[b]	0.47[b]
11	Nocardioidaceae	0.91[a]	0.64[a]	0.66[a]	0.08	0.65[a]	0.84[a]	0.84[a]	0.68[a]	0.69[a]
12	Anaerolineaceae	0.82[a]	0.64[a]	0.81[a]	0.20	0.75[a]	0.86[a]	0.72[a]	0.71[a]	0.71[a]
13	Blrii41	0.86[a]	0.50[b]	0.69[a]	0.18	0.62[a]	0.93[a]	0.71[a]	0.57[a]	0.60[a]
14	Methylophilaceae	−0.55[b]	−0.12	−0.05	0.28	−0.11	−0.38	−0.50[b]	−0.17	−0.14
15	Xanthomonadales_Incertae_Sedis	−0.50[b]	0.19	−0.01	0.44[b]	0.07	−0.36	−0.25	0.10	0.07
16	Gemmatimonadaceae	−0.32	0.37	0.33	0.44[b]	0.39	−0.31	−0.24	0.36	0.39
17	Hyphomicrobiaceae	0.52[b]	0.84[a]	0.83[a]	0.35	0.90[a]	0.56[a]	0.60[a]	0.89[a]	0.93[a]
18	Pseudomonadaceae	0.79[a]	0.74[a]	0.65[a]	0.19	0.68[a]	0.69[a]	0.83[a]	0.73[a]	0.70[a]
19	Cytophagaceae	0.31	0.32	0.56[a]	0.23	0.55[b]	0.48[b]	0.21	0.44[b]	0.59[a]
20	Xanthomonadaceae	−0.44[b]	−0.14	0.12	0.16	0.13	−0.12	−0.46[b]	0.05	0.10
21	Bacillaceae	0.28	−0.13	0.18	−0.05	0.18	0.30	−0.02	0.12	0.22

[a]*Correlation is significant at P < 0.05.* [b]*Correlation is significant at P < 0.01 A, available; T, total; OM, organic matter.*

of pathogenic fungi, with inhibition rates ranging from 44.12 to 77.62%. We also found that HN03 promoted the growth of the banana seedlings, especially the underground parts, even though the banana seedlings were infected with *FOC*4, which always infects banana from the root to the rhizome during the early infection stages (Ploetz, 2006). Moreover, when we studied the effects of organic fertilizer (wormcast or cattle manure), HN03 (10:1, w/w, spores $> 10^8$ CFU/mL), or their combination on banana growth and the suppression of *Fusarium* wilt in pot trials, we found that HN03 and compost reduced the DI; the combination of HN03 and cow dung compost showed a lower DI; and the combination of HN03 and wormcast showed the lowest DI (**Figure 3B**). In this study, different composts differentially impacted the nutrients and community in the soil to induce soil suppression, which may have induced plant resistance. HN03 reestablished the community structure in the soil to induce soil suppression and induced plant resistance by enhancing the POD contents and expression of Zn. When combined with compost, HN03 altered the mechanisms by which the compost acts by changing the types of nutrients, enhancing the POD contents, and modulating the community structure, thereby further inducing soil suppression and plant resistance. The comprehensive mechanisms are discussed below.

Mechanisms Related to the Resistance Activity of the Plant

To determine the differences in the mechanisms for the biocontrol of *Fusarium* wilt in banana plants by HN03 in different environments, we tested for POD in the leaves, as these compound is associated with disease resistance in plants (Su et al., 2016). POD participates in the construction, rigidification, and eventual lignification of cell walls to protect plant tissues from damage (Sun et al., 2012). In a previous study, a resistant cultivar of banana had an inherently higher capacity to stimulate POD production than a susceptible cultivar (Aguilar et al., 2000). Moreover, *B. velezensis* can trigger basal immunity in plants (Jiang et al., 2018) by increasing the expression of plant defense-related genes and the activities of some defense enzymes, such as catalase (CAT) and POD (Jiang et al., 2019). In this study, banana seedlings treated with HN03 had a higher POD content than those in the treatment without HN03, and the highest POD content was detected in the treatment with HN03 combined with wormcast. Thus, HN03 combined with wormcast could induce resistance to pathogen infection by increasing POD activity in banana, which is similar to the mechanism exhibited by disease-resistant cultivars when threatened by the pathogen. To summarize, a high POD content in the plants may be a key factor in suppressing banana vascular wilt disease when HN03 or "HN03 with wormcast" is applied to the soil. Only a suitable carrier can trigger this mechanism, as the POD content is low in the cow dung compost.

Mechanisms Related to Nutrient Element Modulation

In our study, a positive correlation was found between the contents of Zn, Mn, Mg, and P in banana and the suppression of wilt disease severity, which is consistent with the results of Hassan and Abo-Elyousr (2013) and Siddiqui et al. (2015). Some nutrient elements in banana and its associated soil can reduce the severity of plant disease by increasing disease tolerance and resistance against plant pathogens (Dordas, 2008; Siddiqui et al., 2015). Mn, which was significantly higher in the treatments with HN03 than in their controls, can be a highly effective micronutrient in inducing plant resistance against diseases by affecting cell wall composition, lignin biosynthesis, phenol biosynthesis, photosynthesis, and several other functions (Hassan and Abo-Elyousr, 2013). It also suppresses the penetration of pathogens into plant tissue and accumulates in the form of Mn^{4+} at the sites where pathogens attack (Dordas, 2008). High Zn levels in leaf tissues are associated with the strong suppression of wilt disease because of the direct toxic effects of Zn on pathogens (Dordas, 2008). The highest contents of Mn and Zn were found in the "S + F + B + CD" treatment. Mg and P, which were the highest in the "S + F + B + EW" treatment, can affect the suppression of plant diseases both directly by affecting pathogen growth and indirectly by affecting plant defenses and stomatal functions (Walters and Bingham, 2007; Huber and Jones, 2012). Therefore, it can be concluded that HN03 can improve the organic amendment strategy in suppressing *Fusarium* wilt of banana plants by modulating Mn. Wormcast is helpful for the accumulation of Mg, P, and Zn in the banana leaves to enhance plant suppression, while cow dung compost can induce plant suppression to *FOC4* by modulating Mn and Zn. HN03 combined with wormcast or cow dung compost can significantly promote Mg/P or Mn/Zn assimilation, respectively, in the leaves.

The contents of TP, TK, and AN in the soil are correlated with increased production, decreased pathogen infection, and reduced disease severity in susceptible crops (Brennan, 1995; Walters and Bingham, 2007; Shen et al., 2015). High N and K contents in the soil decreased the severity of *F. oxysporum* infection (Dordas, 2008). Similarly, in our study, a positive correlation was found between the AN and TK contents in the soil and the suppression of wilt disease. We also found that the contents of TP and TK were higher in the treatments of "S + F + B + EW" and "S + F + EW" than in the other treatments, and AN was higher in the treatments with wormcast or cow dung compost. Additionally, the contents of TK or AN increased significantly when HN03 was combined with wormcast or cow dung compost, respectively. By contrast, the contents of TN and OM, which are negatively correlated with DI and promote the uptake of Mn and Zn by higher plants (Siddiqui et al., 2015), were higher in the treatments of "S + F + B + CD" and "S + F + CD" than in the other treatments, and were highest in "S + F + B + CD." Notably, the contents of OM and TN in treatment "S + F + B + CD" were even higher than in both the unplanted soil treated with cow dung compost $(S + CD)_0$ and the compost control (S + F + CD). These results indicated that HN03 could decompose some substances, such as microbial or plant residue, and thereby increase the nutrient content in the soil.

We concluded that HN03 regulated soil nutrients according to the soil environment and, as a result, suppressed the

pathogen in the soil and adjusted the uptake of plant nutrients, eventually inducing plant resistance against the pathogen. HN03 increased the contents of Mn and Zn in the plants when used alone. In addition, the contents of Mn and Zn in the plants were maximized when HN03 was combined with cow dung compost. The high content of OM in the soil of treatment "S + F + B + CD" could explain this result, which by facilitating Mn and Zn absorption, ultimately led to *FOC4* resistance. The content of P and Mg in the plants increased in the treatments with wormcast, which could be explained by the higher contents of TP, AP, and Mg in the wormcast. When treated with HN03 combined with wormcast, the soil had higher AN and TK contents; therefore, HN03 may suppress *FOC*4 infection by increasing the N and K contents.

Mechanisms Related to Soil Microbiome Modulation

Reshaping of the soil microbiome is the main mechanism by which soil suppression against *Fusarium* wilt disease is induced and has been widely discussed in bio-control systems (Wang et al., 2016; Xiong et al., 2017). According to the results of our study, in the treatment with HN03, the abundance (ACE and Chao 1 indices) and the diversity (Simpson and Shannon indices) of bacterial communities in the soil increased significantly compared with those in the "S + F" treatment. When HN03 was combined with wormcast, the abundance and the diversity of the bacterial communities in the soil peaked. Moreover, the community structure in the soil samples inoculated with *FOC*4 tended to be similar to that in healthy soil after being treated with HN03 for 90 days. The effect of the wormcast treatment on community structure was greater than that of the cow manure treatment. In addition, HN03 and wormcast application increased the differences in bacterial community structure in the soil samples inoculated with *FOC*4, compared with little influence on the soil samples with cow manure. Therefore, the wormcast was better than the cow manure in reshaping the community structure when the soil was infected with *FOC*4, and HN03 enhanced this effect.

The identification of key microorganisms is proposed as a first step in rebuilding the microbiome of tissue-culture banana plants prior to planting to improve defense responses against *FOC* (Dita et al., 2018). In our study, HN03 influenced the soil community structure and mobilized different dominant strains against pathogens in different soil environments. As reported in the literature, the bacteria families Solibacteraceae and Cytophagaceae are highly abundant in a *F. oxysporum*-resistant cultivar, and Cytophagaceae was identified as a *F. oxysporum*-suppressive bacterial taxon by Mendes et al. (2018). Comamonadaceae are a promising group of biocontrol microbes that are likely contribute to the recovery observed in plant growth (Durán et al., 2018) and were thus observed at higher abundance in all HN03 treatments. Members of Xanthomonadaceae, associated with the suppression of disease in soil (Grunert et al., 2016), and Chitinophagaceae, associated with plant growth promotion (Madhaiyan et al., 2015), were most abundant following treatment by HN03. The results shown in **Figure 7B** indicated that (1) strain HN03, isolated in our laboratory, can facilitate the growth of Comamonadaceae, Methylophilaceae, Cytophagaceae, and Xanthomonadaceae in the soil even under *FOC4* infection, and the function of Methylophilaceae could be enhanced by wormcast; (2) cow dung compost and wormcast can modulate the abundance of Solibacteraceae and Xanthomonadaceae_Incertae Sedis, respectively, and the abundance of Solibacteraceae was higher when cow dung compost was accompanied by HN03; (3) Cytophagaceae abundance can be increased by cow dung compost, wormcast, as well as HN03, and the abundance was highest in the treatment amended with HN03 and wormcast. In addition, some other bacterium families that produce erythromycin or are associated with disease suppression in the natural soil were enhanced by cow dung compost or wormcast, such as Nocardioidaceae (Harrell and Miller, 2016) and Pseudomonadaceae (Grunert et al., 2016). Furthermore, the abundance of Gemmatimonadaceae, which forms calcium carbonate via biomineralization and increases soil pH to improve soil quality (Wang et al., 2014), was found to be regulated by the TK of treatment "S + F + B + EW." Obviously, the abundances of bacteria families associated with the suppression of *Fusarium* wilt of banana could be adjusted according to the different soil nutritional environments.

The mechanisms by which HN03 combined with different environments provided protection were further explained by the functional annotation results. Cell motility measures the capacity of the cells to translocate onto a solid substratum (Jouanneau and Thiery, 2002), and this trait is associated with increased colonization of biocontrol bacteria in the plant roots (Liddell and Parke, 1989). The cell motility in the treatments of "S + F + B" and "S + F + B + EW" was high probably because the biocontrol microbial community colonized the diseased plant roots and soil, which improved the suppression of the disease by the biocontrol microbial community. Signal transduction mechanisms, which can recognize specific signals and convert information into specific transcriptional or behavioral responses and thus help the microbial community to survive and prosper in a wide variety of environments (Fabret et al., 1999), were higher in the treatments with HN03 and were highest in the treatment with HN03 combined with wormcast. Relatively more genes assigned to categories inorganic ion transport and metabolism was found in all HN03 treatments, which may demonstrated the function of HN03 in regulating soil and plant nutrients. Compared with compost treatment only, HN03 or its combination with compost was associated with more functional traits, which are processes related to microorganism vital activities such as evolution (Brown et al., 2001). In our study, relatively more genes affiliated with categories defense mechanisms was contained in "S + F + B + EW" than in the other treatments, and consequently, the combination of HN03 and wormcast could regulate a dynamic community with high adaptation and colonization and therefore reduce DI through an increase in defensive mechanisms.

CONCLUSION

In this work, we unraveled the mechanisms used by a new isolated biocontrol bacterium *B. velezensis* HN03 to fight banana *Fusarium* wilt in three types of soil environments: soil with the pathogen only and soil with the pathogen and cow dung compost or wormcast. The strain HN03 could reshape the soil community structure and microbiota motility, regulate soil nutrients to suppress disease, and induce plant resistance to *Fusarium* wilt, such as defense enzymes and nutrient elements. Furthermore, HN03 could alter the strategy by which compost controls soil-borne disease by enhancing the advantages of the composts and stimulating new mechanisms in the plants and soil (**Figure 10**).

A combination of biocontrol bacterium and carrier should thus be considered for enhancing plant defense and soil suppression when controlling soil-borne diseases. The right combination can stimulate plant defense responses by mobilizing specific plant enzymes and nutrient factors to disease and enhance soil suppression by regulating the microbial community and nutrient environment in the soil.

DATA AVAILABILITY STATEMENT

The datasets presented in this study can be found in online repositories. The names of the repository/repositories and accession number(s) can be found below: https://www.ncbi.nlm.nih.gov/genbank/, MF155192.

AUTHOR CONTRIBUTIONS

CW and XW contributed to the conception and design of the study. XW performed the experiments, organized and analyzed the data, and performed the writing the original draft preparation. CW contributed with conceptualization, writing – review and editing, and funding acquisition. YS and YL contributed to organizing and analyzing the data. QL contributed to the supervision. All authors read and approved the final manuscript.

FUNDING

The Central Public-interest Scientific Institution Basal Research Fund for the Chinese Academy of Tropical Agricultural Sciences (1630022017009) and the National Natural Science Foundation of China (41701307) supported this work.

ACKNOWLEDGMENTS

We thank the staff in our laboratory for their help and feedback to improve this work.

SUPPLEMENTARY MATERIAL

The Supplementary Material for this article can be found online at: https://www.frontiersin.org/articles/10.3389/fpls.2020.599904/full#supplementary-material

REFERENCES

Aguilar, E. A., Turner, D. W., and Sivasithamparam, K. (2000). *Fusarium oxysporum* f.sp. cubense inoculation and hypoxia alter peroxidase and phenylalanine ammonia lyase activities in nodal roots of banana cultivars (*Musa* sp.) differing in their susceptibility to Fusarium wilt. *Aust. J. Bot.* 48, 589–596. doi: 10.1071/bt99009

Bo, M., Sona, A., Astengo, M., Fiandra, U., Quagliotti, E., Brescianini, A., et al. (2009). Metabolic syndrome in older subjects: coincidence or clustering? *Arch. Gerontol. Geriatr.* 48, 146–150. doi: 10.1016/j.archger.2007.12.003

Borriss, R., Chen, X. H., Rueckert, C., Blom, J., Becker, A., Baumgarth, B., et al. (2011). Relationship of *Bacillus amyloliquefaciens* clades associated with strains DSM 7T and FZB42T: a proposal for *Bacillus amyloliquefaciens* subsp. *amyloliquefaciens* subsp. nov. and *Bacillus amyloliquefaciens* subsp. *plantarum* subsp. nov. based on complete genome sequence comparisons. *Int. J. Syst. Evol. Microbiol.* 61, 1786–1801. doi: 10.1099/ijs.0.023267-0

Brennan, R. F. (1995). Effect of levels of take-all and phosphorus fertiliser on the dry matter and grain yield of wheat. *J. Plant Nutr.* 18, 1159–1176. doi: 10.1080/01904169509364970

Brown, E. W., Leclerc, J. E., Kotewicz, M. L., and Cebula, T. A. (2001). Three R's of bacterial evolution: how replication, repair, and recombination frame the origin of species. *Environ. Mol. Mutagen.* 38, 248–260. doi: 10.1002/em.1079

Bubici, G., Kaushal, M., Prigigallo, M. I., Gomez-Lama Cabanas, C., and Mercado-Blanco, J. (2019). Biological control agents against *Fusarium* wilt of banana. *Front. Microbiol.* 10:616. doi: 10.3389/fmicb.2019.00616

Cai, X. C., Liu, C. H., Wang, B. T., and Xue, Y. R. (2017). Genomic and metabolic traits endow *Bacillus velezensis* CC09 with a potential biocontrol agent in control of wheat powdery mildew disease. *Microbiol. Res.* 196, 89–94. doi: 10.1016/j.micres.2016.12.007

De, L. J., Cattoir, H., and Reynaerts, A. (1970). The quantitative measurement of DNA hybridization from renaturation rates. *Eur. J. Biochem.* 12, 133–142. doi: 10.1111/j.1432-1033.1970.tb00830.x

Dita, M., Barquero, M., Heck, D., Mizubuti, E. S. G., and Staver, C. P. (2018). Fusarium wilt of banana: current knowledge on epidemiology and research needs toward sustainable disease management. *Front. Plant Sci.* 9:1468. doi: 10.3389/fpls.2018.01468

Dordas, C. (2008). Role of nutrients in controlling plant diseases in sustainable agriculture. A review. *Agron. Sustain. Dev.* 28, 33–46. doi: 10.1051/agro:2007051

Durán, P., Thiergart, T., Garrido-Oter, R., Agler, M., Kemen, E., Schulze-Lefert, P., et al. (2018). Microbial interkingdom interactions in roots promote *Arabidopsis* survival. *Cell* 175, 973–983.e14.

Edgar, R. C. (2013). UPARSE: highly accurate OTU sequences from microbial amplicon reads. *Nat. Methods* 10, 996–998. doi: 10.1038/nmeth.2604

El-Hassan, S. A., and Gowen, S. R. (2006). Formulation and delivery of the bacterial antagonist *Bacillus subtilis* for management of *Lentil* vascular wilt caused by *Fusarium oxysporum* f. sp. *lentis*. *Phytopathology* 154, 148–155. doi: 10.1111/j.1439-0434.2006.01075.x

Fabret, C., Feher, V. A., and Hoch, J. A. (1999). Two-component signal transduction in *Bacillus subtilis*: how one organism sees its world. *Am. Soc. Microbiol.* 181, 1975–1983. doi: 10.1128/jb.181.7.1975-1983.1999

Fouquier, J., Rideout, J. R., Bolyen, E., Chase, J., Shiffer, A., Mcdonald, D., et al. (2016). Ghost-tree: creating hybrid-gene phylogenetic trees for diversity analyses. *Microbiome* 4:11.

Fu, L., Penton, C. R., Ruan, Y., Shen, Z., Xue, C., Li, R., et al. (2017). Inducing the rhizosphere microbiome by biofertilizer application to suppress banana *Fusarium* wilt disease. *Soil Biol. Biochem.* 104, 39–48. doi: 10.1016/j.soilbio.2016.10.008

Fu, L., Ruan, Y., Tao, C., Li, R., and Shen, Q. (2016). Continous application of bioorganic fertilizer induced resilient culturable bacteria community associated with banana *Fusarium* wilt suppression. *Sci. Rep.* 6:27731.

Gillis, M., De, L. J., and De, C. M. (1970). The determination of molecular weight of bacterial genome DNA from renaturation rates. *FEBS J.* 12, 143–153. doi: 10.1111/j.1432-1033.1970.tb00831.x

Grunert, O., Hernandez-Sanabria, E., Vilchez-Vargas, R., Jauregui, R., Pieper, D. H., Perneel, M., et al. (2016). Mineral and organic growing media have distinct community structure, stability and functionality in soilless culture systems. *Sci. Rep.* 6:18837.

Harrell, E. A., and Miller, E. S. (2016). Genome sequence of *Aeromicrobium erythreum* NRRL B-3381, an erythromycin-producing bacterium of the *Nocardioidaceae*. *Genome Announc.* 4:e00300-16.

Hassan, M. A. E., and Abo-Elyousr, K. A. M. (2013). Impact of compost application on *Fusarium* wilt disease incidence and microelements contents of basil plants. *Arch. Phytopathol. Plant Prot.* 46, 1904–1918. doi: 10.1080/03235408.2013.780696

Huang, J., Pang, Y., Zhang, F., Huang, Q., Zhang, M., Tang, S., et al. (2019). Suppression of *Fusarium* wilt of banana by combining acid soil ameliorant with biofertilizer made from *Bacillus velezensis* H-6. *Eur. J. Plant Pathol.* 154, 585–596. doi: 10.1007/s10658-019-01683-5

Huang, J., Wei, Z., Tan, S., Mei, X., Shen, Q., and Xu, Y. (2014). Suppression of bacterial wilt of tomato by bioorganic fertilizer made from the antibacterial compound producing strain *Bacillus amyloliquefaciens* HR62. *Agric. Food Chem.* 62, 10708–10716. doi: 10.1021/jf503136a

Huang, N., Wang, W., Yao, Y., Zhu, F., Wang, W., and Chang, X. (2017). The influence of different concentrations of bio-organic fertilizer on cucumber *Fusarium* wilt and soil microflora alterations. *PLoS One* 12:e0171490. doi: 10.1371/journal.pone.0171490

Huber, D. M., and Jones, J. B. (2012). The role of magnesium in plant disease. *Plant Soil* 368, 73–85. doi: 10.1007/s11104-012-1476-0

Jiang, C.-H., Liao, M.-J., Wang, H.-K., Zheng, M.-Z., Xu, J.-J., and Guo, J.-H. (2018). *Bacillus velezensis*, a potential and efficient biocontrol agent in control of pepper gray mold caused by *Botrytis cinerea*. *Biol. Control* 126, 147–157. doi: 10.1016/j.biocontrol.2018.07.017

Jiang, C. H., Yao, X. F., Mi, D. D., Li, Z. J., Yang, B. Y., Zheng, Y., et al. (2019). Comparative transcriptome analysis reveals the biocontrol mechanism of *Bacillus velezensis* F21 against *Fusarium* wilt on watermelon. *Front. Microbiol.* 10:652. doi: 10.3389/fmicb.2019.00652

Jiang, H., Ling, Z., Zhang, Y., Mao, H., Ma, Z., Yin, Y., et al. (2015). Altered fecal microbiota composition in patients with major depressive disorder. *Brain Behav. Immun.* 48, 186–194. doi: 10.1016/j.bbi.2015.03.016

Jouanneau, J., and Thiery, J. P. (2002). Tumor cell motility and invasion. *Encycl. Cancer* 4, 467–473. doi: 10.1016/b0-12-227555-1/00252-5

Kämpfer, P., Steiof, M., and Dott, W. (1991). Microbiological characterization of a fuel-oil contaminated site including numerical identification of heterotrophic water and soil bacteria. *Microb. Ecol.* 21, 227–251. doi: 10.1007/bf02539156

Kim, O.-S., Cho, Y.-J., Lee, K., Yoon, S.-H., Kim, M., Na, H., et al. (2012). Introducing EzTaxon-e: a prokaryotic 16S rRNA gene sequence database with phylotypes that represent uncultured species. *Int. J. Syst. Evol. Microbiol.* 62, 716–721. doi: 10.1099/ijs.0.038075-0

Kowalenko, C. G., and Babuin, D. (2007). Interference problems with phosphoantimonylmolybdenum colorimetric measurement of [phosphorus in soil and plant materials. *Commun. Soil Sci. Plant Anal.* 38, 1299–1316. doi: 10.1080/00103620701328594

Kucey, R. M. N., Janzen, H. H., and Leggett, M. E. (1989). Microbially mediated increases in plant-available phosphorus. *Adv. Agron.* 42, 199–228. doi: 10.1016/s0065-2113(08)60525-8

Lang, J., Hu, J., Ran, W., Xu, Y., and Shen, Q. (2011). Control of cotton *Verticillium* wilt and fungal diversity of rhizosphere soils by bio-organic fertilizer. *Biol. Fertil. Soils* 48, 191–203. doi: 10.1007/s00374-011-0617-6

Li, C., Hu, W., Pan, B., Liu, Y., Yuan, S., Ding, Y., et al. (2017). Rhizobacterium *Bacillus amyloliquefaciens* strain SQRT3-mediated induced systemic resistance controls bacterial wilt of tomato. *Pedosphere* 27, 1135–1146. doi: 10.1016/s1002-0160(17)60406-5

Liddell, C. M., and Parke, J. L. (1989). Enhanced colonization of pea taproots by a fluorescent pseudomonad biocontrol agent by water infiltration into soil. *Phytopathology* 79, 1327–1332. doi: 10.1094/phyto-79-1327

Liu, G., Kong, Y., Fan, Y., Geng, C., Peng, D., and Sun, M. (2017). Data on genome analysis of *Bacillus velezensis* LS69. *Data Brief* 13, 1–5. doi: 10.1016/j.dib.2017.04.053

Ma, Y., Zhang, C., Oliveira, R. S., Freitas, H., and Luo, Y. (2016). Bioaugmentation with endophytic bacterium E6S homologous to *Achromobacter piechaudii* enhances metal rhizoaccumulation in host *Sedum plumbizincicola*. *Front. Plant Sci.* 7:75. doi: 10.3389/fpls.2016.00075

Madhaiyan, M., Poonguzhali, S., Kwon, S. W., and Sa, T. M. (2010). *Bacillus methylotrophicus* sp. nov., a methanol-utilizing, plant-growth-promoting bacterium isolated from rice rhizosphere soil. *Int. J. Syst. Evol. Microbiol.* 60, 2490–2495. doi: 10.1099/ijs.0.015487-0

Madhaiyan, M., Poonguzhali, S., Senthilkumar, M., Pragatheswari, D., Lee, J. S., and Lee, K. C. (2015). *Arachidicoccus rhizosphaerae* gen. nov., sp. nov., a plant-growth-promoting bacterium in the family *Chitinophagaceae* isolated from rhizosphere soil. *Int. J. Syst. Evol. Microbiol.* 65, 578–586. doi: 10.1099/ijs.0.069377-0

Mehta, C. M., Palni, U., Franke-Whittle, I. H., and Sharma, A. K. (2014). Compost: its role, mechanism and impact on reducing soil-borne plant diseases. *Waste Manag.* 34, 607–622. doi: 10.1016/j.wasman.2013.11.012

Mendes, L. W., Raaijmakers, J. M., De Hollander, M., Mendes, R., and Tsai, S. M. (2018). Influence of resistance breeding in common bean on rhizosphere microbiome composition and function. *Int. Soc. Microb. Ecol.* 12, 212–224. doi: 10.1038/ismej.2017.158

Moreno-Velandia, C. A., Izquierdo-García, L. F., Ongena, M., Kloepper, J. W., and Cotes, A. M. (2018). Soil sterilization, pathogen and antagonist concentration affect biological control of *Fusarium* wilt of cape gooseberry by *Bacillus velezensis* Bs006. *Plant Soil* 435, 39–55. doi: 10.1007/s11104-018-3866-4

Mori, H., Maruyama, F., Kato, H., Toyoda, A., Dozono, A., Ohtsubo, Y., et al. (2014). Design and experimental application of a novel non-degenerate universal primer set that amplifies prokaryotic 16S rRNA genes with a low possibility to amplify eukaryotic rRNA genes. *DNA Res.* 21, 217–227. doi: 10.1093/dnares/dst052

Muhammad, Z., Mir, A. K., Mushtaq, A., Gul, J., Shazia, S., Kifayat, U., et al. (2010). Elemental analysis of some medicinal plants used in traditional medicine by atomic absorption spectrophotometer (AAS). *J. Med. Plants Res.* 4, 1987–1990. doi: 10.5897/jmpr10.081

Olorunfemi, I. E., Fasinmirin, J. T., and Akinola, F. F. (2018). Soil physico-chemical properties and fertility status of long-term land use and cover changes: a case study in Forest vegetative zone of Nigeria. *Eurasian J. Soil Sci.* 7, 133–150. doi: 10.18393/ejss.366168

Parks, D. H., Tyson, G. W., Hugenholtz, P., and Beiko, R. G. (2014). STAMP: statistical analysis of taxonomic and functional profiles. *Bioinformatics* 30, 3123–3124. doi: 10.1093/bioinformatics/btu494

Ploetz, R. C. (2006). *Fusarium* wilt of banana is caused by several pathogens referred to as *Fusarium oxysporum* f. sp. *cubense*. *Phytopathology* 96, 653–656. doi: 10.1094/phyto-96-0653

Qiu, M., Zhang, R., Xue, C., Zhang, S., Li, S., Zhang, N., et al. (2012). Application of bio-organic fertilizer can control *Fusarium* wilt of cucumber plants by regulating microbial community of rhizosphere soil. *Biol. Fertil. Soils* 48, 807–816. doi: 10.1007/s00374-012-0675-4

Rajaofera, M. J. N., Jin, P. F., Fan, Y. M., Sun, Q. Q., Huang, W. K., Wang, W. B., et al. (2017). Antifungal activity of the bioactive substance from *Bacillus atrophaeus* strain HAB-5 and its toxicity assessment on *Danio rerio*. *Pestic. Biochem. Physiol.* 147, 153–161. doi: 10.1016/j.pestbp.2017.06.006

Raza, W., Ling, N., Zhang, R., Huang, Q., Xu, Y., and Shen, Q. (2017). Success evaluation of the biological control of *Fusarium* wilts of cucumber, banana, and tomato since 2000 and future research strategies. *Crit. Rev. Biotechnol.* 37, 202–212. doi: 10.3109/07388551.2015.1130683

Regassa, A. B., Taegyu, C., Lee, Y. S., and Yong Kil, K. (2018). Supplementing biocontrol efficacy of *Bacillus velezensis* against *Glomerella cingulata*. *Physiol. Mol. Plant Pathol.* 102, 173–179. doi: 10.1016/j.pmpp.2018.03.002

Roiloa, S. R., Li, Q., Jia, Z., Zhu, Y., Wang, Y., Li, H., et al. (2015). Spatial heterogeneity of soil nutrients after the establishment of *Caragana intermedia* plantation on sand dunes in alpine sandy land of the Tibet Plateau. *PLoS One* 10:e0124456. doi: 10.1371/journal.pone.0124456

Ruiz-Garcia, C., Bejar, V., Martinez-Checa, F., Llamas, I., and Quesada, E. (2005). *Bacillus velezensis* sp. nov., a surfactant-producing bacterium isolated from the

river Velez in Malaga, southern Spain. *Int. J. Syst. Evol. Microbiol.* 55, 191–195. doi: 10.1099/ijs.0.63310-0

Ruiz-Romero, P., Valdez-Salas, B., Gonzalez-Mendoza, D., and Mendez-Trujillo, V. (2018). Antifungal effects of silver phytonanoparticles from *Yucca shilerifera* against strawberry soil-borne pathogens: *Fusarium solani* and *Macrophomina phaseolina*. *Mycobiology* 46, 47–51. doi: 10.1080/12298093.2018.1454011

Salehi, M. H., Beni, O. H., Harchegani, H. B., Borujeni, I. E., and Motaghian, H. R. (2011). Refining soil organic matter determination by Loss-on-Ignition. *Pedosphere* 21, 473–482. doi: 10.1016/s1002-0160(11)60149-5

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. Microbiol.* 75, 7537–7541. doi: 10.1128/aem.01541-09

Shen, Z., Ruan, Y., Wang, B., Zhong, S., Su, L., Li, R., et al. (2015). Effect of biofertilizer for suppressing *Fusarium* wilt disease of banana as well as enhancing microbial and chemical properties of soil under greenhouse trial. *Appl. Soil Ecol.* 93, 111–119. doi: 10.1016/j.apsoil.2015.04.013

Shen, Z., Zhong, S., Wang, Y., Wang, B., Mei, X., Li, R., et al. (2013). Induced soil microbial suppression of banana *Fusarium* wilt disease using compost and biofertilizers to improve yield and quality. *Eur. J. Soil Biol.* 57, 1–8. doi: 10.1016/j.ejsobi.2013.03.006

Siddiqui, S., Alamri, S. A., Alrumman, S. A., Meghvansi, M. K., Chaudhary, K. K., Kilany, M., et al. (2015). "Role of soil amendment with micronutrients in suppression of certain soilborne plant fungal diseases: a review," in *Organic Amendments and Soil Suppressiveness in Plant Disease Management*, Vol. 46, eds M. Meghvansi and A. Varma (Cham: Springer), 363–380. doi: 10.1007/978-3-319-23075-7_17

Sneath, P. H. A. (1986). *Bergey's Manual® of Systematic Bacteriology*, Vol. 2. Philadelphia, PA: Lippincott Williams & Wilkins.

Steiner, C., Glaser, B., Geraldes Teixeira, W., Lehmann, J., Blum, W. E. H., and Zech, W. (2008). Nitrogen retention and plant uptake on a highly weathered central Amazonian Ferralsol amended with compost and charcoal. *J. Plant Nutr. Soil Sci.* 171, 893–899. doi: 10.1002/jpln.200625199

Su, Y., Wang, Z., Xu, L., Peng, Q., Liu, F., Li, Z., et al. (2016). Early selection for smut resistance in sugarcane using pathogen proliferation and changes in physiological and biochemical Indices. *Front. Plant Sci.* 7:1133. doi: 10.3389/fpls.2016.01133

Sumpavapol, P., Tongyonk, L., Tanasupawat, S., Chokesajjawatee, N., Luxananil, P., and Visessanguan, W. (2010). *Bacillus siamensis* sp. nov., isolated from salted crab (poo-khem) in Thailand. *Int. J. Syst. Evol. Microbiol.* 60, 2364–2370. doi: 10.1099/ijs.0.018879-0

Sun, J., Zou, L., Li, W., Yang, J., Wang, Y., Xia, Q., et al. (2018). Rhizosphere soil properties and banana *Fusarium* wilt suppression influenced by combined chemical and organic fertilizations. *Agric. Ecosyst. Environ.* 254, 60–68. doi: 10.1016/j.agee.2017.10.010

Sun, J. M., Fu, J. F., Zhou, R. J., Su, W. N., and Yan, X. R. (2012). Effects of methyl jasmonate on *Cylindrocarpon* root rot development on *Ginseng*. *Adv. Mater. Res.* 610–613, 3511–3517. doi: 10.4028/www.scientific.net/amr.610-613.3511

Tan, D., Fu, L., Han, B., Sun, X., Zheng, P., and Zhang, J. (2015). Identification of an endophytic antifungal bacterial strain isolated from the rubber tree and its application in the biological control of banana *Fusarium* wilt. *PLoS One* 10:e0131974. doi: 10.1371/journal.pone.0131974

Walters, D. R., and Bingham, I. J. (2007). Influence of nutrition on disease development caused by fungal pathogens: implications for plant disease control. *Ann. Appl. Biol.* 151, 307–324. doi: 10.1111/j.1744-7348.2007.00176.x

Wang, L., Li, J., Yang, F., E, Y., Raza, W., Huang, Q., et al. (2016). Application of bioorganic fertilizer significantly increased apple yields and shaped bacterial community structure in orchard soil. *Microb. Ecol.* 73, 404–416. doi: 10.1007/s00248-016-0849-y

Wang, L., Lu, X., Yuan, H., Wang, B., and Shen, Q. (2015). Application of bio-organic fertilizer to control tomato *Fusarium* wilting by manipulating soil microbial communities and development. *Commun. Soil Sci. Plant Anal.* 46, 2311–2322. doi: 10.1080/00103624.2015.1081694

Wang, Y., Sheng, H. F., He, Y., Wu, J. Y., Jiang, Y. X., Tam, N. F., et al. (2012). Comparison of the levels of bacterial diversity in freshwater, intertidal wetland, and marine sediments by using millions of Illumina tags. *Appl. Environ. Microbiol.* 78, 8264–8271. doi: 10.1128/aem.01821-12

Wang, Z., Huang, F., Mei, X., Wang, Q., Song, H., Zhu, C., et al. (2014). Long-term operation of an MBR in the presence of zinc oxide nanoparticles reveals no significant adverse effects on its performance. *J. Memb. Sci.* 471, 258–264. doi: 10.1016/j.memsci.2014.08.024

Wayne, L. G. (1986). "The mycobacteria," in *Bergey's Manual of Systematic Bacteriology*, Vol. 2, eds P. H. A. Sneath, N. S. Mair, M. E. Sharp, and J. G. Holt (Baltimore, MD: Williams & Wilkins), 1435–1457.

Winstanley, H. F., Abeln, S., and Deane, C. M. (2005). How old is your fold? *Bioinformatics* 21(Suppl. 1), i449–i458.

Wu, Y., Zhao, C., Farmer, J., and Sun, J. (2015). Effects of bio-organic fertilizer on pepper growth and *Fusarium* wilt biocontrol. *Sci. Hortic.* 193, 114–120. doi: 10.1016/j.scienta.2015.06.039

Xiong, W., Guo, S., Jousset, A., Zhao, Q., Wu, H., Li, R., et al. (2017). Bio-fertilizer application induces soil suppressiveness against *Fusarium* wilt disease by reshaping the soil microbiome. *Soil Biol. Biochem.* 114, 238–247. doi: 10.1016/j.soilbio.2017.07.016

Yergeau, E., Sommerville, D. W., Maheux, E., Vujanovic, V., Hamel, C., Whalen, J. K., et al. (2006). Relationships between *Fusarium* population structure, soil nutrient status and disease incidence in field-grown asparagus. *FEMS Microbiol. Ecol.* 58, 394–403. doi: 10.1111/j.1574-6941.2006.00161.x

Zhao, S., Liu, D., Ling, N., Chen, F., Fang, W., and Shen, Q. (2014). Bio-organic fertilizer application significantly reduces the *Fusarium oxysporum* population and alters the composition of fungi communities of watermelon *Fusarium* wilt rhizosphere soil. *Biol. Fertil. Soils* 50, 765–774. doi: 10.1007/s00374-014-0898-7

Zhao, Y. P., Lin, S., Chu, L., Gao, J., Azeem, S., and Lin, W. (2016). Insight into structure dynamics of soil microbiota mediated by the richness of replanted *Pseudostellaria heterophylla*. *Sci. Rep.* 6:26175.

Zhou, D., Jing, T., Chen, Y., Wang, F., Qi, D., Feng, R., et al. (2019). Deciphering microbial diversity associated with *Fusarium* wilt-diseased and disease-free banana rhizosphere soil. *BMC Microbiol.* 19:161. doi: 10.1186/s12866-019-1531-6

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: 14 December 2020
doi: 10.3389/fpls.2020.590063

Local Responses and Systemic Induced Resistance Mediated by Ectomycorrhizal Fungi

*Steven Dreischhoff, Ishani S. Das, Mareike Jakobi, Karl Kasper and Andrea Polle**

Forest Botany and Tree Physiology, University of Göttingen, Göttingen, Germany

Ectomycorrhizal fungi (EMF) grow as saprotrophs in soil and interact with plants, forming mutualistic associations with roots of many economically and ecologically important forest tree genera. EMF ensheath the root tips and produce an extensive extramatrical mycelium for nutrient uptake from the soil. In contrast to other mycorrhizal fungal symbioses, EMF do not invade plant cells but form an interface for nutrient exchange adjacent to the cortex cells. The interaction of roots and EMF affects host stress resistance but uncovering the underlying molecular mechanisms is an emerging topic. Here, we focused on local and systemic effects of EMF modulating defenses against insects or pathogens in aboveground tissues in comparison with arbuscular mycorrhizal induced systemic resistance. Molecular studies indicate a role of chitin in defense activation by EMF in local tissues and an immune response that is induced by yet unknown signals in aboveground tissues. Volatile organic compounds may be involved in long-distance communication between below- and aboveground tissues, in addition to metabolite signals in the xylem or phloem. In leaves of EMF-colonized plants, jasmonate signaling is involved in transcriptional re-wiring, leading to metabolic shifts in the secondary and nitrogen-based defense metabolism but cross talk with salicylate-related signaling is likely. Ectomycorrhizal-induced plant immunity shares commonalities with systemic acquired resistance and induced systemic resistance. We highlight novel developments and provide a guide to future research directions in EMF-induced resistance.

Keywords: ectomycorrhiza, systemic resistance, mycorrhiza, plant defense, phytohormone, chitin, herbivores

OPEN ACCESS

Edited by:
Paulo José Pereira Lima Teixeira, University of São Paulo, Brazil

Reviewed by:
Philipp Franken, Friedrich Schiller University Jena, Germany
Mika Tapio Tarkka, Helmholtz Centre for Environmental Research (UFZ), Germany

****Correspondence:***
Andrea Polle
apolle@gwdg.de

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

Received: *31 July 2020*
Accepted: *10 November 2020*
Published: *14 December 2020*

Citation:
Dreischhoff S, Das IS, Jakobi M, Kasper K and Polle A (2020) Local Responses and Systemic Induced Resistance Mediated by Ectomycorrhizal Fungi. Front. Plant Sci. 11:590063. doi: 10.3389/fpls.2020.590063

INTRODUCTION

Plants live in close relationship with microbes, which colonize their hosts as symbiotrophic, saprotrophic or pathogenic organisms (Bonfante and Anca, 2009; Vandenkoornhuyse et al., 2015). An important example is the beneficial interaction between certain soil fungi and plant roots, leading to the formation of a new organ, the mycorrhiza (from Greek μύκης mι kçs, "fungus," and ßζα rhiza, "root"). The mycorrhizal symbiosis is well characterized by a bidirectional exchange of nutrients (Smith and Read, 2008). The fungus receives photosynthesis-derived carbohydrates from the plant and supplies essential, often rarely available nutrients like nitrogen or phosphorus from the soil to the plant (van der Heijden et al., 2015; Nehls and Plassard, 2018).

Mycorrhizal symbiosis enhances the performance of plants (Smith and Read, 2008) and, thus, most likely drastically facilitated the evolution of land plants (Wang et al., 2010). Approximately 85 % (˜340,000 species) of all plant species are colonized by mycorrhizal fungi (˜50,000 species) (van der Heijden et al., 2015; Brundrett and Tedersoo, 2018; Genre et al., 2020). The most ancient and widely spread symbiosis is formed by arbuscular mycorrhizal fungi (AMF) (Bonfante and Anca, 2009; Martin et al., 2018). In forests of the temperate and boreal zone, ectomycorrhizal symbioses with the roots of tree species are predominant (Brundrett, 2009). Ectomycorrhizal fungi (EMF) have evolved independently multiple times from saprotrophic clades, making EMF no homogenous group (Martin et al., 2016; Genre et al., 2020). EMF and AMF are the most well studied groups among mycorrhiza-forming fungi, however, exhibiting different lifestyles. While AMF form hyphopodia to invade the plant and grow inside cortical root cells, EMF cover the root tip with a hyphal mantle and grow between the root epidermis and outer layers of cortical cells, forming the Hartig net (Bonfante and Anca, 2009). Both AMF and EMF generate extraradical hyphae as the main structures for nutrient uptake from soil.

There is now growing awareness that mycorrhizas do not only improve plant nutrition but also enhance plant resistance against abiotic and biotic cues. Resistance is the ability of a plant to restrict the growth and development or the damage caused by a specific pest or pathogen. Resistance can be achieved by activation of defense mechanisms or is the result of tolerance, i.e., the ability to endure the stress (Larcher, 1995). The term "mycorrhiza-induced resistance" (MIR) has been used to describe this phenomenon for the interaction of a mycorrhizal fungus with a host plant (Cameron et al., 2013; Mauch-Mani et al., 2017). MIR shares similarities with both systemic acquired resistance (SAR), induced after pathogen attack, while induced systemic resistance (ISR) is conferred by beneficial soil microbes. In this review, we focus on ectomycorrhiza-induced systemic resistance, which is a rapidly expanding research area. We define systemic effects as those effects that occur in distal tissues (here leaves) that are not in direct contact with the mycorrhizal fungus, while local responses occur in tissues (here roots) in contact with the EMF. We discuss local responses to EMF colonization, leading to long-distance signaling, systemic transcriptional rewiring and metabolic changes induced by EMF. We address the role of phytohormones in MIR and discuss commonalities with SAR and ISR. Since MIR by EMF is an emerging field, we also include examples for MIR induced by AMF highlighting similarities in defense activation.

A GLIMPSE ON SYSTEMIC RESISTANCE IN PLANTS—SAR AND ISR

The two major types of systemic resistance intensely studied in plant microbial interactions are SAR (Spoel and Dong, 2012) and ISR (Pieterse et al., 2014). SAR and ISR are based on distinct phytohormonal signals. SAR describes defenses against (hemi-)biotrophic pathogens activated after local challenge by a pathogen in systemic, uninfected tissues. The SAR signaling cascade is triggered by microbe-associated molecular patterns (MAMPs) leading to MAMP-triggered immunity or triggered by pathogen effectors leading to effector-triggered immunity (Jones and Dangl, 2006). Subsequently, the defense in systemic uninfected tissues is induced in an SA dependent manner and acts against a broad range of pathogens (Vlot et al., 2009; Spoel and Dong, 2012). Various compounds have been proposed as potential signals for SAR activation. For instance, methyl salicylate is a phloem-mobile compound that can be transported to systemic plant parts, where it is hydrolyzed to the bio-active SA to induce resistance (Park et al., 2007). For defense induction and in addition for attracting predators of herbivores, methyl SA might also act as a volatile signal (Shulaev et al., 1997; Koo et al., 2007; Ament et al., 2010; Rowen et al., 2017). Recently, the non-proteinogenic amino acid pipecolic acid (Pip) and its derivative N-hydroxypipecolic acid have been identified as essential for SAR signaling (Návarová et al., 2012; Chen et al., 2018; Hartmann et al., 2018; Wang et al., 2018). The mobile signals activate MAPK (MITOGEN-ACTIVATED PROTEIN KINASE) cascades (Conrath et al., 2015) and induce the expression of pathogenesis-related (PR) proteins, especially PR1 (PATHOGENESIS-RELATED 1) involving antagonistic key regulators NPR1 and NPR3/4 [NON-EXPRESSER OF PR GENES (Ding et al., 2018)]. Other compounds invoked as mobile SAR signals are azaleic acid (a C_9 lipid peroxidation product), lipid transfer proteins, and the diterpene dihydroabietinal (Vlot et al., 2017). Ultimately, an enhanced defense is achieved either through direct defenses (e.g., callose deposition) or trough priming, whereby the plant exhibits stronger defenses toward a secondary infection (Conrath et al., 2006; Jung et al., 2009, 2012; Pieterse et al., 2014; Mauch-Mani et al., 2017).

In contrast to SAR induced by pathogens, ISR is conferred by beneficial microbes. They interact with roots and make the whole plant more resistant or tolerant against stressors. The picture for ISR is less specific than for SAR because different microbial species might recruit different compounds for ISR signaling (Haney et al., 2018). In general, jasmonic acid (JA) and its derivatives, in particular JA-Isoleucine (JA-Ile) are the key phytohormones and their signaling pathways are modulated by either ethylene (defense against necrotrophic pathogens) or abscisic acid (against herbivores) (Pieterse et al., 2012). JAZ (JASMONATE-ZIM-DOMAIN PROTEIN), which stabilize the JA receptor COI1 (CORONATINE INSENSITIVE 1), and MYB (MYB DOMAIN PROTEIN) transcription factors are essential in ISR. Similar to SAR, more than one component might act as a long-distance signal (see section "Long-Distance Signaling in Systemic Resistance—Tapping Around in the Dark"). At the cellular level, the pathways for systemic defenses, ISR and SAR often appear to be regulated antagonistically. When SA signaling is upregulated, JA signaling is suppressed, implying trade-off for the resistance against necrotrophic pathogens when the defense against biotrophic pathogens is upregulated and vice versa (Pieterse et al., 2012).

SHEDDING LIGHT ON ECTOMYCORRHIZAL INDUCED DEFENSES

Defense Signaling in Local Root Tissue Interacting With EMF Unveils Commonalty With Pathogen-Triggered Responses

In the process of establishing an active symbiosis, host plant and EMF exchange an array of molecules with different properties, e.g., flavonoids, auxin, and secreted proteins, etc. (Felten et al., 2009; Garcia et al., 2015). Genome, transcriptome, and secretome analyses of EMF from distant phyla (basidiomycota: *Laccaria bicolor,* ascomycota: *Tuber melanosporu* and *Cenococcum geophilum* (Vincent et al., 2012; Doré et al., 2015; Kohler et al., 2015; Pellegrin et al., 2015; de Freitas Pereira et al., 2018) uncovered a huge battery of small secreted proteins, among which a subset was strongly up-regulated during mycorrhizal colonization of the host. Three mycorrhizal-induced small proteins, MiSSP7, 7.6, and 8 (named after their atomic mass in kDa) of *L. bicolor* were closer investigated and found to be essential for symbiosis establishment (Plett et al., 2011; Pellegrin et al., 2019; Kang et al., 2020).

In *Populus* × *canescens, Lb*MiSSP7 interacts locally with JAZ6 to stabilize this protein (Plett et al., 2014). JAZ6 is a key repressor of the F-box protein COI1, which is the receptor for JA-Ile, the active form of JA, in the SCF(COI1) complex (Thines et al., 2007). When COI1 binds JA-Ile, JAZ6 is degraded via the proteasome and the transcription of JA responsive genes is activated (Howe et al., 2018). Thus, by stabilizing *Populus* JAZ6 the JA signaling pathways is locally suppressed. Application of JA acts negatively on the establishment of symbiotic structures (Plett et al., 2014). Because of the JA-SA antagonism (see section "A Glimpse on Systemic Resistance in Plants—SAR and ISR"), this regulation is surprising as it may be intuitively expected to facilitate defenses against biotrophic fungi (including EMF). Plett et al. (2011) demonstrated that MiSSP7 also induces the transcription of auxin-responsive genes in root tissues.

Circumstantial evidence suggests that *Lb*MiSSP7.6 may also interfere with local plant immunity. *Lb*MiSSP7.6 interacts with two *Populus* Trihelix transcription factors (*Pt*Trihelix1 and *Pt*Trihelix2) in the nucleus of plant cells. The closest *Arabidopsis thaliana* homolog of *Pt*Trihelix2 is *At*ASR3 (ARABIDOPSIS SH4-RELATED3) (Kang et al., 2020), which is a phosphorylation substrate of MAPK4 and thus, may negatively regulate immunity. Furthermore, pattern-triggered immunity is negatively regulated through phosphorylation of *At*ASR3 by MAPK4 (Li et al., 2015).

Additional support for the modulation of immune responses by small secreted proteins comes from studies on the AMF-host interactions (*Glomus intraradices* with *Medicago truncatula*) (Kloppholz et al., 2011). The AMF fungal protein *Gi*SP7 (secreted protein 7) interacts with *Mt*ERF19 (ETHYLENE-RESPONSIVE FACTOR) transcription factor in the plant nucleus and interferes with *Mt*ERF19-related and, thus, ethylene-modulated defense (Kloppholz et al., 2011). Moreover, *Mt*ERF19 is induced by pathogens and is involved in activating defense against biotrophic pathogens (Kloppholz et al., 2011). Taken together, these examples show that mycorrhizal fungi interact with parts of the plants defense local machinery involving small secreted protein.

Not only small secreted protein could be responsible for the initiation of defense induction, but also a number of other metabolites. It is known for AMF that chitin oligomers and lipochitooligosaccharides are part of Myc factors, which are used for communication with their host (Maillet et al., 2011; Sun et al., 2015). These compounds are also produced by the EMF *L. bicolor* (Cope et al., 2019). In poplar, lipochitooligosaccharides from *L. bicolor* activate the common symbiosis pathway including calcium-spiking (Cope et al., 2019), which plays a role in activating defense responses to microbes (Yuan et al., 2017). Lipochitooligosaccharides were also found to modulate host immunity (Limpens et al., 2015). Furthermore, chitin and chitin-related components (e.g., chitosan) are known elicitors (MAMPs) for plant defense (Boller and Felix, 2009). Chitosan formulations have been applied as a biological control agents to leaves and roots to boost plant defenses (El Hadrami et al., 2010; Pusztahelyi, 2018) but their mode of action is unclear.

Chitin is a cell wall component of fungi but not of plants. Novel results assign a crucial role to chitin in fungal perception and defense stimulation (Zhang et al., 2015; Vishwanathan et al., 2020). When Arabidopsis roots were treated with chitin enhanced protection against leaf herbivory was observed similar to that found in response to *L. bicolor* inoculation of Arabidopsis roots (Vishwanathan et al., 2020). This finding shows that—at least part of—MIR by EMF does not require formation of a functional mycorrhiza because Arabidopsis is a non-host to mycorrhizal fungi. This result further shows that chitin, an abundant compound in many potentially hazardous organisms (fungi, insects), is sufficient for the defense induction. The plant chitin receptor CHITIN ELICITOR RECEPTOR KINASE 1 (CERK1) (Miya et al., 2007) is well known for its central role in mediating plant immunity (Gong et al., 2020). In Arabidopsis knock-out mutants *cerk1-2* MIR induced by EMF was abolished (Vishwanathan et al., 2020; **Figure 1**), demonstrating the critical role of chitin in the non-host interaction of *Arabidopsis* with *L. bicolor*. Other components such as LYK4 and LYK5 forming a complex with CEKR1 necessary for defense induction (Xue et al., 2019), may also be involved but this assumption has yet to be experimentally tested. Upon chitin or *L. bicolor* exposure, the MAP kinase signaling cascade (MAPK 3, 4, and 6) was activated in Arabidopsis (Vishwanathan et al., 2020). MAPKs belong like calcium influx and oxidative burst to the microbial triggered immunity responses (Boller and Felix, 2009), suggesting that *L. bicolor* activates a general microbial defense pathway via chitin perception. In rice, CERK1 has also a function in defense signaling and AMF symbiosis (Zhang et al., 2015).

Chitin is released from fungal cell walls by plant chitinases as part of defenses against fungal pathogens (Sharma et al., 2011). It was first reported for the interaction of *Amanita muscaria* with *Picea abies* that EMF also induce chitinases in host roots (Sauter and Hager, 1989). Similarly Albrecht et al. (1994a,b) showed that chitinases are induced upon contact of the EMF *Pisolithus tinctorius* with *Eucalyptus globulus* and

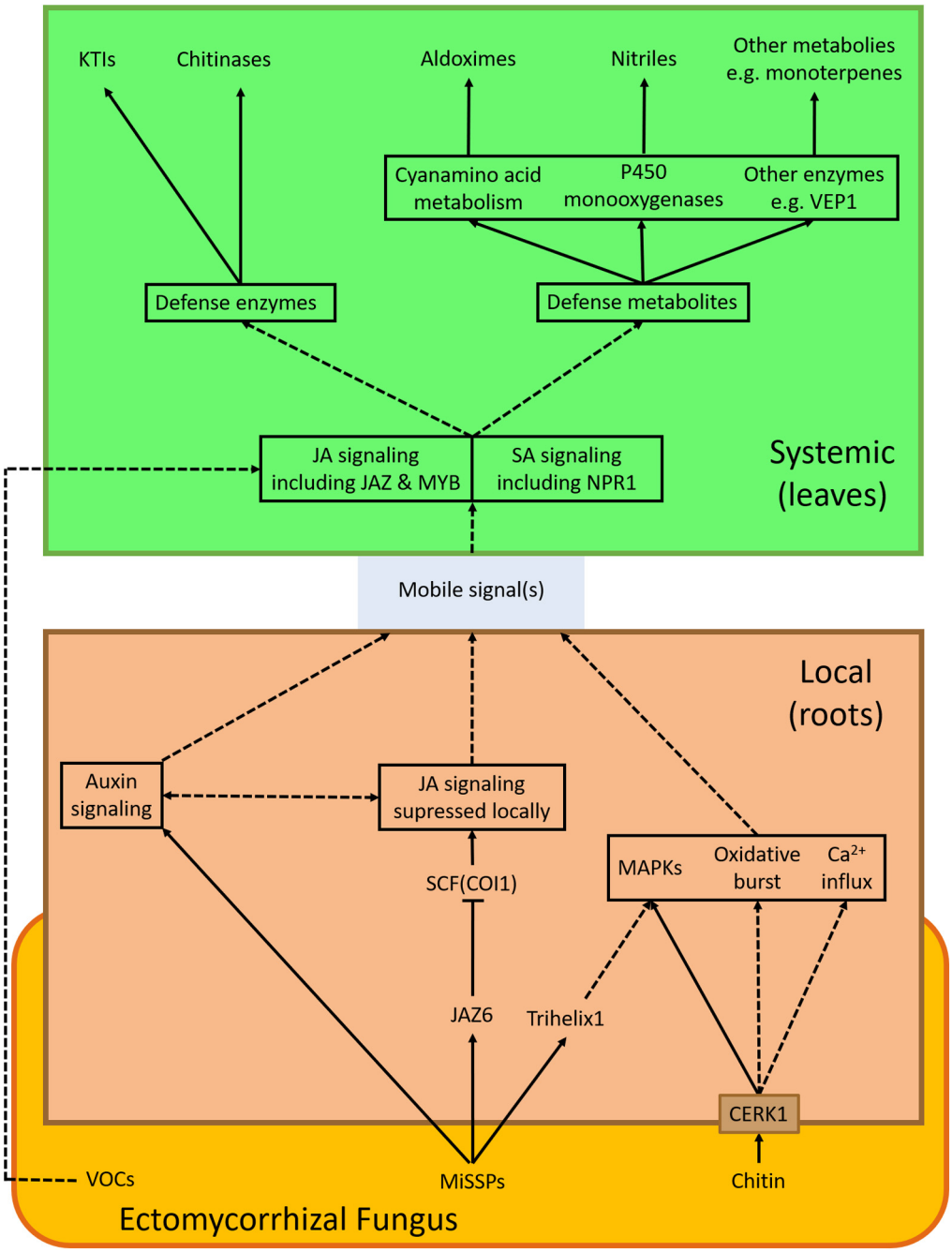

FIGURE 1 | EMF-induced signaling cascade(s). The scheme summarizes signaling components discussed in this review displaying processes induced by an ectomycorrhizal fungus (red) in the root (brown) and in leaves (green). Arrows show interactions/connections (full lines: direct evidence, dashed lines: speculative). VOCs—Volatile organic compounds; JAZ6—JASMONATE-ZIM-DOMAIN PROTEIN 6; CERK1—CHITIN ELICITOR RECEPTOR KINASE 1; SCF(COI1)—CORONATINE INSENSITIVE 1—Skp, Cullin, F-box containing complex; MAPKs—mitogen-activated protein kinases; JA—jasmonic acid; MYB—MYB DOMAIN PROTEIN; KTIs—Kunitz trypsin inhibitors; VEP1—VEIN PATTERNING 1.

that the strength of this defense response correlated with the extent of colonization by the fungus. Many previous studies showed transient transcriptional activation of chitinases in concert with other defenses (e.g., metallothionein-like proteins and glutathione-*S*-transferases) when EMF interacted with host roots (Franken and Gnädinger, 1994; Johansson et al., 2004; Duplessis et al., 2005; Frettinger et al., 2007; Heller et al., 2008).

A number of studies indicate that host colonization by EMF activates local host defenses only transiently. For example, the transcription of defense genes was locally upregulated in

birch roots during the formation of the ectomycorrhizal mantle and the Hartig net by *Paxillus involutus* (Le Quéré et al., 2005). In later developmental stages when the mycorrhiza was mature, plant defense genes were repressed (Le Quéré et al., 2005). In oak colonized by the EMF *Piloderma croceum*, genes of the phenylpropanoid metabolism were down-regulated (Tarkka et al., 2013). A recent study shows that the transcriptional responses in oak vary substantially depending on the ectomycorrhizal fungal species that is colonizing the root, but a common response induced by the tested EMF species was the reduction of defense gene transcript levels, when the roots had been colonized (Bouffaud et al., 2020). It is therefore possible that initially fungal MAMPs induce defenses, which are subsequently suppressed by mechanisms similar to those employed pathogenic fungi (Barsoum et al., 2019).

Altogether, these studies highlight that EMF locally trigger (a subset of) plant defenses against fungal pathogens, at least during the initial stages of colonization. Chitin signaling is required to elicit systemic responses in distant tissues. Intriguing questions for future research are whether MIR is part of the universal non-host response of plants to microbes or whether MIR in a functional mycorrhiza as the result of compatible EMF-host interactions has additional facets.

Long-Distance Signaling in Systemic Resistance—Tapping Around in the Dark

Mobile inter-organ signaling is required to achieve MIR in systemic tissues. The most direct and fastest connection between mycorrhizal roots and the shoot is the xylem. In addition to its function in water and mineral nutrition transport, the composition of the xylem sap is characterized by a plethora of compounds such as phytohormones, proteins, peptides, and amino acids, etc. (Shabala et al., 2016). In response to nodulation by rhizobia or symbiosis with AMF, specific small peptides (CLE) have been found (Okamoto et al., 2013; Le Marquer et al., 2019), which are part of the plant autoregulation of symbiotic interactions (Wang et al., 2018). Given the similarities of the genetic make-up of root symbiotic interactions for EMF, AMF, and rhizobia (Cope et al., 2019), it is tempting to speculate that CLE peptides may also signal the root mycorrhizal status in EMF plants. However, to date neither peptides nor phytohormones or other molecules have been identified in xylem sap that were functionally linked with MIR in EMF plants.

MIR influences the performance of phytophagous insects (Pozo and Azcón-Aguilar, 2007). Therefore, it is conceivable that JA(-derivatives), which are known to mount defenses against wounding and insect feeding (Zhang and Hu, 2017), play a role in long-distance signaling of MIR. JA-derived molecules such methyl-JA can be transported in both the xylem and the phloem (Thorpe et al., 2007). Mutants of tomato, which are unable to mount systemic defenses, revealed that the systemic wound response requires local JA biosynthesis and the ability to perceive a JA signal systemically (Schilmiller and Howe, 2005).

Vascular transport of mobile signals has most intensely been studied for SAR. The phloem was identified as the major signaling route (Shah and Zeier, 2013). Upon interaction with biotrophic pathogens or virulence factors, compounds such as the methyl ester of salicylic acid (Dempsey and Klessig, 2012), JA (Truman et al., 2007), and pipecolic acid (Shah and Zeier, 2013) accumulate in the vasculature and were able to induce SAR independently of other compounds. Azelaic acid (Jung et al., 2009), a glycerol-3-phosphate-derived molecule (Chanda et al., 2011) and the abietane diterpenoid dehydroabietinal (Chaturvedi et al., 2012) are bound to the lipid transport protein DIR1 (DEFECTIVE IN RESISTANCE1) for transport through the vasculature, leading to SAR induction (Isaacs et al., 2016). Most of the potential SAR signaling molecules accumulate in petiole exudates (Maldonado et al., 2002; Thorpe et al., 2007; Truman et al., 2007; Jung et al., 2009; Chanda et al., 2011; Sato et al., 2011; Chaturvedi et al., 2012; Champigny et al., 2013; Isaacs et al., 2016). Feeding petiole exudates of SAR-induced wildtype Arabidopsis to transgenic lines, unable to express the signaling compound glycerol-3-phosphate or *DIR1*, recovered SAR in the mutants (Chanda et al., 2011; Isaacs et al., 2016). In poplar, SA or methyl-SA can induce resistance in systemic tissues (Li et al., 2018). These phytohormones are also required for the activation flavan-3-ols synthesis as defense against rust fungi (Ullah et al., 2019). Whether SA or its derivatives also play a role in the transmission of EMF-induced signals in trees is still unknown.

In addition to the classical pathways through xylem and phloem for the directed transport of molecules, volatile organic compounds (VOCs) are undirected aerial signals, serving inter-kingdom communication between plants and fungi (Werner et al., 2016; Schulz-Bohm et al., 2017). EMF emit a rich spectrum of VOCs, dominated by mono- and sequiterpenes (Müller et al., 2013). Among these VOCs, β-caryophyllene mounts plant defenses against bacterial pathogens (Huang et al., 2012; Hammerbacher et al., 2019). Furthermore, EMF influence the VOC emission pattern of mycorrhizal poplar, leading for example to slightly suppressed ocimene levels (Kaling et al., 2018). The perception of VOCs and stimulation of defenses can be amplified, activating SAR from plant to plant (Wenig et al., 2019). Since direct evidence for genes responsive to VOCs and EMF is missing, we screened the literature for genes regulated in response to VOCs (Godard et al., 2008; Riedlmeier et al., 2017; Lee et al., 2019) overlapping with those responsive to EMF (Luo et al., 2009; Kaling et al., 2018; **Table 1**). Notably, many of these genes are involved in JA signaling and play roles in wounding or pathogen defense (*JAZ1, JAZ7, JAZ8, WRKY40,* β*-1,3-ENDO-GLUCANASE, SIS, CYP94B1, and GSTU1*; **Table 1**). These observations suggest that long-distance signaling by VOCs should be taken into account in future studies of systemic defense activation.

Mycorrhiza Induced Resistance in Systemic Tissues—Signals and Defense Activation

Phytohormones orchestrate the expression of defense-related genes in systemic tissues. In response to biotrophic pathogens,

TABLE 1 | Transcriptional regulation of Arabidopsis genes by volatile organic compounds (VOCs) and their poplar orthologs responsive to ectomycorrhiza symbiosis.

Gene name	Gene function	AGI	Regulation	Host	Treatment	Experimental set-up	Sample tissue	References
GSTU1	Glutathione S-transferase TAU 1, responsive to ME-JA	AT2G29490	Up	*P. x canescens*	*L. bicolor*	Pot, in root contact	Leaves	Kaling et al., 2018
			Up	*A. thaliana*	Monoterpene isolated from plants	Pot, exposed to Ocimene	Rosette leaves, stems, cauline leaves	Godard et al., 2008
GSTU4	Glutathione S-transferase tau 4, involved in defense from necrotrphic pathogens	AT2G29460	Up	*P. x canescens*	*P. involutus*	Pot, in root contact	Roots	Luo et al., 2009
			Up	*A. thaliana*	Monoterpene isolated from plants	Pot, exposed to Ocimene	Rosette leaves, stems, cauline leaves	Godard et al., 2008
JAZ1	JAZ1, involved in jasmonate signaling, defense, wounding. JAZ1 transcript levels rise in response to a jasmonate stimulus.	AT1G19180	Down	*P. x canescens*	*L. bicolor*	Pot, in root contact	Leaves	Kaling et al., 2018
			Up	*A. thaliana*	Monoterpene isolated from plants	Pot, exposed to Ocimene	Rosette leaves, stems, cauline leaves	Godard et al., 2008
JAZ7	Jasmonate-zim-domain protein 7; wounding response	AT2G34600	Up	*A. thaliana*	Monoterpene isolated from plants	Pot, exposed to Ocimene	Rosette leaves, stems, cauline leaves	Godard et al., 2008
			Down	*A. thaliana*	1-decene isolated from Trichoderma	Plants in petri dish, 1-decene added	shoots	Lee et al., 2019
JAZ8	Jasmonate-zim-domain protein 8; wounding response	AT1G30135	Down	*P. x canescens*	*L. bicolor*	Pot, in root contact	Leaves	Kaling et al., 2018
			Down	*A. thaliana*	1-decene isolated from Trichoderma	Plants in petri dish, 1-decene added	Shoots	Lee et al., 2019
WRKY40	Probable WRKY transcription factor 40; Pathogen-induced transcription factor, response to chitin, SA, Me-JA	AT1G80840	Up	*P. x canescens*	*P. involutus*	pot, in root contact	Roots	Luo et al., 2009
			Up	*A. thaliana*	Monoterpene isolated from plants	Pot, exposed to Ocimene	Rosette leaves, stems, cauline leaves	Godard et al., 2008
			Down	*A. thaliana*	1-decene isolated from Trichoderma	Plants in petri dish, 1-decene added	Shoots	Lee et al., 2019
			Up	*A. thaliana*	Monoterpene isolated from plants	Pot, exposed to Pinene	Leaves	Riedlmeier et al., 2017

(Continued)

TABLE 1 | Continued

Gene name	Gene function	AGI	Regulation	Host	Treatment	Experimental set-up	Sample tissue	References
SPX1	SPX domain-containing protein 1; response to phosphate starvation, response to *Pseudomonas syringae*	AT5G20150	up or down (depending on poplar homolog)	*P. x canescens*	*P. involutus*	Pot, in root contact	Roots	Luo et al., 2009
				A. thaliana	Rhizobacteria	Bi-compartmented petri dishes, no contact	Seedlings	Wenke et al., 2012
PAP1	Purple acid phosphatase, response phosphate (Pi) and phosphite (Phi), response to non-host bacteria.	AT1G13750	Down	*P. x canescens*	*P. involutus*	Pot, in root contact	Roots	Luo et al., 2009
			Up	*A. thaliana*	Monoterpene isolated from plants	Pot, exposed to Pinene	Leaves	Riedlmeier et al., 2017
BBE8	FAD-binding Berberine family protein, response avirulent *Pseudomonas synrigae*, response to non-host bacteria	AT1G30700	Up	*P. x canescens*	*P. involutus*	Pot, in root contact	Roots	Luo et al., 2009
			Up	*A. thaliana*	Monoterpene isolated from plants	Pot, exposed to Pinene	Leaves	Riedlmeier et al., 2017
-	Putative β-1,3-endoglucanase, response to nematode, response to fungus	AT4G16260	Up	*P. x canescens*	*L. bicolor*	Pot, in root contact	Leaves	Kaling et al., 2018
			Up	*A. thaliana*	Monoterpene isolated from plants	Pot, exposed to Pinene	Leaves	Riedlmeier et al., 2017
PRX47	Peroxidase superfamily protein,response to oxidative stress	AT4G33420	Down	*P. x canescens*	*L. bicolor*	Pot, in root contact	Leaves	Kaling et al., 2018
			Up	*A. thaliana*	Monoterpene isolated from plants	Pot, exposed to Pinene	Leaves	Riedlmeier et al., 2017
-	Tetratricopeptide repeat (TPR)-like superfamily protein	AT4G37380	Down	*P. x canescens*	*P. involutus*	Pot, in root contact	Roots	Luo et al., 2009
			Down	*A. thaliana*	Monoterpene isolated from plants	Pot, exposed to Pinene	Leaves	Riedlmeier et al., 2017

(Continued)

TABLE 1 | Continued

Gene name	Gene function	AGI	Regulation	Host	Treatment	Experimental set-up	Sample tissue	References
SIS	Salt Induced Serine rich, response to salt, response to virulent *Pseudomonas syringae*	AT5G02020	Up	*P. x canescens*	*P. involutus*	Pot, in root contact	Roots	Luo et al., 2009
			Up	*A. thaliana*	Monoterpene isolated from plants	Pot, exposed to Pinene	Leaves	Riedlmeier et al., 2017
KAT5	3-keto-acyl-CoA thiolase 2 precursor, involved in flavonoid biosynthesis	AT5G48880	Up	*P. x canescens*	*L. bicolor*	Pot, in root contact	Leaves	Kaling et al., 2018
			Down	*A. thaliana*	Monoterpene isolated from plants	Pot, exposed to Pinene	Leaves	Riedlmeier et al., 2017
CCT101	Member of ASML2 family of CCT domain proteins, high expression in eds16 mutants (isochorimate synthase for SA synthesis)	AT5G53420	Down	*P. x canescens*	*L. bicolor*	Pot, in root contact	Leaves	Kaling et al., 2018
			Down	*A. thaliana*	Monoterpene isolated from plants	Pot, exposed to Pinene	Leaves	Riedlmeier et al., 2017
CYP94B1	cytochrome P450, family 94, subfamily B, polypeptide 1, JA metabolic process, wounding	AT5G63450	Down	*P. x canescens*	*L. bicolor*	Pot, in root contact	Leaves	Kaling et al., 2018
			Down	*A. thaliana*	Monoterpene isolated from plants	Pot, exposed to Pinene	Leaves	Riedlmeier et al., 2017

The table summarizes differentially expressed genes overlapping between VOC and EMF response. AGI shows Arabidopsis Gene Identity for the best poplar match. Treatment indicates the EMF used for plant inoculation or the VOC to which plants were exposed to. Experimental set-up indicates non-sterile conditions when plants were grown in pots or sterile growth systems. The gene functions were taken from the TAIR data base (https://www.arabidopsis.org/) and response were also searched via the eFP browser implemented in TAIR.

accumulation of SA is accompanied by the induction of *PR* (Pathogenesis-related) gene expression (Dixon et al., 1994; Hammond-Kosack and Jones, 1997; Brodersen et al., 2005; Radojičić et al., 2018). The most prominent representative of the PR proteins is PR1, which is characteristic for the SA defense pathway (Nimchuk et al., 2003; Durrant and Dong, 2004; Glazebrook, 2005). AMF can activate SA defenses in their host plants (Barea and Jeffries, 1995; García-Garrido and Ocampo, 2002). AMF-colonized crops exhibit enhanced resistance against *Phytophthora infestans* (potato, Gallou et al., 2011), *Magnaporthe oryzae* (rice, Campos-Soriano et al., 2012), and *Alternaria solani* (tomato, Song et al., 2015). The defense induction was attributed to MIR by AMF (**Table 2**) and has similarities with SAR (see section "A Glimpse on Systemic Resistance in Plants—SAR and ISR").

Likewise, the EMF-induced systemic resistance also involves components of SAR signaling. The fitness of caterpillars feeding on SAR signaling mutants of Arabidopsis (*npr1, npr3/4*) was reduced, similar to the effects imposed by *L. bicolor* inoculation (Vishwanathan et al., 2020). In poplar leaves, transcriptional regulation of *NPR1* was detected in EMF-colonized compared to non-colonized plants (Kaling et al., 2018). Pfabel et al. (2012) observed enhanced levels of SA in poplars colonized by the EMF *Hebeloma mesophaeum* as well as in poplars challenged with rust fungi *Melampsora larici-populina.* Therefore, it is likely that similarly to AMF, EMF systemically activate components of the SAR pathway (**Figure 1**).

In AMF, the induction of down-stream defenses against pathogens is often less pronounced than by SAR and therefore, the alerted stage induced by mycorrhizal colonization has been considered as "priming" (Cameron et al., 2013). As defined by Pozo and Azcón-Aguilar (2007), the phenomenon of priming is the pre-conditioning of the plant host for a more efficient activation of plant defenses upon pathogen attack (Jung et al., 2012). "Priming" by AMF involves, for instance, transcriptional regulation of *PR1* and *NPR1*, hallmarks of the SA pathway (Cameron et al., 2013). However, AMF also prime the JA pathway in the host plant as an "alert" signal against necrotrophic pathogens and leaf-chewing insects (Glazebrook, 2005; Pozo and Azcón-Aguilar, 2007; Jung et al., 2012). These responses include transcriptional regulation of *MYBs* (many of these transcription factors are induced by JA), *LOX* (*LIPOXYGENASE*), *OPR* (*12−OXOPHYTODIENOATE REDUCTASE*), *COI* (*CORONATINE−INSENSITIVE*), *AOC* (*ALLENE OXIDE CYCLASE*), and *AOS* (*ALLENE OXIDE SYNTHASE*) etc. (**Table 2**). While most studies tested alleviation of damage by necrotrophic pathogens (**Table 2**), increased resistance against herbivores such as cabbage looper (*Trichoplusia ni*) and cotton bollworm (*Helicoverpa armigera*) was also reported for AMF crops (Song et al., 2013; Schoenherr et al., 2019).

Ecological studies often show beneficial effects of EMF-colonization on the resistance of tree species from different habitats and different phylogenetic origin, e.g., *Larix sibirica, Betula pubescens,* and *Eucalyptus urophylla* against herbivores (*Otiorhyncus* spp., *Anomala cupripes*, and *Strepsicrates* spp. (Halldórsson et al., 2000; Gange et al., 2005; Shen et al., 2015). For example, on the leaves of birch, the birch aphid *Calaphis flava* produces significantly less nymphs when the trees are colonized with EMF (*Paxillus involutus* or *Leccinum versipelle*) compared with non-mycorrhizal trees (Nerg et al., 2008). However, beneficial effects of EMF on the host are not always observed. Larval growth of the autumnal moth *Epirrita autumnata* was not attenuated on EMF-colonized birch trees (Nerg et al., 2008) and EMF colonization of pine roots had no effect on the oviposition of generalist herbivore *Lygus rugulipennis* (Manninen et al., 1998). These studies suggest that the resistance induced by EMF is context-dependent. This idea is also supported by recent transcriptome analyses showing that host defense gene expression of leaves can be diminished when the tree roots are colonized by EMF and depends on the specific host—EMF combination (Maboreke et al., 2016; Bacht et al., 2019; Bouffaud et al., 2020).

Genetic studies exploring the systemic consequences of EMF-plant interaction are scarce. Arabidopsis knock-out mutants of *coi1-16*, which cannot activate the JA pathway, are more susceptible to cabbage looper feeding than the wildtype, indicating that the protective effect of *L. bicolor* is lost when the JA signaling is compromised (Vishwanathan et al., 2020). In poplar, *L. bicolor* induced a transcriptional network characterized by six major gene ontology (GO) terms: "regulation of phytohormones," "immune response," "response to wounding," "flavonoid metabolism," "secondary metabolism," and "response to toxic substance" (**Figure 2**). "Regulation of phytohormones" and "immune response" comprise mainly transcription factors such as *JAZ1* (orthologs of *JAR1* and *JAR8*) and *MYBs* (orthologs of *MYB4, MYB5, MYB14,* and *MYB108*) which are key the regulators of the JA responses (Goossens et al., 2016). Altogether, these studies imply that regulation of MIR by EMF involves both JA and SA signaling pathways (**Figure 1**).

Induction of JA and SA-related gene expression also occurs in beneficial fungi, which do not form mycorrhizal structures such as *Serpendita indica* (formerly known as *Piriformospora indica,* Sebacinales, Basidiomycota), and *Trichoderma* sp. (Basidiomycota) (**Table 2**). *Serpendita indica* activates *PR1* as well as PDF1.2 (defensin) expression in its host (Stein et al., 2008; Molitor et al., 2011). *Trichoderma harzianum* induces JA- and SA-dependent defenses against *Botrytis cineria* by stimulating defense proteins such as PROTEINASE INHIBITOR II and MULTICYSTATIN (Martinez-Medina et al., 2013). *Trichoderma* sp., which is available as commercial inoculum, has often been reported to be a potent biocontrol agent against pathogens (Sharon et al., 2011; Kumar and Ashraf, 2017). For example, in cucumber *Trichoderma harzianum* caused an increased expression of defense genes [*PR*4, *LOX* (lipoxygenase), *GOL* (galactinol synthase)] against the damping-off disease caused by the pathogen *Phytophthora melonis* (Sabbagh et al., 2017). Similar responses were also observed for the AMF *Glomus mosseae*, suggesting that both are effective in diminishing diseases (Sabbagh et al., 2017). Under field conditions, it is also possible that the induction SA and JA-dependent defenses is the consequence of an interaction of AMF (inducing SA defenses) and beneficial rhizobacteria (inducing JA defense) (Cameron et al., 2013). Similar interactions are feasible for

TABLE 2 | Systemic defense activation by mycorrhizal plants.

Gene name	Gene function	Proposed defense pathway	Mycorrhiza Type	Mycorrhiza species	Effects of mycorrhiza	Plant host	Resistance against	Disease/Effect	References
PMR4	Callose synthase	JA pathway	AMF	*Rhizoglomus irregularis*	Fungal biomass- *B. cinerea* reduced to 66%	Tomato- *Solanum lycopersicum*	Fungus- *Botrytis cinerea*	Gray mold	Sanmartín et al., 2020
ATL31	Carbon/Nitrogen insensitive 1(Arabidopsis Toxicos en Levadura 31)								
SYP121	Vesicular trafficking protein								
VCH3	Chitinase	Chitinase induced defense pathway	AMF	*Glomus versiforme*	Significant reduction in *M. incognita* infection	Grapevine- *Vitis amurensis*	Nematode- *Meloidogyne incognita*	Root knot	Li et al., 2006
CHI	Chitinase 1b	JA and SA pathway	AMF	*Glomus intraradices*	*X. index* count in soil and galls reduced significantly (after 35 days)	Grapevine- *Vitis berlandieri* × *Vitis riparia*	Nematode- *Xiphinema index*	Root gall	Hao et al., 2012
PR10	Pathogenesis-related 10								
GST	Glutathione S-transferase								
STS	Stilbene synthase 1								
ESPS	5-enolpyruvyl shikimate-3-phosphate synthase								
PR1-a	Pathogenesis-related 1	SA pathway	AMF	*Glomus mosseae*	74—84% decrease in necroses andintraradical pathogen hyphae of *P. phytophthora*	Tomato- *Solanum lycopersicum*	Pathogen- *Phytophthora parasitica*	Fruit rot	Cordier et al., 1998
OsNPR1	Non-expressor of PR1	JA and SA pathway	AMF	*Glomus intraradices*	Significant reduction in spore count of *M. oryzae*	Rice- *Oryza sativa L.*	Fungus- *Magnaporthe oryzae*	Rice blast	Campos-Soriano et al., 2012
OsAP2	APETALA2								
OsEREBP	Ethylene-responsive element-binding protein								
OsJAmyb	JA-regulated myb transcription factor								
PR	Pathogenesis-related								

(Continued)

TABLE 2 | Continued

Gene name	Gene function	Proposed defense pathway	Mycorrhiza Type	Mycorrhiza species	Effects of mycorrhiza	Plant host	Resistance against	Disease/Effect	References
PR2a	Pathogenesis-related 2a	DIMBOA-phytoalexin based defense and JA pathway	AMF	*Glomus mosseae*	Disease index of *R. solani* reduced by 50%	Corn- *Zea mays*	Fungus- *Rhizoctonia solani*	Sheath blight	Song et al., 2011
PAL	Phenylalanine ammonia-lyase								
AOS	Allene oxide synthase								
BX9	DIMBOA (2,4-dihydroxy-7-methoxy-2 H-1,4-benzoxazin-3(4 H)-one) biosynthesis pathway gene								
PR1, PR2	Pathogenesis-related 1, pathogenesis-related 2	SA pathway	AMF	*Glomus* sp.	Leaf infection index decreased significantly.	Potato- *Solanum tuberosum*	Pathogen- *Phytophthora infestans*	Late blight	Gallou et al., 2011
POX381	Peroxidase	SA pathway	AMF	*Funneliformis mosseae*	*B. graminis* infection on leaves reduced to 78%.	Wheat- *Triticum* sp.	Fungus- *Blumeria graminis f.* sp. *Tritici*	Powdery mildew	Mustafa et al., 2017
PAL	Phenylalanine ammonia lyase								
CHI1	Chitinase 1								
NPR1	Non-expressor of pathogenesis-related proteins 1								
PAL	Phenylalanine ammonia lyase	JA pathway	AMF	*Glomus Macrocarpum; Glomus Fasciculatum*	*F. oxysporum* disease severity reduced to ∼ 75%	Tomato- *Solanum lycopersicum*	Fungus- *Fusarium Oxysporum f.* sp. *Lycopersici*	Fusarium wilt	Kapoor, 2008
LOX	Lipoxygenase	JA pathway	AMF	*Glomus fasciculatum*	Significant decrease in the severity of fusarium wilt disease.	Tomato- *Solanum lycopersicum*	Fungus- *Fusarium Oxysporum f.* sp. *Lycopersici*	Fusarium wilt	Nair et al., 2015

(Continued)

TABLE 2 | Continued

Gene name	Gene function	Proposed defense pathway	Mycorrhiza Type	Mycorrhiza species	Effects of mycorrhiza	Plant host	Resistance against	Disease/Effect	References
LOX	Lipoxygenase	JA pathway	AMF	*Glomus fasciculatum*	Decrease in disease severity of *A. alternata*	Tomato- *Solanum lycopersicum*	Pathogen-*Alternaria alternata*	Fruit rot	Nair et al., 2015
OPR3	12-oxophytodienoate reductase 3								
COI1	Coronatine-insensitive1								
PR1, PR2, PR3	Pathogenesis related1, Pathogenesis related 2, Pathogenesis related 3	JA and SA pathway	AMF	*Funneliformis mosseae*	Disease index of *A. solani* reduced by 54.3%	Tomato- *Solanum lycopersicum*	Pathogen- *Alternaria solani*	Early blight	Song et al., 2015
LOX	Lipoxygenase								
AOC	Allene oxide cyclase								
PAL	Phenylalanine ammonia-lyase								
LOXD	Lipoxygenase D	JA pathway	AMF	*Glomus mosseae*	62.3% less weight gain of *H. arimegera* larvae	Solanum lycopersicum Mill.	Insect- *Helicoverpa arimigera*	Herbivory	Song et al., 2013
AOC	Allene oxide cyclase								
PI-I and PI-II	Serine protease inhibitors I and II								
AOS1	Allene oxide synthase 1	JA pathway, phenylpropanoid pathway and protease inhibitor activity	AMF	*Rhizophagus irregularis*	Larvae weigh ∼40 mg which is significantly lower than the control (after 8 days).	Potato- *Solanum tuberosum*	Insect- *Trichoplusia ni*	Herbivory	Schoenherr et al., 2019
OPR3	12-oxo-phytodienoate reductase 3								
PI-I	Protease inhibitor type								
PAL	Phenylalanine ammonia lyase								

(Continued)

TABLE 2 | Continued

Gene name	Gene function	Proposed defense pathway	Mycorrhiza Type	Mycorrhiza species	Effects of mycorrhiza	Plant host	Resistance against	Disease/Effect	References
JAZ(JAR1, JAR8)	Jasmonate zim domain 1	JA pathway	EMF	*Laccaria bicolor*	Significant less oviposition by beetles on mycorrhizal host plant.	Poplar- *Populus* × *canescens*	Insect- *Chrysomela populi*	Herbivory	Kaling et al., 2018
MYB (MYB4, MYB5, MYB14 and MYB108)	Transcription factors of JA								
NAS3	Nicotianamine synthase								
KPI	Kunitz protease inhibitors								
CHI	Chitinases								
CERK1	Chitin receptor	Both JA and SA pathway	EMF	*Laccaria bicolor*	27% reduction in larval weight on *L. bicolor* colonized host plant.	Arabidopsis	Insect- *Trichoplusia ni*	Herbivory	Vishwanathan et al., 2020
PR1, PR2 and PR5	Pathogenesis related 1, Pathogenesis related 2, Pathogenesis related 5	SA pathway	Endophyte	*Piriformospora indica*	33—59% reduction in colony numbers of *B. graminis*	Barley- *Hordeum vulgare*	Fungus- *Blumeria graminis f.* sp. *Hordei*	Powdery mildew	Molitor et al., 2011
Hsp70, Hsp17.9	Heat shock proteins70; Heat shock proteins17.9								
BCI-7	Barley chemically induced 7								
PR1, PR5	Pathogenesis-related 1, Pathogenesis-related 5	JA and SA pathway	Endophyte	*Piriformospora indica*	∼ 50% reduction in the number of conidia of *G. orontii* formed per mycelium.	Arabidopsis	Fungus- *Golovinomyces orontii*	Powdery mildew	Stein et al., 2008
ERF1	Ethylene response factor 1								
PDF1.2	Plant defensin 1.2								
VSP	Vegetative storage protein								

Symbiotic relationships between mycorrhiza and plants were reviewed with regard to the defense induction by mycorrhiza. Genes were grouped upon their function as well as their effect on the disease of pathogens on their hosts.

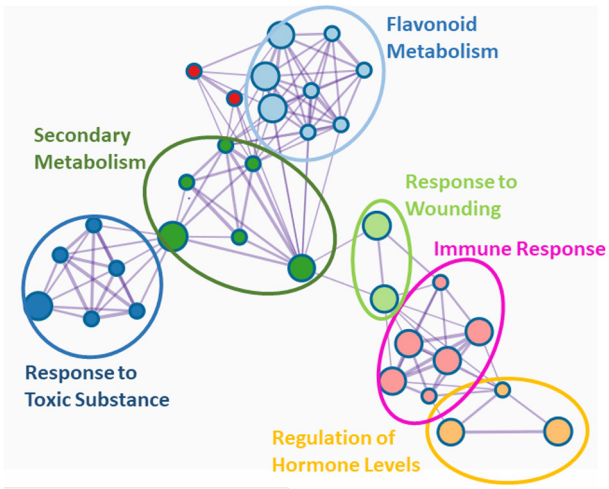

FIGURE 2 | Network of GO terms in systemic leaves after ectomycorrhizal colonization of poplar. Data of differentially expressed genes (DEGs) between mycorrhizal and non-mycorrhizal trees were taken from the Supplemental Table S1 in Kaling et al. (2018). It should be noted that many DEGS were down regulated. The best matches of Arabidopsis orthologs of the poplar genes were uploaded and analyzed in Metascape (Zhou et al., 2019). Significant GO terms ($P_{adjusted} < 0.05$) are shown.

EMF and mycorrhizal helper bacteria, which might be able to boost plant tolerance by growth stimulation (Labbé et al., 2014; Zhao et al., 2014).

Mycorrhiza Induced Resistance in Systemic Tissues—Preparing the Weapons

In practical terms, the production of defense enzymes and defense metabolites including VOCs are important for enhanced resistance. Enzymes such as peroxidases (PRX), polyphenol oxidases, and laccases and their substrates (phenolic compounds) are important to strengthen the cell wall, thereby, erecting barriers against the spreading of pathogens (Carroll and Hoffman, 1980; Darvill and Albersheim, 1984; Baldwin, 1988). Other enzymes (defensins, chitinases, etc.) have antibiotic activities by attenuating pathogens' growth (Freeman and Beattie, 2008; War et al., 2012). In poplar colonized by *L. bicolor* the transcript levels of putative chitinases (Kaling et al., 2018) and in *Eucalyptus* colonized by *Pisolithus tinctorius* the activity of chitinases were increased in systemic leaves (Albrecht et al., 1994c; **Figure 1**). Chitinases hydrolyze glycosidic bonds of chitin, a constituent of the insect exoskeletons and thereby, affect the fitness of herbivores or pathogenic fungi.

An important class of proteins acting as a biocidal compounds against insect-herbivores are the protease inhibitors (PIs) (Conconi et al., 1996; Lawrence and Koundal, 2002; Kim et al., 2009; Dunse et al., 2010). Proteases are vital gut enzymes of insects. PIs disturb the activity of proteases, thus, reducing the overall fitness of herbivorous insects (Zhu-Salzman and Zeng, 2015). PIs have also antimicrobial activities inhibiting the physiological development of pathogens (Jashni et al., 2015). EMF colonization of poplar results in upregulated transcription of Kunitz Trypsin Inhibitors (KTI, a class of PIs) and is accompanied by negative consequences for oviposition (Kaling et al., 2018). AMF colonization of crop plants (potato, tomato) affects *PI* expression, leads to reduced diet quality for larvae of *Trichoplusia ni* and *Helicoverpa armigera,* and reduced growth of the caterpillars (Song et al., 2013; Schoenherr et al., 2019). Therefore, we speculate that PIs are part of the systemically induced defense, irrespective of the mycorrhizal type.

Enzymes commonly induced for biotic defense and involved in MIR are the LOXs (lipoxygenases) (Feussner and Wasternack, 2002; Kawano, 2003; La Camera et al., 2004; Shah, 2005; Baysal and Demirdöven, 2007). LOXs catalyze the hydroperoxidation of polyunsaturated fatty acids (Rosahl, 1996). The resulting hydroperoxides are used as substrates by AOS activating JA-based defenses or by hydroperoxide lyase stimulating "volatile phytoalexins" production (Bate and Rothstein, 1998; Wasternack, 2007; Bruinsma et al., 2009; Lyons et al., 2013; Zhou et al., 2014). In AMF colonized tomato plants upregulation of LOX is associated with defense responses against fungal pathogens (*Alternaria solani*, *Alternaria alternata*, *Fusarium oxysporum*) and cotton bollworm (Song et al., 2013, 2015; Nair et al., 2015).

EMF colonization of roots does not only trigger defense proteins but also results in changes of the leaf metabolome (Pfabel et al., 2012; Cameron et al., 2013; Adolfsson et al., 2017; Hill et al., 2018; Kaling et al., 2018). The compounds mainly involved in enhancing plant tolerance or resistance can be chemically categorized as terpenes, phenolic compounds, nitrogenous, and sulfurous compounds (Mazid et al., 2011; Pedone-Bonfim et al., 2015; Wink, 2018). The terpenes and terpenoids comprise a large class of plant metabolites. Many of these compounds are VOCs, which increase drastically in response to herbivory ("Herbivore-Induced Plant Volatiles", Pieterse et al., 2014). VOCs act as repellents for herbivores or as attractants to other arthropods that prey upon or parasitize herbivores (Loreto and Schnitzler, 2010). These ecologically important VOCs are produced by the plant down-stream the JA signaling pathway (Ament et al., 2004; van Schie et al., 2007). Since a role of VOCs for plant-insect interactions has often been reviewed (Holopainen and Gershenzon, 2010; Bouwmeester et al., 2019), we illustrate this area just by few selected examples: β-ocimene (monoterpene) and β-caryophyllene (sesquiterpene) emissions are enhanced by AMF-colonized bean plants and recruit natural predators of spider mites (Schausberger et al., 2012). In tomato, AMF colonization enhanced terpene levels and defenses against larvae of the beet armyworm (Shrivastava et al., 2015).

Phenolic compounds are part of the plant defense arsenal and often higher in EMF than in non-mycorrhizal plants (Gange and West, 1994; Baum et al., 2009; Fontana et al., 2009; Schweiger et al., 2014). While phenol-based compounds enhance antibiosis, e.g., against rust (Pfabel et al., 2012), they are not effective against adapted herbivores such as lepidopteran species feeding on Salicaceae (Lindroth and St. Clair, 2013; Boeckler et al., 2014). For example, poplar leaf beetle (*Chrysomela populii*) prefers phenolic-rich leaves (Behnke et al., 2010). Therefore, transcriptional down-regulation of enzymes required for production of secondary compounds (e.g., tannins,

flavonoids, phenolic glycosides, proanthocyanidin dimers, and trimers) in EMF-colonized poplar and upregulation of aldoxime production suggests that MIR triggers a metabolic shift from carbon-based to N-based defense (Kaling et al., 2018). Aldoximes and other nitrile-derived compounds are very effective herbivore repellents (Irmisch et al., 2013, 2014; McCormick et al., 2014). The changes induced in systemic tissues by EMF are often subtle or unfold only after biotic attack. Therefore, it will be important to enhance research with a wider range of model systems such as poplar, oak, and conifers, etc., that are amenable to functional studies by transgenic approaches and can be handled under controlled conditions.

CONCLUSION

The field of EMF-induced systemic resistance is still scattered but putting the puzzle pieces together, a picture is starting to emerge. EMF in contact with local tissue (roots) activate systemic induced resistance via chitin receptors in Arabidopsis. Since Arabidopsis is a non-host for any mycorrhizal interaction, it will be important to show whether chitin also plays a fundamental role in triggering MIR in EMF-host interactions. EMF-host interactions often positively influence resistance against biotrophic pathogens and herbivory in leaves. The nature of long-distance signaling from roots to leaves remains elusive. Besides vascular routes, aerial transmission via VOCs cannot be excluded (Ditengou et al., 2015). In addition to other effects, systemic leaves show suppressed expression of JAZ transcription factors, whereby transcription of defense proteins and enzymes for the production of defense metabolites is set on. Collectively, studies on AMF or EMF inoculated plants point to activation of JA-related pathways. Still, the recruitment of SA-related defense cannot be dismissed because an involvement of NPR1 and NPR3/4 (positive and negative regulators of SA) has been shown for EMF-induced systemic resistance. The defense responses are versatile. Most of our current knowledge on EMF-activated defenses stems from poplar. Since poplars can be colonized by both EMF and AMF (Khasa et al., 2002; Liu et al., 2015), an increased understanding of MIR requires comparative studies of AMF- and EMF-induced systemic resistance in this host species as well as additional investigations with tree species that can only be colonized by EMF. Since different tree species exhibit a vast range of secondary compounds, there is much work ahead to better understand pathways, which stimulate tree-specific defenses. This is an important task for the future. Since climate change is affecting plant-pest interactions for the worse (Linnakoski et al., 2019), more insights into resistance mechanisms are urgently needed to guide tree selection and breeding for stable future forests.

AUTHOR CONTRIBUTIONS

AP conceived the study, supervised writing, and revised the manuscript. SD led the writing. ID, KK, and MJ contributed sections to the manuscript. All authors read and approved the final submission.

FUNDING

The work in AP's laboratory is funded by German Science Foundation (DFG). SD, ID, and KK were supported in the framework of the International Research Training Group "PRoTECT" (IRTG 2172, M1 and M2.2) and MJ by project Poplar Communication (PO362/20-2).

ACKNOWLEDGMENTS

We are grateful to our laboratory technicians for permanent excellent support.

REFERENCES

Adolfsson, L., Nziengui, H., Abreu, I. N., Šimura, J., Beebo, A., Herdean, A., et al. (2017). Enhanced secondary- and hormone metabolism in leaves of arbuscular mycorrhizal *Medicago truncatula*. *Plant Physiol.* 175, 392–411. doi: 10.1104/pp.16.01509

Albrecht, C., Asselin, A., Piché, Y., and Lapeyrie, F. (1994a). Chitinase activities are induced in Eucalyptus globulus roots by ectomycorrhizal or pathogenic fungi, during early colonization. *Physiol. Plant.* 91, 104–110. doi: 10.1111/j.1399-3054.1994.tb00665.x

Albrecht, C., Burgess, T., Dell, B., and Lapeyrie, F. (1994b). Chitinase and peroxidase activities are induced in eucalyptus roots according to aggressiveness of Australian ectomycorrhizal strains of Pisolithus sp. *New Phytol.* 127, 217–222. doi: 10.1111/j.1469-8137.1994.tb04273.x

Albrecht, C., Laurent, P., and Lapeyrie, F. (1994c). Eucalyptus root and shoot chitinases, induced following root colonization by pathogenic versus ectomycorrhizal fungi, compared on one- and two-dimensional activity gels. *Plant Sci.* 100, 157–164. doi: 10.1016/0168-9452(94)90071-X

Ament, K., Kant, M. R., Sabelis, M. W., Haring, M. A., and Schuurink, R. C. (2004). Jasmonic acid is a key regulator of spider mite-induced volatile terpenoid and methyl salicylate emission in tomato. *Plant Physiol.* 135, 2025–2037. doi: 10.1104/pp.104.048694

Ament, K., Krasikov, V., Allmann, S., Rep, M., Takken, F. L. W., and Schuurink, R. C. (2010). Methyl salicylate production in tomato affects biotic interactions. *Plant J.* 62, 124–134. doi: 10.1111/j.1365-313X.2010.04132.x

Bacht, M., Tarkka, M. T., López, I. F., Bönn, M., Brandl, R., Buscot, F., et al. (2019). Tree response to herbivory is affected by endogenous rhythmic growth and attenuated by cotreatment with a mycorrhizal fungus. *Mol. Plant Microbe Interect* 32, 770–781. doi: 10.1094/MPMI-10-18-0290-R

Baldwin, I. T. (1988). Short-term damage-induced increases in tobacco alkaloids protect plants. *Oecologia* 75, 367–370. doi: 10.1007/BF00376939

Barea, J. M., and Jeffries, P. (1995). "Arbuscular mycorrhizas in sustainable soil-plant systems," in *Mycorrhiza*, eds A. Varma and B. Hock (Berlin: Springer), 521–560. doi: 10.1007/978-3-662-08897-5_23

Barsoum, M., Sabelleck, B., Spanu, P. D., and Panstruga, R. (2019). Rumble in the effector jungle: candidate effector proteins in interactions of plants with powdery mildew and rust fungi. *Crit. Rev. Plant. Sci.* 38, 255–279. doi: 10.1080/07352689.2019.1653514

Bate, N. J., and Rothstein, S. J. (1998). C6-volatiles derived from the lipoxygenase pathway induce a subset of defense-related genes. *Plant J.* 16, 561–569. doi: 10.1046/j.1365-313x.1998.00324.x

Baum, C., Toljander, Y. K., Eckhardt, K.-U., and Weih, M. (2009). The significance of host-fungus combinations in ectomycorrhizal symbioses for the chemical

quality of willow foliage. *Plant Soil* 323, 213–224. doi: 10.1007/s11104-009-9928-x

Baysal, T., and Demirdöven, A. (2007). Lipoxygenase in fruits and vegetables: a review. *Enzyme Microb. Technol.* 40, 491–496. doi: 10.1016/j.enzmictec.2006.11.025

Behnke, K., Kaiser, A., Zimmer, I., Brüggemann, N., Janz, D., Polle, A., et al. (2010). RNAi-mediated suppression of isoprene emission in poplar transiently impacts phenolic metabolism under high temperature and high light intensities: a transcriptomic and metabolomic analysis. *Plant. Mol. Biol.* 74, 61–75. doi: 10.1007/s11103-010-9654-z

Boeckler, G. A., Towns, M., Unsicker, S. B., Mellway, R. D., Yip, L., Hilke, I., et al. (2014). Transgenic upregulation of the condensed tannin pathway in poplar leads to a dramatic shift in leaf palatability for two tree-feeding Lepidoptera. *J. Chem. Ecol.* 40, 150–158. doi: 10.1007/s10886-014-0383-7

Boller, T., and Felix, G. (2009). A renaissance of elicitors: perception of microbe-associated molecular patterns and danger signals by pattern-recognition receptors. *Annu. Rev. Plant Biol.* 60, 379–406. doi: 10.1146/annurev.arplant.57.032905.105346

Bonfante, P., and Anca, I.-A. (2009). Plants, mycorrhizal fungi, and bacteria: a network of interactions. *Annu. Rev. Microbiol.* 63, 363–383. doi: 10.1146/annurev.micro.091208.073504

Bouffaud, M.-L., Herrmann, S., Tarkka, M. T., Bönn, M., Feldhahn, L., and Buscot, F. (2020). Oak displays common local but specific distant gene regulation responses to different mycorrhizal fungi. *BMC Genomics* 21:399. doi: 10.1186/s12864-020-06806-5

Bouwmeester, H., Schuurink, R. C., Bleeker, P. M., and Schiestl, F. (2019). The role of volatiles in plant communication. *Plant J.* 100, 892–907. doi: 10.1111/tpj.14496

Brodersen, P., Malinovsky, F. G., Hématy, K., Newman, M.-A., and Mundy, J. (2005). The role of salicylic acid in the induction of cell death in Arabidopsis acd11. *Plant Physiol.* 138, 1037–1045. doi: 10.1104/pp.105.059303

Bruinsma, M., Posthumus, M. A., Mumm, R., Mueller, M. J., van Loon, J. J. A., and Dicke, M. (2009). Jasmonic acid-induced volatiles of Brassica oleracea attract parasitoids: effects of time and dose, and comparison with induction by herbivores. *J. Exp. Bot.* 60, 2575–2587. doi: 10.1093/jxb/erp101

Brundrett, M. C. (2009). Mycorrhizal associations and other means of nutrition of vascular plants: understanding the global diversity of host plants by resolving conflicting information and developing reliable means of diagnosis. *Plant Soil* 320, 37–77. doi: 10.1007/s11104-008-9877-9

Brundrett, M. C., and Tedersoo, L. (2018). Evolutionary history of mycorrhizal symbioses and global host plant diversity. *New Phytol.* 220, 1108–1115. doi: 10.1111/nph.14976

Cameron, D. D., Neal, A. L., van Wees, S. C. M., 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.2013.06.004

Campos-Soriano, L., García-Martínez, J., and Segundo, B. S. (2012). The arbuscular mycorrhizal symbiosis promotes the systemic induction of regulatory defence-related genes in rice leaves and confers resistance to pathogen infection. *Mol. Plant Pathol.* 13, 579–592. doi: 10.1111/j.1364-3703.2011.00773.x

Carroll, C. R., and Hoffman, C. A. (1980). Chemical feeding deterrent mobilized in response to insect herbivory and counteradaptation by Epilachna tredecimnotata. *Science* 209, 414–416. doi: 10.1126/science.209.4454.414

Champigny, M. J., Isaacs, M., Carella, P., Faubert, J., Fobert, P. R., and Cameron, R. K. (2013). Long distance movement of DIR1 and investigation of the role of DIR1-like during systemic acquired resistance in Arabidopsis. *Front. Plant Sci.* 4:230. doi: 10.3389/fpls.2013.00230

Chanda, B., Xia, Y., Mandal, M. K., Yu, K., Sekine, K.-T., Gao, Q., et al. (2011). Glycerol-3-phosphate is a critical mobile inducer of systemic immunity in plants. *Nat. Genet.* 43, 421–427. doi: 10.1038/ng.798

Chaturvedi, R., Venables, B., Petros, R. A., Nalam, V., Li, M., Wang, X., et al. (2012). An abietane diterpenoid is a potent activator of systemic acquired resistance. *Plant J.* 71, 161–172. doi: 10.1111/j.1365-313X.2012.04981.x

Chen, Y.-C., Holmes, E. C., Rajniak, J., Kim, J.-G., Tang, S., Fischer, C. R., et al. (2018). N -hydroxy-pipecolic acid is a mobile metabolite that induces systemic disease resistance in Arabidopsis. *Proc. Natl. Acad. Sci. U.S.A.* 115, E4920–E4929. doi: 10.1073/pnas.1805291115

Conconi, A., Smerdon, M. J., Howe, G. A., and Ryan, C. A. (1996). The octadecanoid signalling pathway in plants mediates a response to ultraviolet radiation. *Nature* 383, 826–829. doi: 10.1038/383826a0

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

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

Cope, K. R., Bascaules, A., Irving, T. B., Venkateshwaran, M., Maeda, J., Garcia, K., et al. (2019). The ectomycorrhizal fungus Laccaria bicolor produces lipochitooligosaccharides and uses the common symbiosis Pathway to colonize Populus roots. *Plant Cell* 31, 2386–2410. doi: 10.1105/tpc.18.00676

Cordier, C., Pozo, M. J., Barea, J. M., Gianinazzi, S., and Gianinazzi-Pearson, V. (1998). Cell defense responses associated with localized and systemic resistance to Phytophthora parasitica induced in tomato by an arbuscular mycorrhizal fungus. *Mol. Plant Microbe Interact.* 11, 1017–1028. doi: 10.1094/MPMI.1998.11.10.1017

Darvill, A. G., and Albersheim, P. (1984). Phytoalexins and their elicitors-a defense against microbial infection in plants. *Annu. Rev. Plant Physiol.* 35, 243–275. doi: 10.1146/annurev.pp.35.060184.001331

de Freitas Pereira, M., Veneault-Fourrey, C., Vion, P., Guinet, F., Morin, E., Barry, K. W., et al. (2018). secretome analysis from the ectomycorrhizal ascomycete Cenococcum geophilum. *Front. Microbiol.* 9:141. doi: 10.3389/fmicb.2018.00141

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

Ding, Y., Sun, T., Ao, K., Peng, Y., Zhang, Y., Li, X., et al. (2018). Opposite roles of salicylic acid receptors NPR1 and NPR3/NPR4 in transcriptional regulation of plant Immunity. *Cell* 173, 1454–1467. doi: 10.1016/j.cell.2018.03.044

Ditengou, F. A., Müller, A., Rosenkranz, M., Felten, J., Lasok, H., Doorn, M. M. V., et al. (2015). Volatile signalling by sesquiterpenes from ectomycorrhizal fungi reprogrammes root architecture. *Nat. Commun.* 6:6279. doi: 10.1038/ncomms7279

Dixon, R. A., Harrison, M. J., and Lamb, C. J. (1994). Early events in the activation of plant defense responses. *Annu. Rev. Phytopathol.* 32, 479–501. doi: 10.1146/annurev.py.32.090194.002403

Doré, J., Perraud, M., Dieryckx, C., Kohler, A., Morin, E., Henrissat, B., et al. (2015). Comparative genomics, proteomics and transcriptomics give new insight into the exoproteome of the basidiomycete Hebeloma cylindrosporum and its involvement in ectomycorrhizal symbiosis. *New Phytol.* 208, 1169–1187. doi: 10.1111/nph.13546

Dunse, K. M., Stevens, J. A., Lay, F. T., Gaspar, Y. M., Heath, R. L., and Anderson, M. A. (2010). Coexpression of potato type I and II proteinase inhibitors gives cotton plants protection against insect damage in the field. *Proc. Natl. Acad. Sci. U.S.A.* 107, 15011–15015. doi: 10.1073/pnas.1009241107

Duplessis, S., Courty, P.-E., Tagu, D., and Martin, F. (2005). Transcript patterns associated with ectomycorrhiza development in *Eucalyptus globulus* and *Pisolithus microcarpus*. *New Phytol.* 165, 599–611. doi: 10.1111/j.1469-8137.2004.01248.x

Durrant, W. E., and Dong, X. (2004). Systemic acquired resistance. *Annu. Rev. Phytopathol.* 42, 185–209. doi: 10.1146/annurev.phyto.42.040803.140421

El Hadrami, A., Adam, L. R., El Hadrami, I., and Daayf, F. (2010). Chitosan in plant protection. *Mar. Drugs* 8, 968–987. doi: 10.3390/md8040968

Felten, J., Kohler, A., Morin, E., Bhalerao, R. P., Palme, K., Martin, F., et al. (2009). The ectomycorrhizal fungus Laccaria bicolor stimulates lateral root formation in poplar and Arabidopsis through Auxin transport and signaling. *Plant Physiol.* 151, 1991–2005. doi: 10.1104/pp.109.147231

Feussner, I., and Wasternack, C. (2002). The lipoxygenase pathway. *Annu. Rev. Plant Biol.* 53, 275–297. doi: 10.1146/annurev.arplant.53.100301.135248

Fontana, A., Reichelt, M., Hempel, S., Gershenzon, J., and Unsicker, S. B. (2009). The effects of arbuscular mycorrhizal fungi on direct and indirect defense metabolites of Plantago lanceolata L. *J. Chem. Ecol.* 35, 833–843. doi: 10.1007/s10886-009-9654-0

Franken, P., and Gnädinger, F. (1994). Analysis of parsley arbuscular endomycorrhiza: infection development and mRNA levels of defense-related genes. *Mol. Plant Microbe Interact.* 7, 612–620. doi: 10.1094/mpmi-7-0612

Freeman, B., and Beattie, G. (2008). An overview of plant defenses against pathogens and herbivores. *Plant Health Instruct.* 149, 1–12. doi: 10.1094/PHI-I-2008-0226-01

Frettinger, P., Derory, J., Herrmann, S., Plomion, C., Lapeyrie, F., Oelmüller, R., et al. (2007). Transcriptional changes in two types of pre-mycorrhizal roots and in ectomycorrhizas of oak microcuttings inoculated with Piloderma croceum. *Planta* 225, 331–340. doi: 10.1007/s00425-006-0355-4

Gallou, A., Lucero Mosquera, H. P., Cranenbrouck, S., Suárez, J. P., and Declerck, S. (2011). Mycorrhiza induced resistance in potato plantlets challenged by Phytophthora infestans. *Physiol. Mol. Plant Pathol.* 76, 20–26. doi: 10.1016/j.pmpp.2011.06.005

Gange, A. C., Gane, D. R. J., Chen, Y., and Gong, M. (2005). Dual colonization of Eucalyptus urophylla S.T. Blake by arbuscular and ectomycorrhizal fungi affects levels of insect herbivore attack. *Agric. For. Entomol.* 7, 253–263. doi: 10.1111/j.1461-9555.2005.00268.x

Gange, A. C., and West, H. M. (1994). Interactions between arbuscular mycorrhizal fungi and foliar-feeding insects in *Plantago lanceolata* L. *New Phytol.* 128, 79–87. doi: 10.1111/j.1469-8137.1994.tb03989.x

Garcia, K., Delaux, P.-M., Cope, K. R., and Ané, J.-M. (2015). Molecular signals required for the establishment and maintenance of ectomycorrhizal symbioses. *New Phytol.* 208, 79–87. doi: 10.1111/nph.13423

García-Garrido, J. M., and Ocampo, J. A. (2002). Regulation of the plant defence response in arbuscular mycorrhizal symbiosis. *J. Exp. Bot.* 53, 1377–1386. doi: 10.1093/jexbot/53.373.1377

Genre, A., Lanfranco, L., Perotto, S., and Bonfante, P. (2020). Unique and common traits in mycorrhizal symbioses. *Nat. Rev. Microbiol.* 18, 649–660. doi: 10.1038/s41579-020-0402-3

Glazebrook, J. (2005). Contrasting mechanisms of defense against biotrophic and necrotrophic pathogens. *Annu. Rev. Phytopathol.* 43, 205–227. doi: 10.1146/annurev.phyto.43.040204.135923

Godard, K.-A., White, R., and Bohlmann, J. (2008). Monoterpene-induced molecular responses in *Arabidopsis thaliana*. *Phytochemistry* 69, 1838–1849. doi: 10.1016/j.phytochem.2008.02.011

Gong, B.-Q., Wang, F.-Z., and Li, J.-F. (2020). Hide-and-seek: chitin-triggered plant immunity and fungal counterstrategies. *Trends Plant Sci.* 25, 805–816. doi: 10.1016/j.tplants.2020.03.006

Goossens, J., Fernández-Calvo, P., Schweizer, F., and Goossens, A. (2016). Jasmonates: signal transduction components and their roles in environmental stress responses. *Plant Mol. Biol.* 91, 673–689. doi: 10.1007/s11103-016-0480-9

Halldórsson, G., Sverrisson, H., Eyjólfsdóttir, G. G., and Oddsdóttir, E. S. (2000). Ectomycorrhizae reduce damage to russian larch by Otiorhyncus larvae. *Scand. J. For. Res.* 15, 354–358. doi: 10.1080/028275800447986

Hammerbacher, A., Coutinho, T. A., and Gershenzon, J. (2019). Roles of plant volatiles in defence against microbial pathogens and microbial exploitation of volatiles. *Plant Cell Environ.* 42, 2827–2843. doi: 10.1111/pce.13602

Hammond-Kosack, K. E., and Jones, J. D. G. (1997). Plant disease resistance genes. *Physiol. Mol. Biol. Plants* 48, 575–607. doi: 10.1146/annurev.arplant.48.1.575

Haney, C. H., Wiesmann, C. L., Shapiro, L. R., Melnyk, R. A., O'Sullivan, L. R., Khorasani, S., et al. (2018). Rhizosphere-associated *Pseudomonas* induce systemic resistance to herbivores at the cost of susceptibility to bacterial pathogens. *Mol. Ecol.* 27, 1833–1847. doi: 10.1111/mec.14400

Hao, Z., Fayolle, L., van Tuinen, D., Chatagnier, O., Li, X., Gianinazzi, S., et al. (2012). Local and systemic mycorrhiza-induced protection against the ectoparasitic nematode Xiphinema index involves priming of defence gene responses in grapevine. *J. Exp. Bot.* 63, 3657–3672. doi: 10.1093/jxb/ers046

Hartmann, M., Zeier, T., Bernsdorff, F., Reichel-Deland, V., Kim, D., Hohmann, M., et al. (2018). Flavin monooxygenase-generated N-hydroxypipecolic acid is a critical element of plant systemic immunity. *Cell* 173, 456.e16–469.e16. doi: 10.1016/j.cell.2018.02.049

Heller, G., Adomas, A., Li, G., Osborne, J., van Zyl, L., Sederoff, R., et al. (2008). Transcriptional analysis of *Pinus sylvestris* roots challenged with the ectomycorrhizal fungus Laccaria bicolor. *BMC Plant Biol.* 8:19. doi: 10.1186/1471-2229-8-19

Hill, E. M., Robinson, L. A., Abdul-Sada, A., Vanbergen, A. J., Hodge, A., and Hartley, S. E. (2018). Arbuscular mycorrhizal fungi and plant chemical defence: effects of colonisation on aboveground and belowground metabolomes. *J. Chem. Ecol.* 44, 198–208. doi: 10.1007/s10886-017-0921-1

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

Howe, G. A., Major, I. T., and Koo, A. J. (2018). Modularity in jasmonate signaling for multistress resilience. *Annu. Rev. Plant Biol.* 69, 387–415. doi: 10.1146/annurev-arplant-042817-040047

Huang, M., Sanchez-Moreiras, A. M., Abel, C., Sohrabi, R., Lee, S., Gershenzon, J., et al. (2012). The major volatile organic compound emitted from *Arabidopsis thaliana* flowers, the sesquiterpene (E)-β-caryophyllene, is a defense against a bacterial pathogen. *New Phytol.* 193, 997–1008. doi: 10.1111/j.1469-8137.2011.04001.x

Irmisch, S., McCormick, A. C., Boeckler, G. A., Schmidt, A., Reichelt, M., Schneider, B., et al. (2013). Two herbivore-induced cytochrome P450 Enzymes CYP79D6 and CYP79D7 catalyze the formation of volatile aldoximes involved in poplar defense. *Plant Cell* 25, 4737–4754. doi: 10.1105/tpc.113.118265

Irmisch, S., McCormick, A. C., Günther, J., Schmidt, A., Boeckler, G. A., Gershenzon, J., et al. (2014). Herbivore-induced poplar cytochrome P450 enzymes of the CYP71 family convert aldoximes to nitriles which repel a generalist caterpillar. *Plant J.* 80, 1095–1107. doi: 10.1111/tpj.12711

Isaacs, M., Carella, P., Faubert, J., Champigny, M. J., Rose, J. K. C., and Cameron, R. K. (2016). Orthology analysis and in vivo complementation studies to elucidate the role of DIR1 during systemic acquired resistance in *Arabidopsis thaliana* and *Cucumis sativus*. *Front. Plant Sci.* 7:627. doi: 10.3389/fpls.2016.00566

Jashni, M. K., Mehrabi, R., Collemare, J., Mesarich, C. H., and de Wit, P. J. G. M. (2015). The battle in the apoplast: further insights into the roles of proteases and their inhibitors in plant–pathogen interactions. *Front. Plant Sci.* 6:584. doi: 10.3389/fpls.2015.00584

Johansson, T., Le Quéré, A., Ahren, D., Söderström, B., Erlandsson, R., Lundeberg, J., et al. (2004). Transcriptional responses of *Paxillus involutus* and *Betula pendula* during formation of ectomycorrhizal root tissue. *Mol. Plant Microbe Interact.* 17, 202–215. doi: 10.1094/MPMI.2004.17.2.202

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

Jung, H. W., Tschaplinski, T. J., Wang, L., Glazebrook, J., and Greenberg, J. T. (2009). Priming in systemic plant immunity. *Science* 324, 89–91. doi: 10.1126/science.1170025

Jung, S. C., Martinez-Medina, A., Lopez-Raez, J. A., and Pozo, M. J. (2012). Mycorrhiza-induced resistance and priming of plant defenses. *J. Chem. Ecol.* 38, 651–664. doi: 10.1007/s10886-012-0134-6

Kaling, M., Schmidt, A., Moritz, F., Rosenkranz, M., Witting, M., Kasper, K., et al. (2018). Mycorrhiza-triggered transcriptomic and metabolomic networks impinge on herbivore fitness. *Plant Physiol.* 176, 2639–2656. doi: 10.1104/pp.17.01810

Kang, H., Chen, X., Kemppainen, M., Pardo, A. G., Veneault-Fourrey, C., Kohler, A., et al. (2020). The small secreted effector protein MiSSP7.6 *of Laccaria bicolor* is required for the establishment of ectomycorrhizal symbiosis. *Environ. Microbiol.* 1462-2920, 14959. doi: 10.1111/1462-2920.14959

Kapoor, R. (2008). Induced resistance in mycorrhizal tomato is correlated to concentration of jasmonic acid. *Online J. Biol. Sci.* 8, 49–56. doi: 10.3844/ojbsci.2008.49.56

Kawano, T. (2003). Roles of the reactive oxygen species-generating peroxidase reactions in plant defense and growth induction. *Plant Cell Rep.* 21, 829–837. doi: 10.1007/s00299-003-0591-z

Khasa, P. D., Chakravarty, P., Robertson, A., Thomas, B. R., and Dancik, B. P. (2002). The mycorrhizal status of selected poplar clones introduced in Alberta. *Biomass Bioenergy* 22, 99–104. doi: 10.1016/S0961-9534(01)00072-1

Kim, J.-Y., Park, S.-C., Hwang, I., Cheong, H., Nah, J.-W., Hahm, K.-S., et al. (2009). Protease inhibitors from plants with antimicrobial activity. *Int. J. Mol. Sci.* 10, 2860–2872. doi: 10.3390/ijms10062860

Kloppholz, S., Kuhn, H., and Requena, N. (2011). A secreted fungal effector of Glomus intraradices promotes symbiotic biotrophy. *Curr. Biol.* 21, 1204–1209. doi: 10.1016/j.cub.2011.06.044

Kohler, A., Kuo, A., Nagy, L. G., Morin, E., Barry, K. W., Buscot, F., et al. (2015). Convergent losses of decay mechanisms and rapid turnover of symbiosis genes in mycorrhizal mutualists. *Nat. Genet.* 47, 410–415. doi: 10.1038/ng.3223

Koo, Y. J., Kim, M. A., Kim, E. H., Song, J. T., Jung, C., Moon, J.-K., et al. (2007). Overexpression of salicylic acid carboxyl methyltransferase reduces salicylic

acid-mediated pathogen resistance in *Arabidopsis thaliana*. *Plant. Mol. Biol.* 64, 1–15. doi: 10.1007/s11103-006-9123-x

Kumar, M., and Ashraf, S. (2017). "Role of Trichoderma spp. as a Biocontrol Agent of Fungal Plant Pathogens," in *Probiotics and Plant Health*, eds V. Kumar, M. Kumar, S. Sharma, and R. Prasad (Singapore: Springer), 497–506. doi: 10.1007/978-981-10-3473-2_23

La Camera, S., Gouzerh, G., Dhondt, S., Hoffmann, L., Fritig, B., Legrand, M., et al. (2004). Metabolic reprogramming in plant innate immunity: the contributions of phenylpropanoid and oxylipin pathways. *Immunol. Rev.* 198, 267–284. doi: 10.1111/j.0105-2896.2004.0129.x

Labbé, J. L., Weston, D. J., Dunkirk, N., Pelletier, D. A., and Tuskan, G. A. (2014). Newly identified helper bacteria stimulate ectomycorrhizal formation in Populus. *Front. Plant Sci.* 5:579. doi: 10.3389/fpls.2014.00579

Larcher, W. (1995). *Physiological Plant Ecology: Ecophysiology and Stress Physiology of Functional Groups*. Berlin: Springer, 514.

Lawrence, P. K., and Koundal, K. R. (2002). Plant protease inhibitors in control of phytophagous insects. *Electron. J. Biotechnol.* 5, 93–109.

Le Marquer, M., Bécard, G., and Frei dit Frey, N. (2019). Arbuscular mycorrhizal fungi possess a CLAVATA3/embryo surrounding region-related gene that positively regulates symbiosis. *New Phytol.* 222, 1030–1042. doi: 10.1111/nph.15643

Le Quéré, A., Wright, D. P., Söderström, B., Tunlid, A., and Johansson, T. (2005). Global patterns of gene regulation associated with the development of ectomycorrhiza between birch (*Betula pendula* Roth.) and *Paxillus involutus* (Batsch) Fr. *Mol. Plant Microbe Interact.* 18, 659–673. doi: 10.1094/MPMI-18-0659

Lee, S., Behringer, G., Hung, R., and Bennett, J. (2019). Effects of fungal volatile organic compounds on Arabidopsis thaliana growth and gene expression. *Fungal Ecol.* 37, 1–9. doi: 10.1016/j.funeco.2018.08.004

Li, B., Jiang, S., Yu, X., Cheng, C., Chen, S., Cheng, Y., et al. (2015). Phosphorylation of trihelix transcriptional repressor ASR3 by MAP KINASE4 negatively regulates Arabidopsis immunity. *Plant Cell* 27, 839–856. doi: 10.1105/tpc.114.134809

Li, H.-Y., Yang, G.-D., Shu, H.-R., Yang, Y.-T., Ye, B.-X., Nishida, I., et al. (2006). Colonization by the arbuscular mycorrhizal fungus Glomus versiforme induces a defense response against the root-knot nematode Meloidogyne incognita in the grapevine (*Vitis amurensis* Rupr.), which includes transcriptional activation of the class III chitinase gene VCH3. *Plant Cell Physiol.* 47, 154–163. doi: 10.1093/pcp/pci231

Li, Y., Zhang, W., Dong, H., Liu, Z., Ma, J., and Zhang, X. (2018). Salicylic acid in Populus tomentosa is a remote signalling molecule induced by *Botryosphaeria dothidea* infection. *Sci. Rep.* 8:14059. doi: 10.1038/s41598-018-32204-9

Limpens, E., van Zeijl, A., and Geurts, R. (2015). Lipochitooligosaccharides modulate plant host immunity to enable endosymbioses. *Annu. Rev. Phytopathol.* 53, 311–334. doi: 10.1146/annurev-phyto-080614-120149

Lindroth, R. L., and St. Clair, S. B. (2013). Adaptations of quaking aspen (*Populus tremuloides* Michx.) for defense against herbivores. *Forest. Ecol. Manag* 299, 14–21. doi: 10.1016/j.foreco.2012.11.018

Linnakoski, R., Kasanen, R., Dounavi, A., and Forbes, K. M. (2019). Editorial: forest health under climate change: effects on tree resilience, and pest and pathogen dynamics. *Front. Plant Sci.* 10:1157. doi: 10.3389/fpls.2019.01157

Liu, T., Sheng, M., Wang, C. Y., Chen, H., Li, Z., and Tang, M. (2015). Impact of arbuscular mycorrhizal fungi on the growth, water status, and photosynthesis of hybrid poplar under drought stress and recovery. *Photosynthetica* 53, 250–258. doi: 10.1007/s11099-015-0100-y

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

Luo, Z.-B., Janz, D., Jiang, X., Goebel, C., Wildhagen, H., Tan, Y., et al. (2009). Upgrading toot physiology for stress tolerance by ectomycorrhizas: insights from metabolite and transcriptional profiling into reprogramming for stress anticipation. *Plant Physiol.* 151, 1902–1917. doi: 10.1104/pp.109.143735

Lyons, R., Manners, J. M., and Kazan, K. (2013). Jasmonate biosynthesis and signaling in monocots: a comparative overview. *Plant Cell Rep.* 32, 815–827. doi: 10.1007/s00299-013-1400-y

Maboreke, H. R., Feldhahn, L., Bönn, M., Tarkka, M. T., Buscot, F., Herrmann, S., et al. (2016). Transcriptome analysis in oak uncovers a strong impact of endogenous rhythmic growth on the interaction with plant-parasitic nematodes. *BMC Genomics* 17:627. doi: 10.1186/s12864-016-2992-8

Maillet, F., Poinsot, V., André, O., Puech-Pagès, V., Haouy, A., Gueunier, M., et al. (2011). Fungal lipochitooligosaccharide symbiotic signals in arbuscular mycorrhiza. *Nature* 469, 58–63. doi: 10.1038/nature09622

Maldonado, A. M., Doerner, P., Dixon, R. A., Lamb, C. J., and Cameron, R. K. (2002). A putative lipid transfer protein involved in systemic resistance signalling in Arabidopsis. *Nature* 419, 399–403. doi: 10.1038/nature00962

Manninen, A.-M., Holopainen, T., and Holopainen, J. K. (1998). Susceptibility of ectomycorrhizal and non-mycorrhizal Scots pine (*Pinus sylvestris*) seedlings to a generalist insect herbivore, *Lygus rugulipennis*, at two nitrogen availability levels. *New Phytol.* 140, 55–63. doi: 10.1046/j.1469-8137.1998.00246.x

Martin, F., Kohler, A., Murat, C., Veneault-Fourrey, C., and Hibbett, D. S. (2016). Unearthing the roots of ectomycorrhizal symbioses. *Nat. Rev. Microbiol.* 14, 760–773. doi: 10.1038/nrmicro.2016.149

Martin, F. M., Harrison, M. J., Lennon, S., Lindahl, B., Öpik, M., Polle, A., et al. (2018). Cross-scale integration of mycorrhizal function. *New Phytol.* 220, 941–946. doi: 10.1111/nph.15493

Martinez-Medina, A., Fernandez, 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

Mauch-Mani, B., Baccelli, I., Luna, E., and Flors, V. (2017). Defense priming: an adaptive part of induced resistance. *Annu. Rev. Plant Biol.* 68, 485–512. doi: 10.1146/annurev-arplant-042916-041132

Mazid, M., Khan, T., and Mohammad, F. (2011). Role of secondary metabolites in defense mechanisms of plants. *Biol. Med.* 3, 232–249.

McCormick, A. C., Irmisch, S., Reinecke, A., Boeckler, G. A., Veit, D., Reichelt, M., et al. (2014). Herbivore-induced volatile emission in black poplar: regulation and role in attracting herbivore enemies. *Plant Cell Environ.* 37, 1909–1923. doi: 10.1111/pce.12287

Miya, A., Albert, P., Shinya, T., Desaki, Y., Ichimura, K., Shirasu, K., et al. (2007). CERK1, a LysM receptor kinase, is essential for chitin elicitor signaling in Arabidopsis. *Proc. Natl. Acad. Sci. U.S.A.* 104, 19613–19618. doi: 10.1073/pnas.0705147104

Molitor, A., Zajic, D., Voll, L. M., Pons-Kühnemann, J., Samans, B., Kogel, K.-H., et al. (2011). Barley leaf transcriptome and metabolite analysis reveals new aspects of compatibility and *Piriformospora indica*–mediated systemic induced resistance to powdery mildew. *Mol. Plant Microbe Interact.* 24, 1427–1439. doi: 10.1094/MPMI-06-11-0177

Müller, A., Faubert, P., Hagen, M., zu Castell, W., Polle, A., Schnitzler, J.-P., et al. (2013). Volatile profiles of fungi – Chemotyping of species and ecological functions. *Fungal Genet. Biol.* 54, 25–33. doi: 10.1016/j.fgb.2013.02.005

Mustafa, G., Khong, N. G., Tisserant, B., Randoux, B., Fontaine, J., Magnin-Robert, M., et al. (2017). Defence mechanisms associated with mycorrhiza-induced resistance in wheat against powdery mildew. *Funct. Plant Biol.* 44, 443–454. doi: 10.1071/FP16206

Nair, A., Kolet, S. P., Thulasiram, H. V., and Bhargava, S. (2015). Role of methyl jasmonate in the expression of mycorrhizal induced resistance against Fusarium oxysporum in tomato plants. *Physiol. Mol. Plant. Path.* 92, 139–145. doi: 10.1016/j.pmpp.2015.10.002

Návarová, H., Bernsdorff, F., Döring, A.-C., and Zeier, J. (2012). Pipecolic acid, an endogenous mediator of defense amplification and priming, is a critical regulator of inducible plant immunity. *Plant Cell* 24, 5123–5141. doi: 10.1105/tpc.112.103564

Nehls, U., and Plassard, C. (2018). Nitrogen and phosphate metabolism in ectomycorrhizas. *New Phytol.* 220, 1047–1058. doi: 10.1111/nph.15257

Nerg, A.-M., Kasurinen, A., Holopainen, T., Julkunen-Tiitto, R., Neuvonen, S., and Holopainen, J. K. (2008). The significance of ectomycorrhizas in chemical quality of silver birch foliage and above-ground insect herbivore performance. *J. Chem. Ecol.* 34, 1322–1330. doi: 10.1007/s10886-008-9542-z

Nimchuk, Z., Eulgem, T., Holt, I. I. I. B. F., and Dangl, J. L. (2003). Recognition and response in the plant immune system. *Annu. Rev. Genet.* 37, 579–609. doi: 10.1146/annurev.genet.37.110801.142628

Okamoto, S., Shinohara, H., Mori, T., Matsubayashi, Y., and Kawaguchi, M. (2013). Root-derived CLE glycopeptides control nodulation by direct binding to HAR1 receptor kinase. *Nat. Commun.* 4, 1–7.

Park, S.-W., Kaimoyo, E., Kumar, D., Mosher, S., and Klessig, D. F. (2007). Methyl salicylate is a critical mobile signal for plant systemic acquired resistance. *Science* 318, 113–116. doi: 10.1126/science.1147113

Pedone-Bonfim, M. V. L., da Silva, F. S. B., and Maia, L. C. (2015). Production of secondary metabolites by mycorrhizal plants with medicinal or nutritional potential. *Acta Physiol. Plant.* 37, 27.

Pellegrin, C., Daguerre, Y., Ruytinx, J., Guinet, F., Kemppainen, M., Frey, N. F. D., et al. (2019). Laccaria bicolor MiSSP8 is a small-secreted protein decisive for the establishment of the ectomycorrhizal symbiosis. *Environ. Microbiol.* 21, 3765–3779. doi: 10.1111/1462-2920.14727

Pellegrin, C., Morin, E., Martin, F. M., and Veneault-Fourrey, C. (2015). Comparative analysis of secretomes from ectomycorrhizal fungi with an emphasis on small-secreted proteins. *Front. Microbiol.* 6:1278. doi: 10.3389/fmicb.2015.01278

Pfabel, C., Eckhardt, K.-U., Baum, C., Struck, C., Frey, P., and Weih, M. (2012). Impact of ectomycorrhizal colonization and rust infection on the secondary metabolism of poplar (Populus trichocarpa × deltoides). *Tree Physiol.* 32, 1357–1364. doi: 10.1093/treephys/tps093

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.

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

Plett, J. M., Daguerre, Y., Wittulsky, S., Vayssières, A., Deveau, A., Melton, S. J., et al. (2014). Effector MiSSP7 of the mutualistic fungus Laccaria bicolor stabilizes the Populus JAZ6 protein and represses jasmonic acid (JA) responsive genes. *Proc. Natl. Acad. Sci. U.S.A.* 111, 8299–8304. doi: 10.1073/pnas.1322671111

Plett, J. M., Kemppainen, M., Kale, S. D., Kohler, A., Legué, V., Brun, A., et al. (2011). A secreted effector protein of Laccaria bicolor is required for symbiosis development. *Curr. Biol.* 21, 1197–1203. doi: 10.1016/j.cub.2011.05.033

Pozo, M. J., and Azcón-Aguilar, C. (2007). Unraveling mycorrhiza-induced resistance. *Curr. Opin. Plant Biol.* 10, 393–398. doi: 10.1016/j.pbi.2007.05.004

Pusztahelyi, T. (2018). Chitin and chitin-related compounds in plant–fungal interactions. *Mycology* 9, 189–201. doi: 10.1080/21501203.2018.1473299

Radojičić, A., Li, X., and Zhang, Y. (2018). Salicylic acid: a double-edged sword for programed cell death in plants. *Front. Plant Sci.* 9:1133. doi: 10.3389/fpls.2018.01133

Riedlmeier, M., Ghirardo, A., Wenig, M., Knappe, C., Koch, K., Georgii, E., et al. (2017). Monoterpenes support systemic acquired resistance within and between plants. *Plant Cell* 29, 1440–1459. doi: 10.1105/tpc.16.00898

Rosahl, S. (1996). Lipoxygenases in plants -their role in development and stress response. *Z. Naturforsch. C* 51, 123–138. doi: 10.1515/znc-1996-3-401

Rowen, E., Gutensohn, M., Dudareva, N., and Kaplan, I. (2017). Carnivore attractant or plant elicitor? multifunctional roles of methyl salicylate lures in tomato defense. *J. Chem. Ecol.* 43, 573–585. doi: 10.1007/s10886-017-0856-6

Sabbagh, S. K., Roudini, M., and Panjehkeh, N. (2017). Systemic resistance induced by Trichoderma harzianum and Glomus mossea on cucumber damping-off disease caused by Phytophthora melonis. *Arch. Phytopathol. Pflanzenschutz* 50, 375–388. doi: 10.1080/03235408.2017.1317953

Sanmartín, N., Pastor, V., Pastor-Fernández, J., Flors, V., Pozo, M. J., and Sánchez-Bel, P. (2020). Role and mechanisms of callose priming in mycorrhiza-induced resistance. *J. Exp. Bot.* 71, 2769–2781. doi: 10.1093/jxb/eraa030

Sato, C., Aikawa, K., Sugiyama, S., Nabeta, K., Masuta, C., and Matsuura, H. (2011). Distal transport of exogenously applied jasmonoyl–isoleucine with wounding stress. *Plant Cell Physiol.* 52, 509–517. doi: 10.1093/pcp/pcr011

Sauter, M., and Hager, A. (1989). The mycorrhizal fungus *Amanita muscaria* induces chitinase activity in roots and in suspension-cultured cells of its host *Picea abies*. *Planta* 179, 61–66. doi: 10.1007/BF00395771

Schausberger, P., Peneder, S., Jürschik, S., and Hoffmann, D. (2012). Mycorrhiza changes plant volatiles to attract spider mite enemies. *Funct. Ecol.* 26, 441–449. doi: 10.1111/j.1365-2435.2011.01947.x

Schilmiller, A. L., and Howe, G. A. (2005). Systemic signaling in the wound response. *Curr. Opin. Plant Biol.* 8, 369–377. doi: 10.1016/j.pbi.2005.05.008

Schoenherr, A. P., Rizzo, E., Jackson, N., Manosalva, P., and Gomez, S. K. (2019). Mycorrhiza-induced resistance in potato involves priming of defense responses against cabbage looper (Noctuidae: Lepidoptera). *Environ. Entomol.* 48, 370–381. doi: 10.1093/ee/nvy195

Schulz-Bohm, K., Martín-Sánchez, L., and Garbeva, P. (2017). Microbial volatiles: small molecules with an important role in intra- and inter-kingdom interactions. *Front. Microbiol.* 8:2484. doi: 10.3389/fmicb.2017.02484

Schweiger, R., Baier, M. C., Persicke, M., and Müller, C. (2014). High specificity in plant leaf metabolic responses to arbuscular mycorrhiza. *Nat. Commun.* 5:3886. doi: 10.1038/ncomms4886

Shabala, S., White, R. G., Djordjevic, M. A., Ruan, Y.-L., and Mathesius, U. (2016). Root-to-shoot signalling: integration of diverse molecules, pathways and functions. *Funct. Plant Biol.* 43, 87. doi: 10.1071/FP15252

Shah, J. (2005). Lipids, lipases, and lipid-modifying enzymes in plant disease resistance. *Annu. Rev. Phytopathol.* 43, 229–260. doi: 10.1146/annurev.phyto.43.040204.135951

Shah, J., and Zeier, J. (2013). Long-distance communication and signal amplification in systemic acquired resistance. *Front. Plant Sci.* 4:30. doi: 10.3389/fpls.2013.00030

Sharma, N., Sharma, K., Gaur, R., and Gupta, V. (2011). Role of chitinase in plant defense. *Asian J. Biochem.* 6, 29–37. doi: 10.3923/ajb.2011.29.37

Sharon, E., Chet, I., and Spiegel, Y. (2011). "Trichoderma as a Biological Control Agent," in *Biological Control of Plant-Parasitic Nematodes: Building Coherence between Microbial Ecology and Molecular Mechanisms Progress in Biological Control*, eds K. Davies and Y. Spiegel (Dordrecht: Springer), 183–201. doi: 10.1007/978-1-4020-9648-8_8

Shen, Z., Sun, J., Yao, J., Wang, S., Ding, M., Zhang, H., et al. (2015). High rates of virus-induced gene silencing by tobacco rattle virus in Populus. *Tree Physiol.* 35, 1016–1029.

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

Shulaev, V., Silverman, P., and Raskin, I. (1997). Airborne signalling by methyl salicylate in plant pathogen resistance. *Nature* 385, 718–721. doi: 10.1038/385718a0

Smith, S. E., and Read, D. J. (2008). *Mycorrhizal symbiosis*. San Diego, CA: Academic Press, 800.

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

Song, Y. Y., Cao, M., Xie, L. J., Liang, X. T., Zeng, R. S., Su, Y. J., et al. (2011). Induction of DIMBOA accumulation and systemic defense responses as a mechanism of enhanced resistance of mycorrhizal corn (*Zea mays* L.) to sheath blight. *Mycorrhiza* 21, 721–731. doi: 10.1007/s00572-011-0380-4

Song, Y. Y., Ye, M., Li, C. Y., Wang, R. L., Wei, X. C., Luo, S. M., et al. (2013). Priming of anti-herbivore defense in tomato by arbuscular mycorrhizal fungus and involvement of the jasmonate pathway. *J. Chem. Ecol.* 39, 1036–1044. doi: 10.1007/s10886-013-0312-1

Spoel, S. H., and Dong, X. (2012). How do plants achieve immunity? Defence without specialized immune cells. *Nat. Rev. Immunol.* 12, 89–100. doi: 10.1038/nri3141

Stein, E., Molitor, A., Kogel, K.-H., and Waller, F. (2008). Systemic resistance in Arabidopsis conferred by the mycorrhizal fungus Piriformospora indica requires jasmonic acid signaling and the cytoplasmic function of NPR1. *Plant Cell Physiol.* 49, 1747–1751. doi: 10.1093/pcp/pcn147

Sun, J., Miller, J. B., Granqvist, E., Wiley-Kalil, A., Gobbato, E., Maillet, F., et al. (2015). Activation of symbiosis signaling by arbuscular mycorrhizal fungi in legumes and rice. *Plant Cell* 27, 823–838. doi: 10.1105/tpc.114.131326

Tarkka, M. T., Herrmann, S., Wubet, T., Feldhahn, L., Recht, S., Kurth, F., et al. (2013). OakContigDF159.1, a reference library for studying differential gene expression in *Quercus robur* during controlled biotic interactions: use for quantitative transcriptomic profiling of oak roots in ectomycorrhizal symbiosis. *New Phytol.* 199, 529–540. doi: 10.1111/nph.12317

Thines, B., Katsir, L., Melotto, M., Niu, Y., Mandaokar, A., Liu, G., et al. (2007). JAZ repressor proteins are targets of the SCF(COI1) complex during jasmonate signalling. *Nature* 448, 661–665. doi: 10.1038/nature05960

Thorpe, M. R., Ferrieri, A. P., Herth, M. M., and Ferrieri, R. A. (2007). 11C-imaging: methyl jasmonate moves in both phloem and xylem, promotes transport of jasmonate, and of photoassimilate even after proton transport is decoupled. *Planta* 226, 541–551. doi: 10.1007/s00425-007-0503-5

Truman, W., Bennett, M. H., Kubigsteltig, I., Turnbull, C., and Grant, M. (2007). Arabidopsis systemic immunity uses conserved defense signaling pathways and is mediated by jasmonates. *Proc. Natl. Acad. Sci. U.S.A.* 104, 1075–1080. doi: 10.1073/pnas.0605423104

Ullah, C., Tsai, C., Unsicker, S. B., Xue, L., Reichelt, M., Gershenzon, J., et al. (2019). Salicylic acid activates poplar defense against the biotrophic rust fungus Melampsora larici-populina via increased biosynthesis of catechin and proanthocyanidins. *New Phytol.* 221, 960–975. doi: 10.1111/nph.15396

van der Heijden, M. G. A., Martin, F. M., Selosse, M.-A., and Sanders, I. R. (2015). Mycorrhizal ecology and evolution: the past, the present, and the future. *New Phytol.* 205, 1406–1423. doi: 10.1111/nph.13288

van Schie, C. C., Haring, M. A., and Schuurink, R. C. (2007). Tomato linalool synthase is induced in trichomes by jasmonic acid. *Plant Mol. Biol.* 64, 251–263. doi: 10.1007/s11103-007-9149-8

Vandenkoornhuyse, P., Quaiser, A., Duhamel, M., Van, A. L., and Dufresne, A. (2015). The importance of the microbiome of the plant holobiont. *New Phytol.* 206, 1196–1206. doi: 10.1111/nph.13312

Vincent, D., Kohler, A., Claverol, S., Solier, E., Joets, J., Gibon, J., et al. (2012). Secretome of the free-living mycelium from the ectomycorrhizal basidiomycete Laccaria bicolor. *J. Proteome Res.* 11, 157–171. doi: 10.1021/pr200895f

Vishwanathan, K., Zienkiewicz, K., Liu, Y., Janz, D., Feussner, I., Polle, A., et al. (2020). Ectomycorrhizal fungi induce systemic resistance against insects on a nonmycorrhizal plant in a CERK1-dependent manner. *New Phytol.* 228, 728–740. doi: 10.1111/nph.16715

Vlot, A. C., Dempsey, D. A., and Klessig, D. F. (2009). Salicylic acid, a multifaceted hormone to combat disease. *Annu. Rev. Phytopathol.* 47, 177–206. doi: 10.1146/annurev.phyto.050908.135202

Vlot, A. C., Pabst, E., and Riedlmeier, M. (2017). *"Systemic signalling in plant defence." in eLS*. Chichester: John Wiley& Sons, Ltd, doi: 10.1002/9780470015902.a0001322.pub3

Wang, B., Yeun, L. H., Xue, J.-Y., Liu, Y., Ané, J.-M., and Qiu, Y.-L. (2010). Presence of three mycorrhizal genes in the common ancestor of land plants suggests a key role of mycorrhizas in the colonization of land by plants. *New Phytol.* 186, 514–525. doi: 10.1111/j.1469-8137.2009.03137.x

Wang, C., Liu, R., Lim, G.-H., Lorenzo, L. D., Yu, K., Zhang, K., et al. (2018). Pipecolic acid confers systemic immunity by regulating free radicals. *Sci. Adv.* 4:eaar4509. doi: 10.1126/sciadv.aar4509

War, A. R., Paulraj, M. G., Ahmad, T., Buhroo, A. A., Hussain, B., Ignacimuthu, S., et al. (2012). Mechanisms of plant defense against insect herbivores. *Plant Signal. Behav.* 7, 1306–1320. doi: 10.4161/psb.21663

Wasternack, C. (2007). Jasmonates: an update on biosynthesis, signal transduction and action in plant stress response, growth and development. *Ann. Bot.* 100, 681–697. doi: 10.1093/aob/mcm079

Wenig, M., Ghirardo, A., Sales, J. H., Pabst, E. S., Breitenbach, H. H., Antritter, F., et al. (2019). Systemic acquired resistance networks amplify airborne defense cues. *Nat. Commun.* 10, 3813. doi: 10.1038/s41467-019-11798-2

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

Werner, S., Polle, A., and Brinkmann, N. (2016). Belowground communication: impacts of volatile organic compounds (VOCs) from soil fungi on other soil-inhabiting organisms. *Appl. Microbiol. Biotechnol.* 100, 8651–8665. doi: 10.1007/s00253-016-7792-1

Wink, M. (2018). Plant secondary metabolites modulate insect behavior-steps toward addiction? *Front. Physiol.* 9:364. doi: 10.3389/fphys.2018.00364

Xue, D.-X., Li, C.-L., Xie, Z.-P., and Staehelin, C. (2019). LYK4 is a component of a tripartite chitin receptor complex in Arabidopsis thaliana. *J. Exp. Bot.* 70, 5507–5516. doi: 10.1093/jxb/erz313

Yuan, P., Jauregui, E., Du, L., Tanaka, K., and Poovaiah, B. (2017). Calcium signatures and signaling events orchestrate plant–microbe interactions. *Curr. Opin. Plant Biol.* 38, 173–183. doi: 10.1016/j.pbi.2017.06.003

Zhang, H., and Hu, Y. (2017). Long-distance transport of prosystemin messenger RNA in Tomato. *Front. Plant Sci.* 8:1894. doi: 10.3389/fpls.2017.01894

Zhang, X., Dong, W., Sun, J., Feng, F., Deng, Y., He, Z., et al. (2015). The receptor kinase CERK1 has dual functions in symbiosis and immunity signalling. *Plant J.* 81, 258–267. doi: 10.1111/tpj.12723

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

Zhou, G., Ren, N., Qi, J., Lu, J., Xiang, C., Ju, H., et al. (2014). The 9-lipoxygenase Osr9-LOX1 interacts with the 13-lipoxygenase-mediated pathway to regulate resistance to chewing and piercing-sucking herbivores in rice. *Physiol. Plant.* 152, 59–69. doi: 10.1111/ppl.12148

Zhou, Y., Zhou, B., Pache, L., Chang, M., Khodabakhshi, A. H., Tanaseichuk, O., et al. (2019). Metascape provides a biologist-oriented resource for the analysis of systems-level datasets. *Nat. Commun.* 10, 1523. doi: 10.1038/s41467-019-09234-6

Zhu-Salzman, K., and Zeng, R. (2015). Insect response to plant defensive protease inhibitors. *Annu. Rev. Entomol* 60, 233–252. doi: 10.1146/annurev-ento-010814-6

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

ORIGINAL RESEARCH
published: 22 December 2020
doi: 10.3389/fpls.2020.604898

Microbial Inoculation for Productivity Improvements and Potential Biological Control in Sugar Beet Crops

Gonzalo Sacristán-Pérez-Minayo[1], Domingo Javier López-Robles[2], Carlos Rad[2] and Luis Miranda-Barroso[3]*

[1] Microbiology Section, Faculty of Sciences, University of Burgos, Burgos, Spain, [2] Edaphology and Agricultural Sciences Section, Faculty of Sciences, University of Burgos, Burgos, Spain, [3] Sustainable Agriculture Department, Syngenta-Spain, Madrid, Spain

Used mainly for sucrose production, sugar beet is one of the most important crops in Castilla y León (Spain). Several studies have demonstrated the benefits of microorganisms in different crop management programs, among which Plant Growth Promoting Rhizobacteria (PGPR). This research aims to assess the beneficial effects of two PGPRs strains (*Pseudomonas fluorescens* Pf0-1 and *Pseudomonas chlororaphis* CECT 462) on sugar beet (*Beta vulgaris*) production. Three treatments: a PGPRs co-inoculation assay of untreated seeds without any chemical treatment (TB), a conventional treatment with commercial seeds and fungicide application (TT); and a control with seeds without protective coating, bacterial inoculation and chemical treatment (ST). The efficacy of PGPRs inoculation on sugar beet production was determined measuring periodically the photosynthetic status of plants, and the final yield and quality of tubers. Aerial and root plant biomass, maximum beet perimeter, polarization, and sugar values of the sugar beet plants inoculated with PGPRs showed higher values and significant differences to sugar beet subjected to other treatments. We could see that PGPRs inoculation (TB treatment) produced significant differences in the quantum yield of PSII (ΦPSII). TB showed the highest value for ΦPSII and the NPQ (non-photochemical quenching), the lowest value, even though the PSII (maximum quantum yield of photosystem II) was very similar in all treatments. The two assayed PGPR strains triggered a significant increase in sugar beet production yield and quality. PGPRs inoculation techniques could be used in different crops and they could be applied as biofertilizers, improving the agricultural production.

Keywords: integrated crop management, PGPR, sugar beet, photosynthesis parameters, sucrose

OPEN ACCESS

Edited by:
Ioannis Stringlis, Utrecht University, Netherlands

Reviewed by:
Claire Prigent-Combaret, UMR 5557 Ecologie Microbienne (LEM), France
Dilfuza Egamberdieva, Leibniz Center for Agricultural Landscape Research (ZALF), Germany

****Correspondence:***
Gonzalo Sacristán-Pérez-Minayo
gsacristan@ubu.es

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

Received: *10 September 2020*
Accepted: *04 December 2020*
Published: *22 December 2020*

Citation:
Sacristán-Pérez-Minayo G, López-Robles DJ, Rad C and Miranda-Barroso L (2020) Microbial Inoculation for Productivity Improvements and Potential Biological Control in Sugar Beet Crops. Front. Plant Sci. 11:604898. doi: 10.3389/fpls.2020.604898

INTRODUCTION

Sugar beet (*Beta vulgaris* L. var. saccharifera) is an important root crop in moderate climates and the main source of sugar (Dohm et al., 2014). The worldwide cropping area covers over approximately 4.5 million Ha, with roughly 70% of sugar beet production in Europe (FAOSTAT, 2019). Annual world sucrose production stands 175.6 million tons in 2017, of which 28% is extracted from sugar

beet (*Beta vulgaris* L.), and the remainder from sugar cane OECD/FAO (2020). Sugar production in 2018 was approximately 2,870,907 tons (FAOSTAT, 2019). In Spain, sugar beet cultivation is reported to cover about 53,000 Ha. The present research was conducted in the region of Castilla y León, where sugar beet is one of the most important industrial crops, providing over 50% of all Spanish beet sugar. Sugar beet crop profitability is valued in terms of sugar production, which basically refers to its sucrose purity factor, as sucrose content is made up of more than 99.5% in the final white crystalline sugar (Pan et al., 2015).

Several research studies have noted the importance of soil microbiome on plant health, in particular in sugar beet crops (Berendsen et al., 2012; Bakker et al., 2013, 2020; Weijuan et al., 2020; Wolfgang et al., 2020). In fact because the genome and breeding history is known, sugar beet is an interesting model crop for microbiome studies (Zachow et al., 2008; Mendes et al., 2011; Würschum et al., 2013; Dohm et al., 2014; Kusstatscher et al., 2019a,b). Hiltner (1904) established the importance of the rhizosphere microbiome for growth promotion in crops and omics technologies allow in-depth analysis, nowadays (Mendes et al., 2011; Raaijmakers and Mazzola, 2016).

Bacteria with multiple beneficial traits can be advantageous in commercial agriculture and are relevant to the bio-economy (Backer et al., 2018). Recently, research on Plant Growth Promoting Rhizobacteria (PGPR) for crop improvements are gaining prominence and thousands of research works have been published so far (Compant et al., 2005; Mia et al., 2010; Backer et al., 2018). The term PGPR was first defined by Kloepper et al. (1980) in the 1980s, later Compant et al. (2005) subsequently described PGPR as microorganisms from the rhizosphere that can positively influence plant growth and plant health. These PGPRs have ability to protect the plants from drought, salts and heavy metal stresses and play significant role in the plant growth promotion, yields, nutrient acquisition and as well as minimizing the use of chemical fertilizers (Kumar et al., 2019). In particular, PGPRs could promote directly plant growth by various mechanisms, including: (i) the production of phytohormomes such as auxins, cytokinins and gibberellins (Santner et al., 2009); (ii) the production of plant growth promoting (PGP) substances such as indole-3-acetic acid (IAA) and/or siderophores which can provide soluble iron to plants (Scher and Baker, 1982); (iii) the increase of the solubilization of phosphorus and other trace element for plant uptake (Gyaneshwar et al., 2002); (iv) the supply of nutrients to plants, by asymbiotic nitrogen fixation (Doberein and Campelo, 1971) and v) the secretion of enzymes that can modulate plant growth and development, such as reducing ethylene level by synthesis of 1-aminocyclopropane-1-carboxylic acid (ACC) (Yang and Hoffman, 1984; Penrose et al., 2001). The use of PGPRs as biological control agents have been found effective and are being increasingly applied in the field (Pooja et al., 2019). Indirectly, some PGPRs are able to reduce the disease development in plant system by various mechanisms that include among others: production of antimicrobial metabolites, volatile compounds and induced systemic resistance (ISR) (Hassani et al., 2018; Stringlis et al., 2018).

Sugar beet is known to be affected by several pathogens, including bacteria, fungi, viruses and nematodes. *Cercospora beticola* Sacc. and *Erysiphe betae* (Vañha) Weltz are the causative agents of Cercospora Leaf Spot (CLS) and powdery mildew, respectively, and they are the most damaging foliar diseases for sugar beet crops (Jay et al., 2020). CLS occurs in sugar beet-growing areas worldwide and can lead to reductions in recoverable sucrose of 30–48% (Khan et al., 2001). CLS is a polycyclic disease whose severity depends on weather conditions *C. beticola*'s full disease cycle can occur in as few as 10 days under suitable climatic conditions of high relative humidity and high temperatures, thus resulting in multiple infection cycles in each growing season. Fungus conidia infect leaves, resulting in the appearance of millimeter-scale brown round spots. These necrotic spots then expand and coalesce, eventually defoliating the whole plant and requiring new leaves to grow. Beet powdery mildew is also another worldwide fungal disease of sugar beet. Powdery mildew is characterized by a white dust-like mycelium that develops over the leaf surface. Heavily infected tissues develop chlorosis and suffer early senescence, with infection being especially damaging in areas with arid climates, such as in Mediterranean countries (Fernández-Aparicio et al., 2009). Fungal diseases incidence can occur on sugar beet in one season, consecutively or simultaneously, and outbreaks can result in a significant loss of the crop in years with high disease pressure (Heick et al., 2020). The severity and frequency of fungal disease attacks vary considerably every year, depending on factors as weather conditions, microclimate, and agronomic practices (Heick et al., 2020). The traditional treatment for these fungal diseases involves prevention, in other words treatment with highly fungicidal phytosanitary products may be effective in controlling the development of both pathogens incidence. However, a significant reduction of the use of fungicides is highly desired since, some of them, affect the environment while being expensive (Van Zwieten et al., 2004).

There is therefore a need for complementary agricultural practices, such as the use of microorganism-based biological control methods (Compant et al., 2005; Naureen et al., 2009). *Pseudomonas* and *Bacillus* are the most commonly described genera possessing plant growth promoting activities (Munees and Mohammad, 2011). *Pseudomonas* are frequently found across all life stages of sugar beet and that several strains with promising biocontrol potential were found in sugar beet endosphere (Zachow et al., 2008, 2010).

Pseudomonas chlororaphis is capable of promoting the growth of plants such as wheat and corn (Agaras et al., 2020) or millet (Niu et al., 2018). It has been possible to verify the protective capacity against different pathogens such as fungi (*Rhizoctonia solani*) where it induced the plant to increase the expression of certain genes that influenced its protection (Kamou et al., 2020). Indirectly, *Pseudomonas fluorescens* Pf0-1 stimulates plant growth by protecting it from attack by *Pythium ultimum*, *Gaeumannomyces graminis* var. *tritici*, and *Fusarium oxysporum*, in addition to the motility and degree of chemotaxis that it possesses, which are essential properties in the colonization of vegetable roots (Oku et al., 2012).

In the present work, we evaluated the successive application of a mixed PGPRs culture (*Pseudomonas fluorescens* Pf0-1 and *P. chlororaphis* CECT 462) on the productivity of sugar beet

evaluated in terms of production yield and sucrose content, and physiological changes in the whole cycle of the plant. Additionally, we also monitored the biocontrol effects of PGPRs against two fungal pathogens, *Cercospora beticola* and *Erysiphe betae*, throughout the sugar beet production cycle.

MATERIALS AND METHODS

Plant Material and Bacterial Strains

Seeds of *Beta vulgaris* var. Turbata, tolerant to the fungal pathogens *Cercospora beticola* and *Erysiphe betae,* were provided by Koipesol Semillas, S.A. These commercial seeds have a protective coating containing fungicide and insecticide formulations. The sugar beet standard planting density was 100,000 plants per hectare.

The present research was conducted in the region of Castile and Leon, where sugar beet is one of the most important industrial crops, providing over 50% of all Spanish beet sugar (Esteban Baselga, 1993). The present experiment was performed in the 2018 sugar beet campaign, from April to November.

In the present experiment, we applied a combination of two PGPRs strains, *Pseudomonas fluorescens* Pf0-1 and *Pseudomonas chlororaphis* CECT 462. The first of the *Pseudomonas* strains was isolated from an agricultural soil in Pampliega (42°12′N; 3°58′W; altitude 809 m asl), Burgos (Spain) (Sacristán Pérez-Minayo et al., 2011). We used the Fasta Nucleotide Similarity Search Database available from the European Bioinformatics Institute (EMBL-EBI)[1] and we obtained a percentage of similarity of 100% and a percentage of identity of 99.107% compared with *Pseudomonas fluorescens* Pf0-1 (EMBL: CP000094). *Pseudomonas chlororaphis* CECT 462 was provided by the Spanish Collection of Type Cultures (CECT — Colección Española de Cultivos Tipo, 2020). To determine the compatibility between PGPR strains, a cross-streak method of inoculation was done checking the appearance of inhibition zones at the intersection of the paired strains (Santiago et al., 2017). The bacterial strains were maintained at −80°C in nutrient broth with 20% glycerol. Inocula were prepared, separately, by streaking strains at −80°C onto King A medium (Cultimed, Spain), incubating plates at 30°C for 24 h. After incubation, the plates were scrapped off into a sterile 10 mM SO_4Mg buffer at a suspension of 10^8 CFU/ml. This final suspension contained both PGPR strains and was prepared repeatedly before each PGPRs spraying applications.

Experimental Design

The present experiment was performed in the 2018 sugar beet campaign, from 8th April to 26th November. The experimental plot occupied 252 m^2 (18 m long and 14 m wide) of an irrigated field (32.30 Ha) located on the outskirts of Pedrosa del Rey, Valladolid. The site is placed at 706 m of altitude. Annual mean temperature was 12°C, minimum temperature (−0.3°C) was found in January and the maximum (29.4°C) in July. In relation with frost days, the last frost day of spring was 13rd April and first frost day of autumn was 8th November. Hence, we had 208 free frost days. The mean annual rainfall was 374 mm with 61 rainfall days per year (ITACYL — Instituto Tecnológico Agrario de Castilla y León, 2019). The field had corn as precedent crop and is classified as LVk Calcaric Luvisol (IUSS Working Group WRB, 2015). Main soil properties are: texture, pH, EC, SOM, Total N. The soil pH and the organic matter content were 8.3 and closely to 2%, respectively. The texture of the soil in the experimental plots was, in general, loamy, except some small area was found as loamy-clayey. The mean clay content was 25.65%. We performed three different treatments in a completely randomized block design: TB, without seed coating, with PGPRs inoculum application and chemical spraying; TT, with seed coating and chemical spraying and without PGPRs inoculum and ST, without seed coating, chemical spraying and PGPRs inoculum. The chemical spraying consisted of a mixture of "Karate King" insecticide (0.5 Kg/Ha), "Tilt" fungicide (1.25 l/Ha) and *boron-molybdenum* fertilizer (2.50 l/Ha). Each treatment had four replicates of 2 × 2 m^2 subplots, in which 40 plants were introduced, and with 2 m interrow spaces between them, to avoid border effect. Irrigation was performed using a central pivot system (30–50 l/m^2 per week) throughout the whole production cycle, according local irrigation schedule.

The seeds protective coating was removed in TB and ST treatment, with thorough washing and stirring of seeds in sterile distilled water, with the purpose of check the inoculum effect without the presence of fungicide or herbicides. After coating removing, the seeds in the TB trial were inoculated by immersion for 6 h at 30°C with the mixed culture of PGPR strains, *Pseudomonas fluorescens* Pf0-1 and *Pseudomonas chlororaphis* CECT 462. Immediately after sowing, the seeds for the TB replicates were irrigated once with the mixed PGPRs inoculum (1 ml per seed). The plant leaves in the TB replicates were sprayed six times with the PGPRs suspension (1,500 ml each sprayed time). Foliar spray application was performed to promote microorganism-plant interactions during the production cycle. The dates of the PGPRs spraying applications were: first PGPRs spraying, 23 May 2018; second PGPRs spraying, 19 June 2018; third PGPRs spraying, 01 July 2018; fourth PGPRs spraying, 31 July 2018; fifth PGPRs spraying, 02 September 2018, and sixth PGPRs spraying, 08 October 2018. The TT subplots were initially irrigated with water and thereafter, sprayed with water and a mixture of insecticide, fungicide and fertilizer, at the same time as the TB treatment. ST subplots were irrigated and sprayed with water six times. The timelines for the three treatments are shown schematically in **Supplementary Figure 1**.

Sugar Beet: Crop Production Yield and Quality

At the end of the production cycle (232 days after sowing), 10 plants were harvested per subplot and the following parameters analyzed: sugar content (kg), polarization (%), corrected sucrose, N-amino, potassium (K) and sodium (Na) content and industrial loss and yield (%) according to the International Sugar Scale. The total, aerial and root biomass (kg) were also recorded, as well as the root maximum beet perimeter and length. Sucrose content

[1]http://www.ebi.ac.uk/Tools/sss/fasta/nucleotide.html

was measured by polarization (Schmidt and Haensch Mod. 14220), Na and K content by flame photometry (Model NAK-1 Pacisa), and α-amino-nitrogen content (α-N) according to the Stanek and Pavlas (1934) blue index method, as modified by the Swedish Sugar Company with the values given by the Wieninger and Kubadinow (1973) formula. The corrected sucrose, industrial loss and yield values were also calculated with the Wieninger and Kubadinow formulae.

Soil properties were determined after harvest. In each plot, a composite soil sample was obtained at three different point in the crop row in each subplot using an auger (Ø 5 cm), mixed and placed in labeled bags. Texture, pH, conductivity and organic matter content were then determined using standard methods (MAPA., 1994).

Levels of available phosphorus, exchangeable sodium and magnesium and soluble boron were also determined (**Table 1**).

Photosynthesis Parameters Measurement

The photosynthetic status of a plant can be used as an indicator of its physiological status with respect to biostimulation or after a pathogenic attack. Foliar pathogens can cause the reduction of photosynthetic active leaf area, because of the leaf damage and the disturbance of photosynthesis in the remaining or surrounding leaf area (Berger et al., 2004; Robert et al., 2006). For instance, when a CLS disease severity on sugar beet of reached 3–6%, photosynthesis is reduced (Levall and Bornman, 2000). Thus, photosynthesis was measured in 10 leaves from 10 healthy plants with similar vegetative state in each subplot, 2 days after the third PGPRs inoculation (03 July 2018). This date was chosen because the physiological status of the plants was at the highest stage of sugar production.

A portable FMS2 fluorimeter (Fluorescence Monitoring System, Hansatech, Norfolk, United Kingdom) was used to measure fluorescence emission of chlorophyll in leaves previously adapted to darkness, to determine the efficiency of photosynthesis and to diagnose the presence of stress factors that decrease it (Krause and Weis, 1991; Baker, 2008). It also has the advantage of being a non-destructive technique. Two consecutive measurements were performed on the same leaf. The first one, corresponding to minimal fluorescence (Fo), is taken with the leaf adapted to dark conditions using a clamp for 20 min; thereafter the measure was repeated after a saturating light pulse, corresponding to the maximum fluorescence (Fm). With these two parameters, we calculated the maximum quantum yield of photosystem II (PSII) that indicates the maximum amount of energy that PSII could potentially expend in photochemical processes, which is calculated as Fv/Fm, where Fv is variable fluorescence = Fm − Fo. In the second step, we measured fluorescence emitted by the leaves adapted to light (Fs), and fluorescence when subjected to a saturating light pulse (Fm′ = maximum fluorescence measured in a state adapted to light). These parameters allowed us to calculate the quantum yield of PSII (ΦPSII), as ΦPSII = (Fm′ − Fs)/Fm′, and to quantify the proportion of energy absorbed by PSII that is used in photosynthetic electron transport (Genty et al., 1989), which therefore reveals the actual amount of energy that may be used for photochemical processes. Finally, we calculated NPQ (non-photochemical quenching) parameter. It was calculated as NPQ = (Fm − Fm′)/Fm′, a parametric indicator of the proportion of energy received that is dissipated as heat and therefore not used for photochemical processes (Ögren and Baker, 1985; Baker, 2008; Rodríguez-Moreno et al., 2008). All data were processed with MODFL2 software.

Determination of Resistance to Plant Pathogens

The incidence of *Cercospora beticola* and *Erysiphe betae* was recorded throughout the production cycle at four timepoints: after third, fifth, and six PGPRs spraying applications and after the final harvest. Visual assessment of diseases was scored on a four-point scale, where 1 = 0–25% of the replicated area that was affected, 2 = 26–50% of the replicated area that was affected, 3 = 51–75%, and 4 = 76–100%, for each fungal pathogen (**Supplementary Figure 2**).

Infection index was calculated as the percentage of affected plants in each replicate as Index (%) = N/N_t, where N is the number of affected plants in each replicate and N_t is the total number of plants per replicate.

Severity was determined at pathogen assessment-time 2 of the production cycle, the day on which both fungal pathogens reached their highest growth. The severity index was calculated as the percentage of affected leaves on a randomly selected sugar beet plant. The sugar beet plant selected in each replicate was a representative plant of medium size located at the center of the plot. Severity (%) = L/L_t, where L is the number of affected leaves and L_t the total number of leaves. The visual assessment was evaluated on all four timepoints, the infection index was evaluated at timepoints 2 and 3, and the severity index, at timepoints 2.

Statistical Analysis

One-way analysis of variance (ANOVA) using treatment as fixed factor was performed after checking for normality and homogeneity of variances with Kolmogorof-Smirnof's and Levene's test, respectively, LSD test was used to calculate significative differences between treatments. These analyses were carried out using STATGRAPHICS Plus 4.0 software. One and two-ways ANOVA was performed to evaluate the differences between treatments in each fungal disease and between sampling days.

RESULTS

Sugar Beet: Crop Production Yield and Quality

Percentage plant survival rates per replicate were: 92, 96, and 98% for the ST, the TB and the TT assays, respectively. The plant populations of the three assays were very similar and there were no significant differences between the ST, the TB,

TABLE 1 | Soil parameters measured at the end of the sugar beet productive cycle.

	Sand (%)	Silt (%)	Clay (%)	Texture	pH	Conductivity (mmhos/cm)	Organic matter (%)	Phosphorus, P (ppm)	Potassium, K (ppm)
ST1	43.84	32	24.16	Loam	8.3	0.44	2.05	50	251
ST2	39.84	34	26.16	Loam	8.2	0.5	1.9	51	177
ST3	49.84	24	26.16	Loamy-clay-sand	8.5	0.5	1.94	49	409
ST4	49.84	28	22.16	Loam	8.3	0.43	1.82	47	265
TB1	47.84	30	22.16	Loam	8.3	0.84	1.74	54	281
TB2	45.84	28	26.16	Loam	8.5	0.46	1.74	50	276
TB3	43.84	32	24.16	Loam	8.2	0.55	1.78	46	283
TB4	39.84	34	26.16	Loam	8.3	0.48	2.32	45	247
TT1	39.84	34	26.16	Loam	8.2	0.44	1.59	53	276
TT2	41.84	30	28.16	Loamy-clay	8.3	0.49	1.59	45	246
TT3	43.84	28	28.16	Loamy-clay	8.3	0.47	1.86	41	354
TT4	41.84	30	28.16	Loamy-clay	8.3	0.48	2.01	46	355

	Magnesium, Mg (ppm)	Carbonates (%)	Active lime (%)	Exchangeable calcium (ppm)	Exchangeable sodium (ppm)	Boron, B (ppm)
ST1	496	0.15	x	x	468	0.95
ST2	583	0.23	x	x	373	0.88
ST3	510	0.38	x	x	495	0.95
ST4	503	0.31	x	x	206	0.75
TB1	523	0.31	x	x	420	0.88
TB2	542	0.15	x	x	398	0.83
TB3	598	0.23	x	x	493	0
TB4	561	0.38	x	x	300	0.83
TT1	501	0.31	x	x	323	0.83
TT2	537	0.15	x	x	323	1.2
TT3	581	0.23	x	x	233	1.13
TT4	630	0.31	x	x	232	1

Six applications of a mixed PGPRs culture (Pseudomonas fluorescens Pf0-1 and P. chlororaphis CECT 462) on the productivity of sugar beet were performed. Three different treatments, with four replicates, were performed: TB, without seed coating and with bacterial inoculum application; TT, with seed coating and ST, without seed coating in a completely randomized block design.

and the TT plant populations. Sugar beet yield and quality measurement were noted at the end of the production cycle. **Table 2** shows the biometric parameters reported from the three treatments (TB, ST, and TT). Both the total biomass (plant weight) and the sugar content of the beets from the TB plots were significantly higher than those for the other treatments. There were no significant differences between those values for the ST and the TT treatments, although the values of the ST treatment were somewhat higher (**Table 2**). Root weight and maximum beet perimeter values of the beets given the TB treatment were significantly higher than for the ST and the TT treatments, between which there was no significant variation. The highest aerial biomass and root length values were found in the beets given the TB treatment, although differences with regard to beets given the other two treatments were not significant ($p \geq 0.05$) (**Supplementary Figures 3**, **4**). Significant differences were found for both corrected and total sucrose content (polarization) values between TB treatment (with the lowest value) and the other two and no significant differences were found between the latter two (ST and TT) (**Table 2**). The results of soil parameters (edaphic characteristics), at the end of the productive cycle (upon harvest), showed no significant differences between the ST, the TT and the TB treatments.

Photosynthesis Parameters Measurement

Figure 1 shows the quantum yield of PSII (ΦPSII). The beet showed significantly higher mean values (0.70) after the TB treatment than after the ST (0.64) and the TT (0.64) treatments. In relation to maximum quantum yields of photosystem II (PSII) and the non-photochemical quenching (NPQ) parameters, we observed no significant differences between either the ST, or the TT and the TB treatments (**Supplementary Figures 5**, **6**).

Determination of Resistance to Plant Pathogens

Visual assessment revealed that the evolution of *Erysiphe betae* (powdery mildew) and *Cercospora beticola* (cercospora) infection was similar for all three treatments (**Supplementary Figure 7** and **Figure 2**). For powdery mildew, the highest degree of infection was found on timepoint 2, although it subsequently decreased progressively. All values from timepoint 2 were above 3 points on the established 4 point-scale. The values from timepoints 1 and 3 were very similar and yet very different with respect to timepoints 2 and 4 (**Supplementary Figure 7**). The results for cercospora infection were similar to those for mildew, although

TABLE 2 | Sugar beet physiological parameters measured at the end of the productive cycle.

Parameters	ST	TB	TT
Total biomass (kg)	17.55 ± 0.99[a]	20.35 ± 1.47[b]	13.85 ± 1.02[a]
Sugar (kg/10 plants)	2.30 ± 0.17[a]	2.73 ± 0.22[b]	2.61 ± 0.18[a]
Root biomass (g/plant)	1414.17 ± 113.71[a]	1694.17 ± 73.14[b]	1164.17 ± 86.77[a]
Maximum beet			
Perimeter (cm/plant)	44.42 ± 1.47[a]	52.02 ± 1.35[b]	41.92 ± 1.24[a]
Polarization (%/plant)	17.83 ± 0.12[a]	16.78 ± 0.25[b]	18.15 ± 0.08[a]
Corrected sucrose (%/plant)	15.11 ± 0.17[a]	13.79 ± 0.28[b]	15.80 ± 0.06[a]
N-amino	0.47 ± 0.07[a]	0.63 ± 0.11[a]	0.35 ± 0.05[a]
Potassium, K	5.60 ± 0.20[a]	5.68 ± 0.17[a]	5.05 ± 0.13[a]
Sodium, Na	1.34 ± 0.28[ab]	2.00 ± 0.28[a]	0.85 ± 0.11[b]
Industrial Loss%	15.23 ± 0.52[a]	17.80 ± 0.66[b]	12.92 ± 0.34[c]
Industrial Yield%	84.77 ± 0.52[a]	82.20 ± 0.66[b]	87.08 ± 0.34[c]

Six applications of a mixed PGPRs culture (Pseudomonas fluorescens Pf0-1 and P. chlororaphis CECT 462) on the productivity of sugar beet were performed. Three different treatments, with four replicates, were performed: TB, without seed coating and with bacterial inoculum application; TT, with seed coating and ST, without seed coating in a completely randomized block design. A simple ANOVA was performed; letters show significant differences ($p \leq 0.05$) between treatments. Values are means (10 replicates) ± S.E.

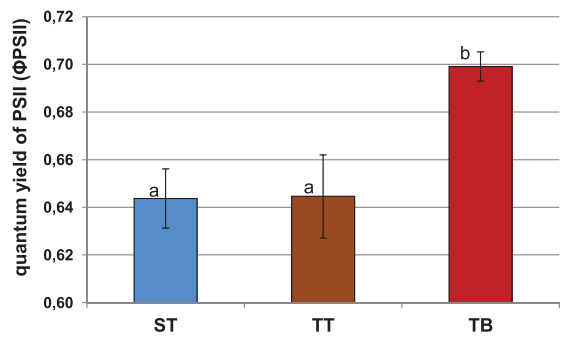

FIGURE 1 | Quantum yield of photosystem II (ΦPSII) for 12-weeks-old sugar beet plants (2 days after the third PGPRs inoculation, 03 July 2018). Six applications of a mixed PGPRs culture (*Pseudomonas fluorescens* Pf-01 and *P. chlororaphis* CECT 462) on the productivity of sugar beet were performed. Three different treatments, with four replicates in a completely randomized block design, were performed: TB, without seed coating, with PGPRs inoculum application and chemical spraying; TT, with seed coating and chemical spraying and without PGPRs inoculum and ST, without seed coating, chemical spraying and PGPRs inoculum. A simple ANOVA was performed; letters show significant differences ($p \leq 0.05$).

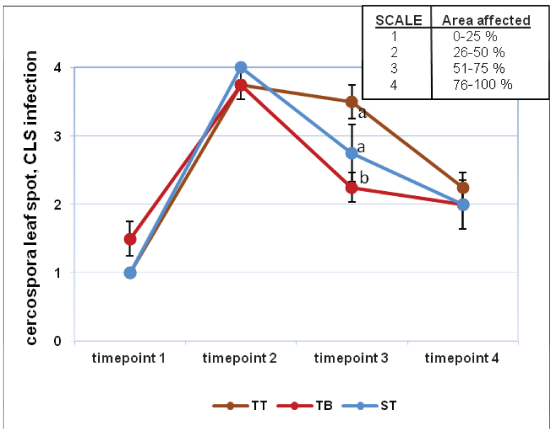

FIGURE 2 | Visual infection evaluation (cercospora leaf spot, CLS) during the experiment at four timepoints. Fungal diseases were scored on a four-point scale (1–4). Six applications of a mixed PGPRs culture (*Pseudomonas fluorescens* Pf-01 and *P. chlororaphis* CECT 462) on the productivity of sugar beet were performed. Three different treatments, with four replicates in a completely randomized block design, were performed: TB, without seed coating, with PGPRs inoculum application and chemical spraying; TT, with seed coating and chemical spraying and without PGPRs inoculum and ST, without seed coating, chemical spraying and PGPRs inoculum. Two-way ANOVA was carried out for CLS infection values of three treatments along four evaluation times.

the values obtained over the four evaluated timepoints differed greatly between each other. All values from timepoint 2 were very near to 4 points on the established 4 point-scale. On timepoint 3, the highest degree of cercospora infection was found in the TT treatment (3.5), whereas the values for ST and TB were 2.75 and 2.25, respectively (**Figure 2**). The index values for these two pathogens on timepoints 2 and 3 did not differ significantly between the three treatments (**Supplementary Figures 8A,B**). The degree of infection was higher on timepoint 2, and this value differed significantly from that found for timepoint 3. On timepoint 3, the highest index for mildew infection was found for the TB treatment (41.33%), while the ST and the TT treatments gave values of 22.31 and 12.42%, respectively (**Supplementary Figure 8A**). In contrast, on timepoint 3, the TT treatment had the highest cercospora infection index at 67.45%, while the ST and the TB treatments had 59.37 and 56.37%, respectively (**Supplementary Figure 8B**). The mean severity for mildew and cercospora did not differ significantly between any of the three treatments (**Supplementary Figure 9**), although a significant difference in the severity of these two pathogens was found for the ST treatment (**Supplementary Figure 9**).

DISCUSSION

The present work has studied the effect of PGPRs inoculation of sugar beet on agronomic performance, photosynthetic process changes and biocontrol effects on two main fungal pathogens.

In the 2018 campaign, the Sociedad Cooperativa General Agropecuaria (ACOR) received 3,183,974 tons of sugar beet, with a mean purity of 17.50%, from the north of the region of Spain. The results of our treatments fall within the purity interval obtained by the ACOR, with values ranging between 16 and 18.4%. The mean purity obtained with the PGPRs inoculation assays (TB treatment) was 16.77%, a value close to that obtained by the ACOR (**Table 2**). The beets given the TB treatment had the highest sugar levels, total biomass, root biomass, maximum beet perimeter, N-amino, Potassium and Sodium content values.

In all cases, those same values following the ST treatment are at an intermediate point between those for the TB and the TT treatments (**Table 2**). From these results, PGPRs inoculations appear to enhance the previously discussed biometric parameters of sugar beet plants. The PGPR strains applied in the present experiment belong to *Pseudomonas* genus, one of the most effective phosphate solubilizing bacteria and is considered as optimal specie for their stable P-solubilizing effects (Huang et al., 2010). Therefore, this biostimulant effect could be explained by the ability of the applied PGPR strains to solubilize phosphate. This mobilization of insoluble soil phosphate into bioavailable forms that can be taken up by the plant root (Monds et al., 2006). Previous related field trials have also reported significantly increased production yields for sugar beet crops upon application of various microorganisms (Cakmakci et al., 2001). Indeed, this latter study reported increases in sugar beet root production of between 6.1 and 13%, with an increase in sugar content of between 2.3 and 7.8%, in plants inoculated with *Bacillus polymyxa*, *Burkholderia* sp. and *Pseudomonas* sp. From the results of our experiment, we suggest that the two assayed PGPRs triggered growth promotion in the treated sugar beet plants. This PGPRs-induced plant growth was also reported in similar studies, with plants treated with these strains growing taller and more vigorously (Suslow and Schroth, 1982; Nandakumar et al., 2001). Bakker et al. (2020) have reviewed in depth the root-associated microbiota and their functions in plant health and especially on how modern microbiomics technologies can help to decipher complex processes that govern the assembly and functioning of the root microbiome.

Nowadays, the use of photosynthetic parameters as metabolic markers of systemic induction by bacterial agents is increasing. Our results were similar to those obtained by Zou et al. (2005), who studied these variations in photosynthetic parameters. From our results, we could see that PGPRs inoculation (TB treatment) produced significant differences in the quantum yield of PSII (ΦPSII) (**Figure 1**). This parameter indicates the real energy that the plants are using in the photochemical processes, at any given time. It seems that PGPRs inoculation could exert a beneficial effect on promoting the physiological stage of sugar beet plants, with regard to the other treatments (ST and TT). These photosynthetic modifications induced by PGPRs have been confirmed by other authors (Van Loon et al., 1998). The NPQ values for the ST, the TT and the TB treatments were also very similar, which means that the three treatments have, *a priori*, the same energy loss at the measurement stage (**Supplementary Figure 6**). Normally, NPQ reduction is observed in plants subject to different stress conditions (Whalen et al., 1991; Yamane et al., 2008). The results of the photosynthetic parameters provided evidence that PGPRs inoculation (TB treatment) showed the highest value for ΦPSII and the NPQ, the lowest value, even though the PSII was very similar in all treatments. Besides, the TT treatment showed a very low quantum yield of PSII, the highest NPQ value, and was the treatment with the highest amount of energy loss (**Figure 1** and **Supplementary Figure 6**). The changes that occur in the photosynthetic parameters due to the use of PGPRs are not surprising because these PGPRs could be recognized as pathogen agents by plants and promote some plant-microorganisms interactions in relation to the Systemic Resistance Induced in sugar beet plants.

Although the results obtained with the biological control of cercospora and mildew were not the most successful for the TB treatment, they should nevertheless be closely analyzed, in order to design subsequent studies to perfect the application of PGPRs to sugar beet. Researchers at Montana State University (Bargabus et al., 2002, 2004) obtained a similar reduction of CLS in sugar beet plants applying a mixed microbial suspension. CLS and mildew infection indexes were higher on timepoint 2 with respect to timepoint 3 of the experiment for all the treatments (**Supplementary Figure 8**). In relation to the CLS infection index on timepoint 3, TB trials showed lower values than those obtained in TT and ST trials (**Supplementary Figure 8B**). The TB mean severity showed an intermediate value compared to the other treatments (**Supplementary Figure 9**). Bargabus et al. (2002, 2004) found that the application of *Bacillus pumilus* (strains 203-6 and 203-7) and *Bacillus mycoides* Bac J reduced the severity of CLS in sugar beet. We could conclude that *Cercospora beticola* infection was slightly lower in those PGPRs inoculated plants with respect to non-treated plants (timepoint 3) (**Figure 2**).

Therefore, co-inoculation of PGPR strains exerts a beneficial effect on sugar beet production, in such a way that physiological modifications inside the sugar beet plants increase its agricultural productivity. Qingxiao et al. (2016) have demonstrated that *B. velezensis* BAC03 can significantly enhance plant growth. Results showed that multiple applications of BAC03 were better than a single application in enhancing radish growth. This might be due to a combination of survival of the bacterium and prolonged period of maintaining the bacterial population at a high level by multiple applications. Similarly to our several PGPRs inoculations, in the research of Qingxiao et al. (2016), BAC03 was applied at five different timepoints during radish growth, including five days before planting (DBP), 1, 10, 20, and 30 DAP with the same concentration of 10^5 CFU cm^3 potting mix. Fresh and dry weight of leaves and roots were determined at harvest, 6 weeks after planting. Similar to our *Pseudomonas* inoculation, sugar beet seeds were treated with the mix bacterial suspensions for 30 min and also during sowing (Fikrettin et al., 2004).

Hence, PGPR efficacy depends on several factors, but it is assessed according to the specific PGPR strains that are used, the amount of inoculum (CFU/ml) and the plant inoculation method. As Munees and Mohammad (2011) indicated, the use of PGPR to augment crop productivity has been limited largely due to the variability and inconsistency of results observed under laboratory, greenhouse and field trials. Soil is an unpredictable environment and an intended result is sometimes difficult to achieve. Climatic variations has also a large impact on the effectiveness of PGPR but sometimes unfavorable growth conditions in the field are to be expected as a normal functioning of agriculture (Zaidi et al., 2009). Despite all these factors, there are many studies that prove the increase in crop yields following PGPR applications in the growth chambers and field trials (Munees and Mohammad, 2011).

The two assayed PGPR strains, *Pseudomonas fluorescens* Pf0-1 and *Pseudomonas chlororaphis* CECT 462, triggered a

significant increase in sugar beet production yield and quality. Our results have shown that, on the whole, the beneficial effects of PGPRs are directly observable. There were increases of sugar beet physiological and photosynthetic parameters. Indirectly, PGPRs co-inoculation did not exert a desirable biocontrol against powdery mildew and cercospora infections.

Finally, PGPRs inoculation techniques used with different crops can be complemented with more traditional agricultural techniques, as far as may be required to ensure sustainable agricultural production.

DATA AVAILABILITY STATEMENT

The original contributions presented in the study are included in the article/**Supplementary Material**, further inquiries can be directed to the corresponding author/s.

AUTHOR CONTRIBUTIONS

All authors listed have made a substantial, direct and intellectual contribution to the work, and approved it for publication.

FUNDING

This research has contributed to H2020 Project "Integrated Novel Strategies for Reducing the Use and Impact of Pesticides, Toward Sustainable Mediterranean Vineyards and Olive Groves" (Project NOVATERRA, Commission's Horizon 2020, Grant Agreement no. 101000554).

ACKNOWLEDGMENTS

We would like to express our thanks to *Koipesol SEMILLAS, S.A.* for the generous supply of the seeds; to *Sociedad Cooperativa General Agropecuaria, ACOR* for technical collaboration, especially to J. Redondo; our thanks also goes to L.M. Palencia, J.M. Palencia, E.M. García-Martínez, and M. Miranda for their generous collaboration in the field assays; and to D. Pérez-Alonso, from the University of Burgos for their technical collaboration.

SUPPLEMENTARY MATERIAL

The Supplementary Material for this article can be found online at: https://www.frontiersin.org/articles/10.3389/fpls.2020.604898/full#supplementary-material

Supplementary Figure 1 | Timelines for ST, TB and TT treatments. Six applications of a mixed PGPRs culture (*Pseudomonas fluorescens* Pf-01 and *P. chlororaphis* CECT 462) on the productivity of sugar beet were performed. Three different treatments, with four replicates in a completely randomized block design, were performed: TB, without seed coating, with PGPRs inoculum application and chemical spraying; TT, with seed coating and chemical spraying and without PGPRs inoculum and ST, without seed coating, chemical spraying and PGPRs inoculum.

Supplementary Figure 2 | Evaluation of the incidence of **(A)** Cercospora Leaf Spot, CLS and **(B)** Powdery Mildew throughout the productive cycle of sugar beet. The fungal incidences of both pathogens were scored on a four-point scale. For the assessment of fungal incidences, the following parameters were recorded: for CLS incidence, extent of necrotic leaf area with characteristic brown round and necrotic spots and for Powdery Mildew, extent of white dust-like mycelium on leaf surface. Both fungal incidences were rated on a 1–4 scale according to the extent of the affected leaf area (1: between 0 and 25%, 2: between 26 and 50%, 3: between 51 and 75% and 4: between 76 and 100%).

Supplementary Figure 3 | Aerial biomass measured at the end of the sugar beet productive cycle. Six applications of a mixed PGPRs culture (*Pseudomonas fluorescens* Pf-01 and *P. chlororaphis* CECT 462) on the productivity of sugar beet were performed. Three different treatments, with four replicates in a completely randomized block design, were performed: TB, without seed coating, with PGPRs inoculum application and chemical spraying; TT, with seed coating and chemical spraying and without PGPRs inoculum and ST, without seed coating, chemical spraying and PGPRs inoculum. A simple ANOVA was performed; letters show significant differences ($p \leq 0.05$).

Supplementary Figure 4 | Root length measured at the end of the sugar beet productive cycle. Six applications of a mixed PGPRs culture (*Pseudomonas fluorescens* Pf-01 and *P. chlororaphis* CECT 462) on the productivity of sugar beet were performed. Three different treatments, with four replicates in a completely randomized block design, were performed: TB, without seed coating, with PGPRs inoculum application and chemical spraying; TT, with seed coating and chemical spraying and without PGPRs inoculum and ST, without seed coating, chemical spraying and PGPRs inoculum. A simple ANOVA was performed; letters show significant differences ($p \leq 0.05$).

Supplementary Figure 5 | Maximum quantum yield of photosystem II (PSII) calculated as Fv/Fm for 12-weeks-old sugar beet plants (2 days after the third PGPRs inoculation, 03 July 2018).Three different treatments, with four replicates in a completely randomized block design, were performed: TB, without seed coating, with PGPRs inoculum application and chemical spraying; TT, with seed coating and chemical spraying and without PGPRs inoculum and ST, without seed coating, chemical spraying and PGPRs inoculum. A simple ANOVA was performed; letters show significant differences ($p \leq 0.05$).

Supplementary Figure 6 | Non-photochemical quenching (NPQ) for 12-weeks-old sugar beet plants (2 days after the third PGPRs inoculation, 03 July 2018). Three different treatments, with four replicates in a completely randomized block design, were performed: TB, without seed coating, with PGPRs inoculum application and chemical spraying; TT, with seed coating and chemical spraying and without PGPRs inoculum and ST, without seed coating, chemical spraying and PGPRs inoculum. A simple ANOVA was performed; letters show significant differences ($p \leq 0.05$).

Supplementary Figure 7 | Visual infection evaluation (powdery mildew) during the experiment at four timepoints. Six applications of a mixed PGPRs culture (*Pseudomonas fluorescens* Pf-01 and *P. chlororaphis* CECT 462) on the productivity of sugar beet were performed. Three different treatments, with four replicates in a completely randomized block design, were performed: TB, without seed coating, with PGPRs inoculum application and chemical spraying; TT, with seed coating and chemical spraying and without PGPRs inoculum and ST, without seed coating, chemical spraying and PGPRs inoculum. Two-way ANOVA was performed; letters show significant differences ($p \leq 0.05$). ST, TB, and TT treatments showed no significant differences (same letters are not shown).

Supplementary Figure 8 | Evaluation of the infection index, (a) powdery mildew and (b) cercospora leaf spot, CLS on timepoints 2 and 3 of the experiment. Six applications of a mixed PGPRs culture (*Pseudomonas fluorescens* Pf-01 and *P. chlororaphis* CECT 462) on the productivity of sugar beet were performed. Three different treatments, with four replicates in a completely randomized block design, were performed: TB, without seed coating, with PGPRs inoculum application and chemical spraying; TT, with seed coating and chemical spraying and without PGPRs inoculum and ST, without seed coating, chemical spraying and PGPRs inoculum. A simple ANOVA was performed; different capital letters for treatments and lowercase letters for time show significant differences ($p \leq 0.05$). In CLS infection, ST, TB and TT treatments showed no significant differences (same letters are not shown). Vertical error bars are S.E.D.

Supplementary Figure 9 | Evaluation of the severity of infection of powdery mildew and cercospora leaf spot (CLS) on timepoint two of the experiment. Six applications of a mixed PGPRs culture (*Pseudomonas fluorescens* Pf-01 and *P. chlororaphis* CECT 462) on the productivity of sugar beet were performed. Three different treatments, with four replicates in a completely randomized block design, were performed: TB, without seed coating, with PGPRs inoculum application and chemical spraying; TT, with seed coating and chemical spraying and without PGPRs inoculum and ST, without seed coating, chemical spraying and PGPRs inoculum. A simple ANOVA was performed between treatments; asterisk indicates significant differences between treatments ($p \leq 0.05$). In powdery mildew, ST, TB, and TT treatments did not shown significant differences (same letters are not shown). Vertical error bars are S.E.D.

REFERENCES

Agaras, B. C., Noguera, F., González Anta, G., Wall, L., and Valverde, C. (2020). Biocontrol potential index of pseudomonads, instead of their direct-growth promotion traits, is a predictor of seed inoculation effect on crop productivity under field conditions. *Biol. Control.* 143:104209. doi: 10.1016/j.biocontrol.2020.104209

Backer, R., Rokem, J. S., Ilangumaran, G., Lamont, J., Praslickova, D., Ricci, E., et al. (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

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

Bakker, P. A. H. M., Berendsen, R. L., Doornbos, R. F., Wintermans, P. C. A., and Pieterse, C. M. J. (2013). The rhizosphere revisited: root microbiomics. *Front. Plant Sci.* 4:165. doi: 10.3389/fpls.2013.00165

Bakker, P. A. H. M., Berendsen, R. L., Van Pelt, J. A., Vismans, G., Yu, K., Li, E., et al. (2020). The soil-borne identity and microbiome-assisted agriculture: looking back to the future. *Mol. Plant* 13, 1394–1401. doi: 10.1016/j.molp.2020.09.017

Bargabus, R. L., Zidack, N. K., Sherwood, J. W., and Jacobsen, B. J. (2002). Characterization of systemic resistance in sugar beet elicited by a non-pathogenic, phyllosphere-colonizing *Bacillus mycoides*, biological control agent. *Physiol. Mol. Plant Pathol.* 61, 289–298. doi: 10.1006/pmpp.2003.0443

Bargabus, R. L., Zidack, N. K., Sherwood, J. W., and Jacobsen, B. J. (2004). Screening for the identification of potential biological control agents that induce systemic acquired resistance in sugar beet. *Biol. Control* 30, 342–350. doi: 10.1016/j.biocontrol.2003.11.005

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

Berger, S., Papadopoulos, M., Schreiber, U., Kaiser, W., and Roitsch, T. (2004). Complex regulation of gene expression, photosynthesis and sugar levels by pathogen infection in tomato. *Physiol. Plant* 122, 419–428. doi: 10.1111/j.1399-3054.2004.00433.x

Cakmakci, R., Kantar, F., and Sahin, F. (2001). Effect of N_2-fixing bacterial inoculations on yield of sugar beet and barley. *J. Plant Nutr. Soil Sci.* 164, 527–531. doi: 10.1002/1522-2624(200110)164:5<527::aid-jpln527>3.0.co;2-1

CECT — Colección Española de Cultivos Tipo (2020). *Universidad de Valencia, Spain*. Available online at https://www.uv.es/uvweb/coleccion-espanola-cultivos-tipo/es/catalogo-cepas/medios-cultivo/buscador-cepas-1285892802374.html (accessed July 7, 2020).

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

Doberein, J., and Campelo, A. B. (1971). Non-symbiotic nitrogen fixing bacteria in tropical soils. *Plant Soil* 35, 457–470. doi: 10.1007/bf02661871

Dohm, J. C., Minoche, A. E., Holtgräwe, D., Capella-Gutiérrez, S., Zakrzewski, F., Tafer, H., et al. (2014). The genome of the recently domesticated crop plant sugar beet (*Beta vulgaris*). *Nature* 505, 546–549. doi: 10.1038/nature12817

Esteban Baselga, J. A. (1993). La remolacha azucarera en Castilla y León. *Agricul. Rev. Agropecu.* 732, 604–610.

FAOSTAT (2019). *Crops – Production/Yield Quantities of Sugar Beet*. Available online at: http://www.fao.org/faostat/en/#data/QC (accessed October 30, 2020)

Fernández-Aparicio, M., Prats, E., Emeran, A. A., and Rubiales, D. (2009). Characterization of resistance mechanisms to powdery mildew (*Erysiphe betae*) in beet (*Beta vulgaris*). *Phytopathology* 99, 385–389. doi: 10.1094/phyto-99-4-0385

Fikrettin, S., Çakmakçi, R., and Kantar, F. (2004). Sugar beet and barley yields in relation to inoculation with N2-fixing and phosphate solubilizing bacteria. *Plant Soil* 265, 123–129. doi: 10.1007/s11104-005-0334-8

Genty, B., Briantais, J. M., and Baker, R. N. (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

Gyaneshwar, P., Kumar, G. N., Parekh, L. J., and Poole, P. S. (2002). Role of soil microorganisms in improving P nutrition of plants. *Plant Soil* 245, 83–93.

Hassani, M. A., Durán, P., and Hacquard, S. (2018). Microbial interactions within the plant holobiont. *Microbiome* 6:58.

Heick, T. M., Hansen, A. L., Munk, L., Labouriau, R., Wu, K., and Jørgensen, L. N. (2020). The effect of fungicide sprays on powdery mildew and rust and yield of sugar beet in Denmark. *Crop Prot.* 135:105199. doi: 10.1016/j.cropro.2020.105199

Hiltner, L. (1904). Über neuere Erfahrungen und Probleme auf dem Gebiete der bodenbakteriologie unter besonderer berücksichtigung der gründüngung und brache. *Arb. der Dtsch. Lanwirtschaftlichen Gesellschaft* 98, 59–78.

Huang, J., Sheng, X., and He, L. (2010). Biodiversity of phosphate-dissolving and plant growth promoting endophytic bacteria of two crops. *Acta Microbiol. Sinica* 50, 710–716.

ITACYL — Instituto Tecnológico Agrario de Castilla y León (2019). *Consejería de Agricultura y Ganadería, Junta de Castilla y León*. Available online at: http://www.atlas.itacyl.es/visor (accessed November 5, 2020).

IUSS Working Group WRB (2015). *World Reference Base for Soil Resources 2014, update 2015 International Soil Classification System for Naming Soils and Creating Legends for Soil Maps*. World Soil Resources Reports No. 106. Rome: FAO.

Jay, S., Comar, A., Benicio, R., Beauvois, J., Dutartre, D., Daubige, G., et al. (2020). Scoring cercospora leaf spot on sugar beet: comparison of UGV and UAV phenotyping systems. *Plant Phenom.* 2020:9452123, 1–18.

Kamou, N. N., Cazorla, F., Kandylas, G., and Lagopodi, A. L. (2020). Induction of defense-related genes in tomato plants after treatments with the biocontrol agents *Pseudomonas chlororaphis* ToZa7 and *Clonostachys rosea* IK726. *Arch. Microbiol.* 202, 257–267. doi: 10.1007/s00203-019-01739-4

Khan, M., Smith, L., Bredehoeft, M., Roehl, S., and Fischer, J. (2001). *Cercospora Leaf Spot Control in Eastern North Dakota and Minnesota in 2000, Sugar Beet Research and Extension Report*. Fargo, ND: North Dakota State University & University of Minnesota.

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

Krause, G. H., and Weis, E. (1991). Chlorophyll fluorescence and photosynthesis: the basics. *Annu. Rev. Plant Phys.* 42, 313–349.

Kumar, A., Patelc, J. S., Meenad, V. S., and Ramtekee, P. W. (2019). Plant growth-promoting rhizobacteria: strategies to improve abiotic stresses under sustainable agriculture. *J. Plant Nutr.* 42, 1402–1415. doi: 10.1080/01904167.2019.1616757

Kusstatscher, P., Cernava, T., Harms, K., Maier, J., Eigner, H., Berg, G., et al. (2019a). Disease incidence in sugar beet fields is correlated with microbial diversity and distinct biological markers. *Phytobiomes J.* 3, 22–30. doi: 10.1094/pbiomes-01-19-0008-r

Kusstatscher, P., Zachow, C., Harms, K., Maier, J., Eigner, H., Berg, G., et al. (2019b). Microbiome-driven identification of microbial indicators for postharvest diseases of sugar beets. *Microbiome* 7:112.

Levall, M. W., and Bornman, J. F. (2000). Differential response of a sensitive and tolerant sugarbeet line to *Cercospora beticola* infection and UV-B radiation. *Physiol. Plant* 109, 21–27. doi: 10.1034/j.1399-3054.2000.100104.x

MAPA. (1994). *Métodos Oficiales de Análisis, : Suelos y Aguas*, Vol. 3. Madrid: MAPA.

Mendes, R., Kruijt, M., de Bruijn, I., Dekkers, E., van der Voort, M., Schneider, J. H. M., et al. (2011). Deciphering the rhizosphere microbiome for disease suppressive bacteria. *Science* 332, 1097–1100. doi: 10.1126/science.1203980

Mia, M. A. B., Shamsuddin, Z. H., and Mahmood, M. (2010). Use of plant growth promoting bacteria in banana: a new insight for sustainable banana production. *Int. J. Agric. Biol.* 12, 459–467.

Monds, R. D., Newell, P. D., Schwartzman, J. A., and O'Toole, G. A. (2006). Conservation of the Pho regulon in *Pseudomonas fluorescens* Pf0-1. *Appl. Environ. Microbiol.* 72, 1910–1924. doi: 10.1128/aem.72.3.1910-1924.2006

Munees, A., and Mohammad, S. K. (2011). Functional aspects of plant growth promoting rhizobacteria: recent advancements. *Insight Microbiol.* 1, 39–54. doi: 10.5567/imicro-ik.2011.39.54

Nandakumar, R., Viswanathan, R., Babu, S., Shella, J., Raghuchander, T., and Samiyappan, R. (2001). A new bio-formulation containing plant growth promoting rhizobacterial mixture for the management of sheath blight and enhanced grain yield in rice. *Biocontrol* 46, 493–510.

Naureen, Z., Price, A. H., Hafeez, F., and Roberts, M. R. (2009). Identification of rice blast disease-suppressing bacterial strains from the rhizosphere of rice grown in Pakistan. *Crop Prot.* 28, 1052–1060. doi: 10.1016/j.cropro.2009.08.007

Niu, X., Song, L., Xiao, Y., and Ge, W. (2018). Drought-tolerant plant growth-promoting rhizobacteria associated with foxtail millet in a semi-arid and their potential in alleviating drought stress. *Front. Microbiol.* 8:2580.

OECD/FAO (2020). "Table C.3 – world sugar projections," in *OECD-FAO Agricultural Outlook 2020-2029*, (Paris: OECD Publishing). Available online at: https://www.oecd-ilibrary.org/agriculture-and-food/world-sugar-projections_b62e023c-en (accessed June 23, 2020).

Ögren, E., and Baker, N. R. (1985). Evaluation of a technique for the measurement of chlorophyll fluorescence from leaves exposed to continuous white light. *Plant Cell Environ.* 8, 539–547. doi: 10.1111/j.1365-3040.1985.tb01691.x

Oku, S., Komatsu, A., Tajima, T., Nakashimada, Y., and Kato, J. (2012). Identification of chemotaxis sensory proteins for amino acids in *Pseudomonas fluorescens* Pf0-1 and their involvement in chemotaxis to tomato root exudate and root colonization. *Microbes Environ.* 27, 462–469. doi: 10.1264/jsme2.me12005

Pan, L., Zhu, O., Lu, R., and McGrath, J. M. (2015). Determination of sucrose content in sugar beet by portable visible and near-infrared spectroscopy. *Food Chem.* 167, 264–271. doi: 10.1016/j.foodchem.2014.06.117

Penrose, D. M., Moffatt, B. A., and Glick, B. R. (2001). Determination of 1-aminocycopropane-1-carboxylic acid (ACC) to assess the effects of ACC deaminase-containing bacteria on roots of canola seedlings. *Can. J. Microbiol.* 47, 77–80. doi: 10.1139/w00-128

Pooja, K., Krishna, K. C., Akhileshwar, K. S., and Amit, K. S. (2019). "PGPR bioelicitors: induced systemic resistance (ISR) and proteomic perspective on biocontrol," in *PGPR Amelioration in Sustainable Agriculture -Food Security and Environmental Management*, eds K. S. Amit, K. Ajay, and K. S. Pawan (Cambridge: Woodhead Publishing), 67–84. doi: 10.1016/b978-0-12-815879-1.00004-5

Qingxiao, M., Helen, J., and Jianjun, H. (2016). Effects of *Bacillus velezensis* strain BAC03 in promoting plant growth. *Biol. Control* 98, 18–26. doi: 10.1016/j.biocontrol.2016.03.010

Raaijmakers, J. M., and Mazzola, M. (2016). Soil immune responses. *Science* 352, 1392–1394. doi: 10.1126/science.aaf3252

Robert, C., Bancal, M. O., Lannou, C., and Ney, B. (2006). Quantification of the effects of *Septoria tritici* blotch on wheat leaf gas exchange with respect to lesion age, leaf number, and leaf nitrogen status. *J. Exp. Bot.* 57, 225–234.

Rodríguez-Moreno, L., Pineda, M., Soukupová, J., Macho, A. P., Beuzón, C. R., Barón, M., et al. (2008). Early detection of bean infection by *Pseudomonas syringae* in asymptomatic leaf areas using chlorophyll fluorescence imaging. *Photosynth. Res.* 96, 27–35. doi: 10.1007/s11120-007-9278-6

Sacristán Pérez-Minayo, G., Reguera-Useros, J. I., López-Robles, D. J., García-Villaraco, A., and Gutiérrez-Mañero, F. J. (2011). Evaluation of biocontrol agro-techniques against *R. solani*: study of microbial communities catabolic profile modifications. *J. Agric. Sci.* 149, 595–607. doi: 10.1017/s0021859611000025

Santiago, C. D., Yagi, S., Ijima, M., Nashimoto, T., Sawada, M., Ikeda, S., et al. (2017). Bacterial compatibility in combined inoculations enhances the growth of potato seedlings. *Microbes Environ.* 32, 14–23. doi: 10.1264/jsme2.me16127

Santner, A., Calderon-Villalobos, L. I. A., and Estelle, M. (2009). Plant hormones are versatile chemical regulators of plant growth. *Nat. Chem. Biol.* 5, 301–307. doi: 10.1038/nchembio.165

Scher, F. M., and Baker, R. (1982). Effect of *Pseudomonas putida* and a synthetic iron chelator on induction of soil suppressiveness to *Fusarium* wilt pathogens. *Phytopathology* 72, 1567–1573. doi: 10.1094/phyto-72-1567

Stanek, V., and Pavlas, P. (1934). Über eine schnelle, informative methode zur bestimmung des schädlichen stickstoffes, der amide und der aminosäuren in der rübe. *Zuckerindustrie Czechoslov. Republic* 59, 129–142.

Stringlis, I., Zhang, H., Pieterse, C., Bolton, M., and de Jonge, R. (2018). Microbial small molecules – weapons of plant subversion. *Nat. Prod. Rep.* 35, 410–433. doi: 10.1039/c7np00062f

Suslow, T. V., and Schroth, M. N. (1982). Rhizobacteria of sugar beets: effects of seed application and root colonization on yield. *Phytopathology* 72, 199–206. doi: 10.1094/phyto-72-199

Van Loon, L. C., Bakker, P. A. H. M., and Pieterse, C. M. J. (1998). Systemic resistance induced by rhizosphere bacteria. *Annu. Rev. Phytopathol.* 36, 453–483. doi: 10.1146/annurev.phyto.36.1.453

Van Zwieten, L., Rust, J., Kingston, T., Merrington, G., and Morris, S. (2004). "Influence of copper fungicide residues on occurrence of earthworms in avocado orchard soils. *Sci. Total Environ.* 329, 29–41. doi: 10.1016/j.scitotenv.2004.02.014

Weijuan, H., Donglei, S., Jiantao, F., Huanhuan, Z., Ronghua, W., and Yuxing, A. (2020). Effects of continuous sugar beet cropping on rhizospheric microbial communities. *Genes* 11:13. doi: 10.3390/genes11010013

Whalen, M. C., Innes, R. W., Bent, A. F., and Staskawicz, B. J. (1991). Identification of *Pseudomonas syringae* pathogens of *Arabidopsis* and a bacterial locus determining avirulence on both *Arabidopsis* and soybean. *Plant Cell* 3, 49–59. doi: 10.2307/3869199

Wieninger, L., and Kubadinow, N. (1973). Die Stickstoffdüngung und ihre auswirkungen auf technologische qualitätsmerkrnale der zuckerrübe. *Zucker* 26, 65–70.

Wolfgang, A., Zachow, C., Müller, H., Grand, A., Temme, N., Tilcher, R., et al. (2020). Understanding the impact of cultivar, seed origin, and substrate on bacterial diversity of the sugar beet rhizosphere and suppression of soil-borne pathogens. *Front. Plant Sci.* 11:560869. doi: 10.3389/fpls.2020.560869

Würschum, T., Reif, J. C., Kraft, T., Janssen, G., and Zhao, Y. (2013). Genomic selection in sugar beet breeding populations. *BMC Genet.* 14:85. doi: 10.1186/1471-2156-14-85

Yamane, K., Kawasaki, M., Taniguchi, M., and Miyake, H. (2008). Correlation between chloroplast ultrastructure and chlorophyll fluorescence characteristics in the leaves of rice (*Oryza sativa* L.) grown under salinity. *Plant Prod. Sci.* 11, 139–145. doi: 10.1626/pps.11.139

Yang, S. F., and Hoffman, N. E. (1984). Ethylene biosynthesis and its regulation in higher plants. *Annu. Rev. Plant Physiol. Plant Mol. Biol.* 35, 155–189. doi: 10.1146/annurev.pp.35.060184.001103

Zachow, C., Fatehi, J., Cardinale, M., Tilcher, R., and Berg, G. (2010). Strain specific colonization pattern of *Rhizoctonia* antagonists in the root system of sugar beet. *FEMS Microbiol. Ecol.* 74, 124–135. doi: 10.1111/j.1574-6941.2010.00930.x

Zachow, C., Tilcher, R., and Berg, G. (2008). Sugar beet-associated bacterial and fungal communities show a high indigenous antagonistic potential against plant pathogens. *Microb. Ecol.* 55, 119–129. doi: 10.1007/s00248-007-9257-7

Zaidi, A., Khan, M. S., Ahemad, M., and Oves, M. (2009). Plant growth promotion by phosphate solubilizing bacteria. *Acta Microbiol. Immunol. Hung.* 56, 263–284. doi: 10.1556/amicr.56.2009.3.6

Zou, J., Rodriguez-Zas, S., Aldea, M., Li, M., Zhu, J., Gonzalez, D., et al. (2005). Expression profiling soybean response to *Pseudomonas syringae* reveals new defence-related genes and rapid HR specific downregulation of photosynthesis. *Mol. Plant Microbe Interact.* 18, 1161–1174. doi: 10.1094/mpmi-18-1161

Conflict of Interest: LM-B was employed by the company Syngenta.

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: 11 January 2021
doi: 10.3389/fmicb.2020.599829

Endosphere Microbiome and Metabolic Differences Between the Spots and Green Parts of *Tricyrtis macropoda* Leaves

*Yan Wang[1], Huyin Cheng[2], Fan Chang[1], Le Zhao[3], Bin Wang[4], Yi Wan[1] and Ming Yue[5]**

[1] Microbiology Institute of Shaanxi, Xi'an, China, [2] College of Pharmacy, Shaanxi University of Chinese Medicine, Xianyang, China, [3] School of Biological Sciences and Engineering, Shaanxi University of Technology, Hanzhong, China, [4] College of Chemistry, Biology and Materials Science, East China University of Technology, Nanchang, China, [5] School of Life Sciences, Northwest University, Xi'an, China

OPEN ACCESS

Edited by:
Christos Zamioudis, Democritus University of Thrace, Greece

Reviewed by:
Yi Song, University of British Columbia, Canada
Karin E. Groten, Max Planck Institute for Chemical Ecology, Germany

***Correspondence:**
Ming Yue
yueming@nwu.edu.cn

Specialty section:
This article was submitted to Microbe and Virus Interactions with Plants, a section of the journal Frontiers in Microbiology

Received: *28 August 2020*
Accepted: *24 November 2020*
Published: *11 January 2021*

Citation:
Wang Y, Cheng H, Chang F, Zhao L, Wang B, Wan Y and Yue M (2021) Endosphere Microbiome and Metabolic Differences Between the Spots and Green Parts of Tricyrtis macropoda Leaves. Front. Microbiol. 11:599829. doi: 10.3389/fmicb.2020.599829

Background: Plant leaves are important organs for photosynthesis and biological energy production. The leaves of *Tricyrtis macropoda* have an unusual spotted pattern. However, whether the spots of *T. macropoda* affect the plant microbiome and metabolites is unclear. In this study, we compared differences in the endosphere microbiome and plant metabolites in green parts and spots and the effects of spots on the photosynthesis of leaves.

Methods: 16S/ITS sequences and metabolite spectra were obtained by high-throughput amplicon sequencing and ultra-high-performance liquid chromatography–high-resolution mass spectrometry, respectively. Changes in the diversity of the endophytic microbial community and metabolites were studied, and the effect of *T. macropoda* leaf spots on photosynthesis was examined by chlorophyll fluorescence.

Results: The results showed that the relative abundance of *Cercospora* fungi in the leaf spots of *T. macropoda* was significantly higher than that in the green parts ($P < 0.05$) while *Colletotrichum* fungi showed low abundance in the spots. Alkaloid and ketone metabolites were decreased in the green parts compared with the spots, and amino acids, organic acids, lipids, and other compounds were increased in the green parts compared with the spots. A combined analysis of microbial communities and metabolites showed a significant correlation between the endophytic fungal communities and metabolite production. The changes in these metabolites may cause changes in local leaf color. In addition, we found that the spot areas of *T. macropoda* can be photosynthetically normal.

Conclusion: This research showed the relationship between endophytic microorganisms and metabolites, and the findings advance our understanding of endophyte–plant interactions and provide a new direction for investigating the relationship between endophytes and phenotypes.

Keywords: *Tricyrtis macropoda*, microbiome, metabolomics, chlorophyll fluorescence, diversity

INTRODUCTION

The leaf spots of *Tricyrtis macropoda* have a peculiar pattern. In the natural environment, the adaxial side of the leaf presents dark brown irregular spots that are usually 5–15 mm in diameter and nearly round, and these leaf spots only occur in three to six leaves of the plant after germination. Leaves far above the ground produce few or no spots (**Supplementary Figures 1A,B**). In addition, patterns can be formed on the leaves of many angiosperms, such as stripes, spots, or complex designs [e.g., *Orchidaceae* (*Goodyera schlechtendaliana* Rchb. f.), *Liliaceae* (*Drimiopsis kirkii* Baker, *Chlorophytum comosum* f. variegata), *Begoniaceae* (*Begonia cathayana* Hemsl, *Begonia masoniana* Irmsch.), and *Euphorbiaceae* (*Codiaeum variegatum* Juss.)], which are one of the factors that characterize angiosperm biodiversity (Glover, 2014). Moreover, the leaf spots exhibit specific arrangements. Variations in the leaf color in plants will inevitably cause changes in the photosynthetic physiological indices (Wang Z. X. et al., 2016; Chen K. Y. et al., 2018; Du et al., 2019).

Tricyrtis macropoda is a perennial herb in the genus *Tricyrtis* Wall in Liliaceae. This plant is found in regions of China, Korea, and Japan in East Asia and mainly distributed throughout forests, grassy areas, or rock crevices in mountainous areas at altitudes of 800–2,400 m (National Pharmacopoeia Commission, 2015). Because of the limited global distribution and lack of a good reference genome for this species, the mechanism of leaf spot formation in *T. macropoda* and its physiological significance in plants are unclear.

Hara (1957) studied the leaf spots of 55 species of plants in 24 families and divided the causes of leaf spots into two categories, each including two types: structural types (including the epidermal type and interstitial type) and pigment types (including the chlorophyll type and pigment type). The structural type of leaf spot results from variation in epidermal cells, causing light interference, diffraction, refraction, and void structures and causing light to reflect twice, with both of these reflections changing the path of incident light on the surface and inside the leaves and then affecting the absorption and reflection spectra of the leaves. These phenomena cause the leaves to appear blue, white, silvery white, light green, or silvery green and form structural leaf spots that affect color (Sheue et al., 2012). Chlorophyll in leaves is an important photosynthetic pigment (Pilar et al., 2016). The chlorophyll type of leaf spot is mainly caused by variations in the chloroplast structure, and the obstruction of chlorophyll synthesis leads to white or yellow leaves. Significant differences are not observed in the tissue structure between leaf spots and the normal green parts of the leaves, although the photosynthetic rate is significantly lower in the leaf spots (Yang, 2015; Li et al., 2017). Finally, the pigment type of leaf spot is caused by anthocyanins, which yield red, purple, and other colors in decorative patterns (Du et al., 2017).

Recent studies have also shown that the leaf color of *Blastus cochinchinensis* Lour. results from a variety of mechanisms, such as epidermal cells, intercellular space, mesophyll cells, chloroplast variation and crystal interaction, which strengthen the white spots in the seedlings (Wang Z. X. et al., 2016; Chen et al., 2017). Gene expression or inhibition often leads to variations in chlorophyll and anthocyanin synthesis (Cho et al., 2016; Gu et al., 2019). Plant microbiota, which is often called the second or extended genome of the host, may directly affect the metabolic activity of plants (Khan et al., 2011; Brader et al., 2014; Huang et al., 2018), and it provides plants with a large number of functional capabilities that can aid in the metabolic processes of host plants encoded by their genomes (Berendsen et al., 2012; Berg et al., 2014; Chen H. H. et al., 2018; Huang et al., 2018). In addition, some microorganisms may infect leaves, thus leading to the formation of plant leaf spots that are often harmful to plants (Khizar et al., 2020; Lin et al., 2020). For example, *Pseudocercospora fuligena* will cause tomato leaves to show melatonin spots (Kang et al., 2019). *Pestalotioid* fungi are one of the major agents underlying leaf spots on mango, and their early foliar symptoms on leaves are small yellow-to-brown lesions. These spots expand with uneven borders, turn from white to gray, and coalesce to form larger gray patches (Shu et al., 2020). *Colletotrichum spaethianum* leads to leaf spots in *Polygonatum odoratum* (Liu et al., 2020). However, the mechanisms of leaf spot formation in *T. macropoda* are currently unclear. Lynch and Hsiao (2019) reviewed the powerful influences of microbial communities associated with animals on host physiology. These microbes regulate metabolism and immune function as well as complex host behaviors. Whether microbial communities associated with plants also affect host physiology, phenotypes, metabolism, and complex immune functions to some extent is of considerable interest.

In this study, the differences in the endophytic microbial community and metabolites between spots and non-spot areas are discussed. We investigated the correlations between the microbiome and metabolites. In this study, we asked three main questions: (1) Are leaf spots related to colonization by microorganisms? (2) Is microbial colonization related to changes in plant metabolites? (3) What is the effect of leaf spots on plant photosynthesis?

MATERIALS AND METHODS

Study Location and Processing of Samples

Tricyrtis macropoda was collected from the northern slope of the Qinling Mountains in China at 107°29′40″E, 34°01′38″N and an altitude of 1,644 at 10 m interval. Complete and healthy *T. macropoda* plants were collected and brought back to the laboratory as soon as possible (total of six plant samples). In the laboratory, among the six plant samples, the leaves of each plant sample were divided into three groups (**Supplementary Figure 1C**). In the first group, the leaves were dark-adapted for 20 min, washed with sterile water to remove surface dust, and placed on a flat tray with the adaxial surface facing upward for a chlorophyll fluorescence experiment. In the second group, the leaves were collected from six samples, washed with sterile water to remove surface dust, separated into spotted and non-spotted parts, and frozen in liquid nitrogen (30 s). After the liquid nitrogen treatment, the tissues were used to extract metabolites. In the third group, the leaves from six samples were collected, washed in 75% alcohol for 2 min, treated with 5% hypochlorite

for 3 min, washed with sterile water three times, and cleared of surface microbes. Then, the spotted and non-spotted parts of the leaves were separated and frozen in liquid nitrogen (30 s). After the liquid nitrogen treatment, total DNA was extracted with a DNA extraction kit. All samples and backup samples were stored at –80°C for further experiments.

DNA Extraction and Sequencing

DNA was extracted from 100-mg samples from the spotted and non-spotted parts of the *T. macropoda* leaves using magnetic beads and a plant genomic DNA extraction kit (Tiangen Plant Genomic DNA Extraction Kit DP342) following the kit instructions. The internal transcribed spacer regions of the fungal ribosomal RNA gene were amplified by PCR using the primers ITS1-1F-F CTTGGTCATTTAGAGGAAGTAA and ITS1-1F-R GCTGCGTTCTTCATCGATGC (Xiong et al., 2016). The bacterial 16S ribosomal RNA genes were amplified by PCR using the primers 341F-CCTAYGGGRBGCASCAG and 806R-GGACTACNNGGGTATCTAAT (Charlotte et al., 2014). PCR was carried out using a 20-μl mixture containing 4 μl of 5 × FastPfu buffer, 0.8 μl of primer (5 mm), 2 μl of 2.5 mM dNTPs, 0.4 μl of Fast Pfu polymerase, and 10 ng of template DNA. The amplification products were extracted from 2% agarose gel, and the AxyPrep DNA gel extraction kit (Axygen Bioscience, United City, CA, United States) was used. Purifications were carried out according to the manufacturer's instructions and quantified by QuantiFluor-St (Promega, Durham, NC, United States).

The purified PCR products were measured by Qubit 3.0 (Life Invitrogen, Waltham, MA, United States). The Illumina library was constructed using polymerized DNA products according to the preparation process of the Illumina genomic DNA library. The amplified library was paired and sequenced on the Illumina MiSeq platform (Beijing Novosource Bioinformation Technology Co., Ltd., Beijing, China) according to the standard protocol. The original data are stored in the National Center for Biotechnology Information (NCBI) sequence and the archived database (SRA: SAMN14490841), and they are accessible via the link https://www.ncbi.nlm.nih.gov/biosample/14490841.

Sequence Processing

Using the analytical platform of the research center, the original 16S sequences were first obtained by FastQC software, the sequences of fungi less than 200 bp and bacteria less than 400 bp in length were filtered, and the primers were deleted by the Cutadapt 1.18 program. Then, Usearch (version 11)[1] was used for follow-up analysis of biological information. The fastq_mergepairs command of Usearch was used to merge paired end sequences, the fastq_filter command was used to control sequence quality, the Unoise3 algorithm was used for operational taxonomic unit (OTU)-like (sub-OTU) non-parametric clustering, and the fastx_uniques command was used to remove redundant and singleton sequences (the minimum parameter was eight). After removing chimeras with Usearch (Edgar, 2010), the similarity of OTUs was 97%. An OTU table was generated. After clustering, the sequences were annotated with the UNITE database, and a cutoff value of 0.8 obtained by the Sintax method was used. The OTU table was constrained by using the smallest number of sequences in the grouping. Annotation was performed with the Ribosomal Database Project (RDP) and UNITE reference databases (v7.1) for the bacterial and fungal communities, respectively, (Abarenkov et al., 2010). Mitochondrial, chloroplast, plant, and protoplast entries were deleted, and the unclassified contaminant sequences were filtered out. There were 916 OTU sequences in the final community data set. After analyzing the complete data set, the leaf spots and green parts were separated to assess the differences between them.

[1] http://www.drive5.com/usearch/

Microbiome Statistical Analysis

Statistical analyses were performed using R 3.5.1 (R Foundation for Statistical Computing, Vienna, Austria) (30). The parameters of alpha diversity and beta diversity were calculated by Usearch (Lozupone et al., 2006). Analysis of variance (ANOVA) was performed to analyze the overall differences, Student's *t*-test was used to analyze the differences between groups, and the differences in the alpha diversity index between leaf spots and the green part of leaves were studied. Beta diversity was calculated using the binary Jaccard algorithm for principal coordinate analysis (PCoA) and then visualized using PERMANOVA ordinations to illustrate compositional differences. The unweighted pair group method with arithmetic mean (UPGMA) procedure was used in the cluster analysis to measure the evolutionary distances between samples. Venn diagrams were used to show the numbers of common and unique OTUs among samples (Hanbo and Paul, 2011) and intuitively visualize the coincidence of OTUs among samples. The error rate of each type of ANOVA model was corrected by the false discovery rate (FDR). Quantitative Insights Into Microbial Ecology (QIIME) software was used to select the OTU sequence with the highest abundance at the taxonomic level for the species analysis to determine the frequency of bacteria and fungi in different parts of the leaves.

Metabolite Extraction

Fifty milligrams of each sample were weighed, and then 1,000 μl of extract [methanol:acetonitrile:water = 2:2:1 (V/V)] was added. The samples were vortexed for 30 s, homogenized at 40 Hz for 4 min, and sonicated for 5 min in an ice-water bath. The homogenization and sonication cycle was repeated three times, followed by incubation at −20°C for 1 h and centrifugation at 12,000 rpm and 4°C for 15 min. The resulting supernatants were transferred to liquid chromatography–mass spectrometry (LC–MS) vials and stored at −80°C until ultra-high-performance liquid chromatography (UHPLC)–quadrupole/electrostatic field (QE) Orbitrap/MS analysis was performed. The quality control (QC) sample was prepared by mixing equal aliquots of the supernatants from all of the samples (Doppler et al., 2016) and used for the metabolomic analysis.

Metabolite Profiling

LC-MS/MS analyses were performed using a UHPLC system (1,290, Agilent Technologies) with a UPLC HSS T3 column

(2.1 mm × 100 mm, 1.8 μm) coupled to a Q Exactive instrument (Orbitrap MS, Thermo). Mobile phase A was 0.1% formic acid in water (positive mode) and 5 mmol/L ammonium acetate in water (negative mode), and mobile phase B was acetonitrile. The elution gradient was as follows: 0 min, 1% B, 1 min, 1% B, 8 min, 99% B, 10 min, 99% B, 10.1 min, 1% B, and 12 min, 1% B. The flow rate was 0.5 ml/min (16 min, 1% B), and the injection volume was 3 μl. A QE mass spectrometer was used to acquire MS/MS spectra on an information-dependent basis (IDB) during the LC/MS experiment. In this mode, the acquisition software (Xcalibur 4.0.27, Thermo) continuously evaluated the full-scan survey MS data during data collection and triggered the acquisition of MS/MS spectra. Electrospray ionization (ESI) source conditions were set as follows: sheath gas flow rate of 45 Arb, aux gas flow rate of 15 Arb, capillary temperature of 400°C, full MS resolution of 70,000, MS/MS resolution of 17,500, collision energy of 20/40/60 eV in the chemical non-equilibrium (NCE) model, and a spray voltage of 4.0 kV (positive mode) or −3.6 kV (negative mode) (Wang J. L. et al., 2016).

Data Preprocessing and Annotation

To explore the composition of the metabolites of the leaf spot areas of *T. macropoda*, Simca software (v15.0.2, Sartorius Stedim Data Analytics AB, Umeå, Sweden) was used to process the data via log conversion and centralized treatment, and automatic modeling was then carried out to perform principal component analysis (PCA) (Wiklund et al., 2008). The raw data were converted to mzXML format using Proteo Wizard. MAPS software (version 1.0) was used to correct the retention time, mass-to-charge ratio (m/z), peak intensity, peak extraction, peak integral, and peak alignment. An in-house MS2 database and R were used for metabolite identification.

Multivariate Statistical Analysis

The peak mass intensity of each sample was normalized and Pareto-scaled with Simca P software (version 12.0, Umetrics, Umeå, Sweden). PCA and orthogonal partial least squares discriminant analysis (OPLS–DA) were used to study the differences in metabolite composition among 12 samples (2 leaf parts × 6 biological replicates). The first component was used to extract the reliability [P(corr)] value of all metabolites in the OPLS–DA. We selected metabolites satisfying the following criteria as potential markers: (1) high confidence [$|P(\text{corr})| > 0.6$] in discrimination between the spots and green parts of leaves, (2) mean intensities in leaf spots that were significantly different from those in the green parts of leaves ($P < 0.05$), and (3) a minimum two-fold change in level between leaves and green spots. The P-value was calculated using an independent two-sample t-test.

Integrative Analysis of the Metabolome and Microbiome

Based on the endophyte community annotation, at the genus level, the fungal community members with an abundance greater than 0.5% and the identified differential metabolites were screened. A correlation analysis was carried out using the Spearman algorithm, and the correlation P-value was less than 0.05. Based on these results, the relationship between the microbial and metabolite groups was determined by visualization in R.

RESULTS

Metabolic Differences Between the Spots and Green Parts of Leaves

To compare the metabolite pattern between the spots and green parts, we first performed a PCA for the features obtained in positive ion mode. The cumulative amount of variation explained by the X variable (PC1) was 39.5%, while that explained by the Y variable (PC2) was 24.1% (**Figure 1**), and the corresponding values for the results obtained in negative ion mode were 46.1 and 24.6%, respectively. Furthermore, the spots and green parts of leaves were significantly separated by the PCA (**Figure 1**), which showed that the composition of metabolites was different.

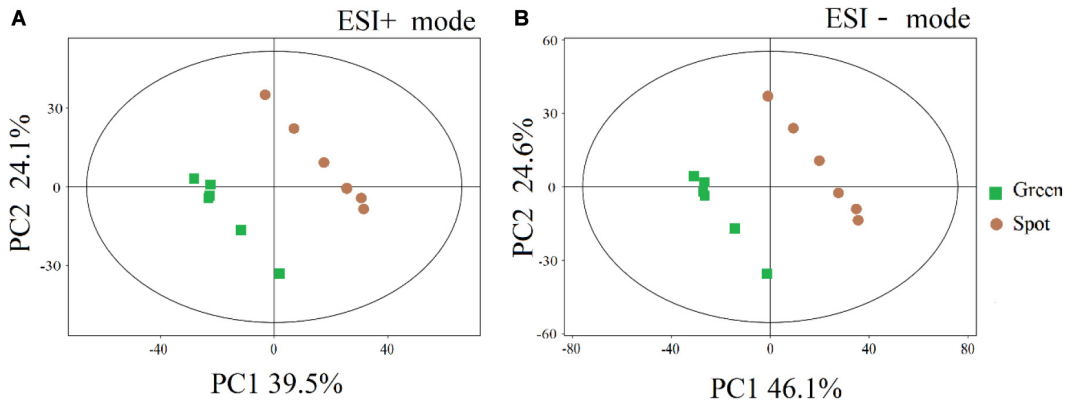

FIGURE 1 | PCA scores of different parts of the leaves. **(A)** Electrospray ionization positive mode. **(B)** Electrospray ionization negative mode. The abscissa PC1 and ordinate PC2 represent the scores of the first and second principal components, respectively, and the color and shape of points represent the experimental groups of samples. All samples are within the 95% confidence intervals.

In addition, a plot of OPLS-DA scores was used to examine the difference between the two parts (**Supplementary Figures 2A,B**), and the prediction value of the model was more than 95%. The OPLS-DA permutation test similarly proved this separation (**Supplementary Figure 2C,D**).

Screening of Differential Metabolites

Based on the inherent characteristics of QE metabolomics data, 28,570 mass features were measured and 527 (positive 351, negative 176) metabolites were identified from mass spectrometry. We screened 450 different metabolites with significant differences ($P < 0.05$). There were 244 upregulated species and 196 downregulated species (**Supplementary Figure 3**). The detected differential metabolites often had similar/complementary results and biological functions or were positively/negatively regulated by the same metabolic pathway, thus leading to similar or opposite expression characteristics between experimental groups. We identified 138 metabolites with such characteristics (**Supplementary Table 1**). A hierarchical cluster analysis of identified substances and a quantitative calculation with a Euclidean distance matrix were carried out. The metabolites with the same characteristics were classified into one group. Hierarchical clustering of the metabolite patterns revealed two clusters (**Figure 2**). In the first cluster, most of the alkaloids (5/7) and ketones (8/11) were detected. These ions were less abundant in the spots than in the green parts. In addition, 5 polar amino acids, 20 organic acids, and 4 lipids were less abundant in the spots than in the green parts (**Supplementary Table 2**). The second cluster was mainly composed of amino acids (6 types), aldehydes (4 types), sugars (4 types), organic acids (16 types), and lipids (5 types). These ions were more abundant in the spots than in the green parts. The dynamic changes in these metabolites may be related to changes in metabolic pathways, and the significantly increased metabolites may be the cause of the change in leaf color.

Analysis of Microbial Community Diversity in Spotted and Non-Spotted Parts

We analyzed the diversity and community composition of bacteria and fungi in different parts of the leaves (spotted and non-spotted parts). We found similar bacterial species richness ($P = 0.658$) and fungal species richness ($P = 0.645$) between the spotted and non-spotted parts. Similarly, alpha diversity parameters (ACE, Chao1, Shannon, and Simpson indices) of the microbiome of the green leaf parts and spots were not significantly different. The abundances were also similar between the two parts of leaves. We also calculated the community diversity of the two groups of samples. The fungal and bacterial diversities in the green parts were greater than those in the spots (**Figure 3**).

We also evaluated the beta diversity of endophytes in the spotted and non-spotted parts of the leaves, compared and determined the composition of the endophytes in the different parts of leaves, calculated a binary Jaccard dissimilarity matrix, and showed the overall similarity in microbial community structure among the samples by PCoA (**Figures 4A,B**). In addition, we used the UPGMA cluster analysis to reveal changes in community composition (**Figures 4C,D**). The PCoA showed no significant clustering of the bacterial community between the spotted and non-spotted parts (**Figure 4A**), although the fungal community displayed stronger clustering (**Figure 4B**). At the OTU level, PC1 explained 37.2% of the total variation, PC2 explained 14.8%, and the cumulative variance explained by the two variables was 52%. Hierarchical clustering of the samples was based on the binary Jaccard dissimilarity values, which were

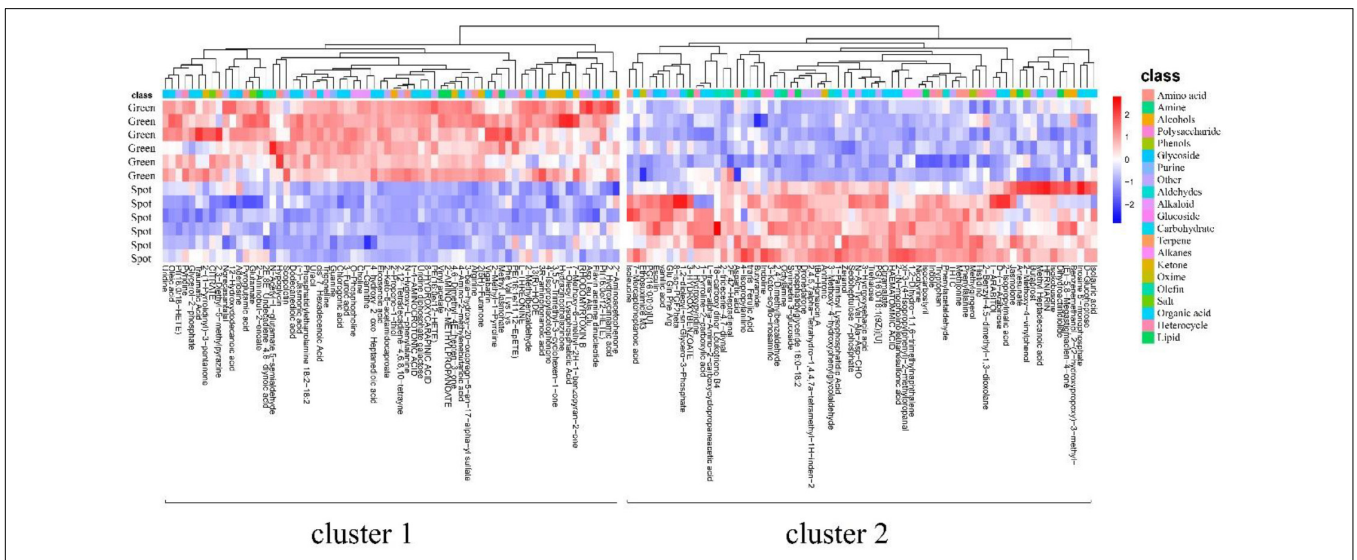

FIGURE 2 | Metabolite patterns detected in two clusters. The abscissa represents the different experimental groups, the ordinate represents metabolites compared between the groups, and the colored blocks at different positions represent the relative expression of metabolites at those positions. The metabolites for which VIP > 1 and $P < 0.05$ were considered to be significantly changed.

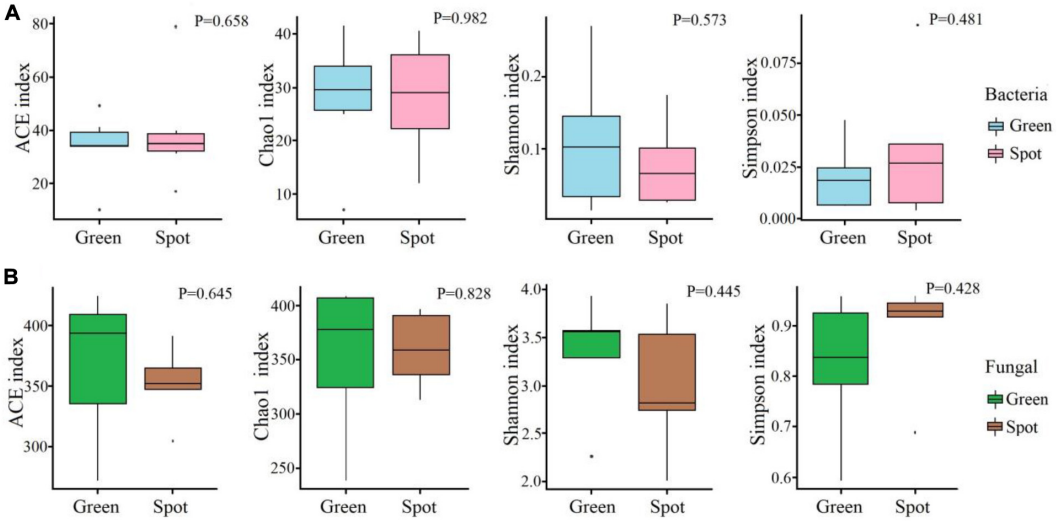

FIGURE 3 | Alpha diversity of the microbiome communities in different parts. **(A)** Alpha diversity index of the bacterial communities. **(B)** Alpha diversity index of the fungal communities. The *t*-test method was used to study the alpha diversity. The box diagram shows the first (25%) and third (75%) quartiles, median values, and maximum and minimum observational values in each data set. The alpha diversity estimation is presented for the samples of green areas and spots on leaves.

superimposed on the PCoA plot. The hierarchical clustering of fungi (at the OTU level) revealed complete clustering. To support the clustering results of the leaf fungal community obtained via the PCoA, an analysis of similarities (ANOSIM) was performed, and it indicated a significant difference between leaf spots and non-spots areas ($R = 0.804$, $P = 0.009$) (**Figures 4E,F**).

Differences in Microbial Community Composition Between the Spotted and Non-Spotted Parts

In this study, we analyzed the division of fungi and bacteria at different levels. The results revealed that the bacteria in the green and spotted areas were mainly Proteobacteria (green 99.49%, spot 99.63%) (**Figure 5A**) while the fungi were mainly concentrated in Ascomycota (80.27%), Basidiomycota (7.92%), and Mortierellomycota (0.22%) (**Figure 5B**). There was no significant difference in the species of dominant organisms (fungi and bacteria) between the spotted and non-spotted parts. In addition, we analyzed the differences in microorganisms at different levels. Bacteria did not exhibit significant differences between the two parts (**Figures 5A,C**). However, for fungi at the genus level (**Figure 5D**), *Cercospora* exhibited a higher relative abundance (34.66%) in the leaf spots than in the green parts ($P = 0.015$). In addition, at the genus level, *Colletotrichum* fungi were less abundant in the spots (25.68% in the spots and 8.62% in the non-spot area), which indicated that symbiosis with *Colletotrichum* fungi in the spot areas may have been inhibited. To better show the distribution of microbial differences in plant leaves, we calculated the proportion of OTUs in specific areas of plant leaves and the OTUs shared by different areas (**Figures 5E,F**). For fungi, a total of 7.33% of the OTUs were unique to the spots and 10.78% were unique to the green parts. However, bacteria had few such differences (**Figure 5E**).

Combined Analysis of Microbial and Metabolite Groups

At the genus level, we used the Spearman algorithm to calculate correlations between fungal community members with an abundance greater than 0.5% and the identified differential metabolites (**Supplementary Figure 5**). Then, we calculated *P*-values for the correlations. The data with a correlation *P*-value less than 0.05 are shown in **Figure 6**, which reveals that the endophytic fungi significantly related to the differences in metabolites (correlation $P < 0.05$) were mainly *Cercospora* and *Diaporthe* of Ascomycota, *Holtermanniella* and *Dioszegia* of Basidiomycota, and related taxa (see **Supplementary Table 2** for additional classification information). In the previous analysis, the abundance of endophytic fungi in leaf spots was also different from that in green parts. Thus, there is a close relationship between plant metabolites and endophytes, which may be caused by the production of metabolites by the endophytes, an influence on host secondary metabolite production, or more complex host–microorganism interactions.

Analysis of Chlorophyll Fluorescence Parameter Differences

F0 is the fluorescence yield when photosystem II (PSII) reaction centers are completely open in the dark-adapted state. The value of F0 is closely related to the light-catching antenna system, the state of PSII reaction centers (Sun et al., 2015), and the concentration of chlorophyll (Shasmita et al., 2019; Zhang, 2019). After dark adaptation, the initial fluorescence (F0), maximum fluorescence (Fm), and variable fluorescence (Fv) were obtained at the onset of illumination. The results showed significant differences in F0 and Fm between the leaf spots and non-spot parts ($P < 0.001$). The F0 value of leaf spots and non-spot parts decreased by 19.6%, and the Fm value

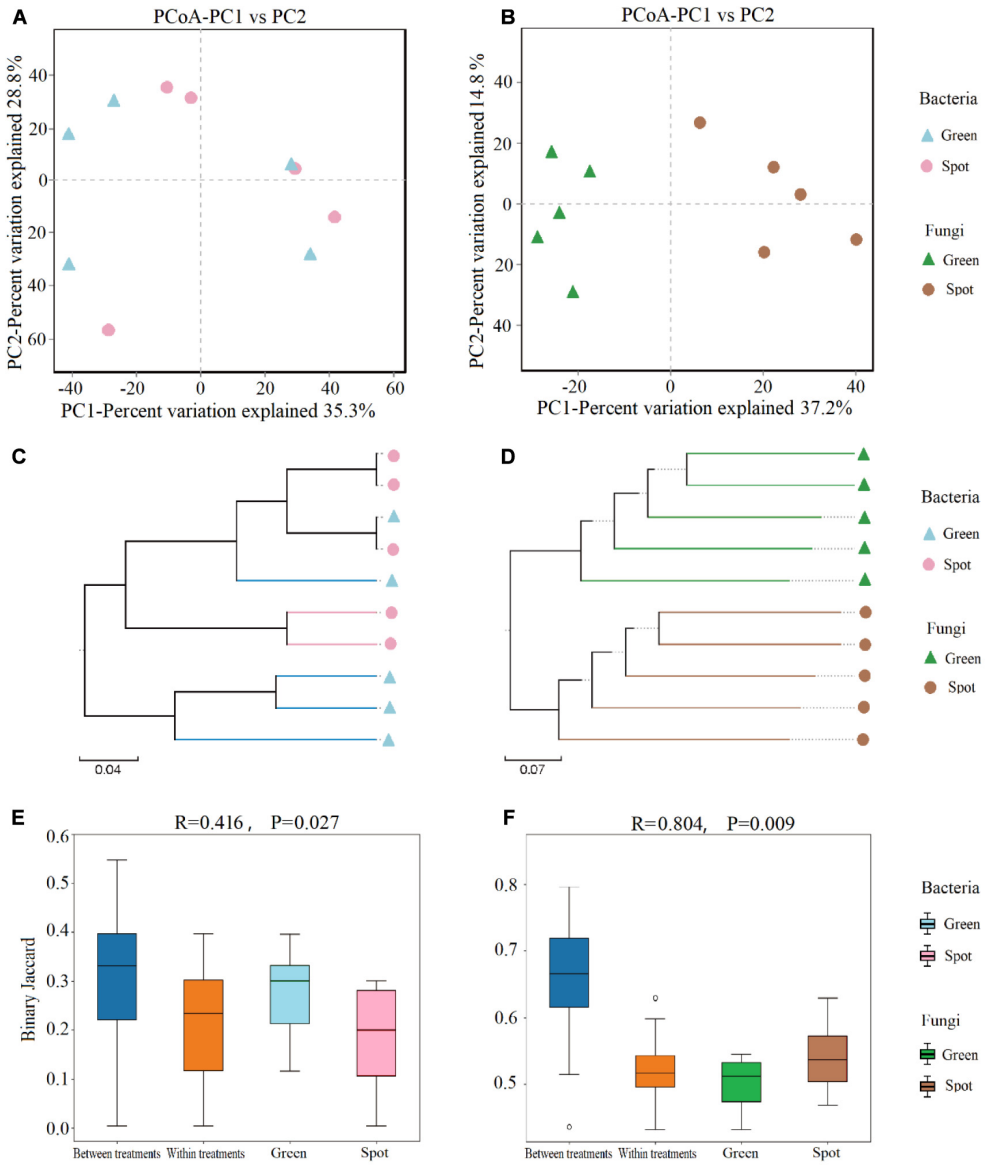

FIGURE 4 | Analysis of endophytic microbial community diversity in leaves. **(A)** PCoA of the bacterial community at the OTU level based on the binary Jaccard algorithm. The horizontal and vertical coordinates are the two characteristic values that explain most of the variation between samples, and the amount of variation explained is expressed as a percentage. **(B)** PCoA of the fungal community at the OTU level based on the binary Jaccard algorithm. Each OTU is shown as a vector in the PCoA map, and the fungal communities of the green parts and spots are distinct. **(C)** UPGMA hierarchical clustering of bacteria. Based on the binary Jaccard algorithm, UPGMA hierarchical clustering of different samples was carried out. Closer samples correspond to shorter branch lengths, which indicates that the species composition of the two samples is more similar. **(D)** UPGMA hierarchical clustering of fungi. **(E)** Beta distance data for bacteria, based on the binary Jaccard algorithm. **(F)** Beta distance data for fungi.

decreased by 17.91%. Significant differences were not observed in PSII potential activity (Fv/F0) or the maximum quantum efficiency of PSII photochemistry (Fv/Fm) between the spotted and non-spotted parts of leaves (P = 0.77 and P = 0.532, respectively) (**Table 1**). Moreover, significant differences were not observed in the fluorescence decay index (FDI) between the spotted and non-spotted parts (P = 0.36), which indicated that the formation of leaf spots had no significant effect on the photosynthetic capacity of the leaves (**Figure 7A**). In addition, micrography revealed the structural integrity of the spotted tissue (**Figures 7B–E**).

DISCUSSION

The formation of pigment patterns depends on pigment (metabolite) biosynthesis in cells (Davies et al., 2012). In this study, we examined the different metabolites between the spot

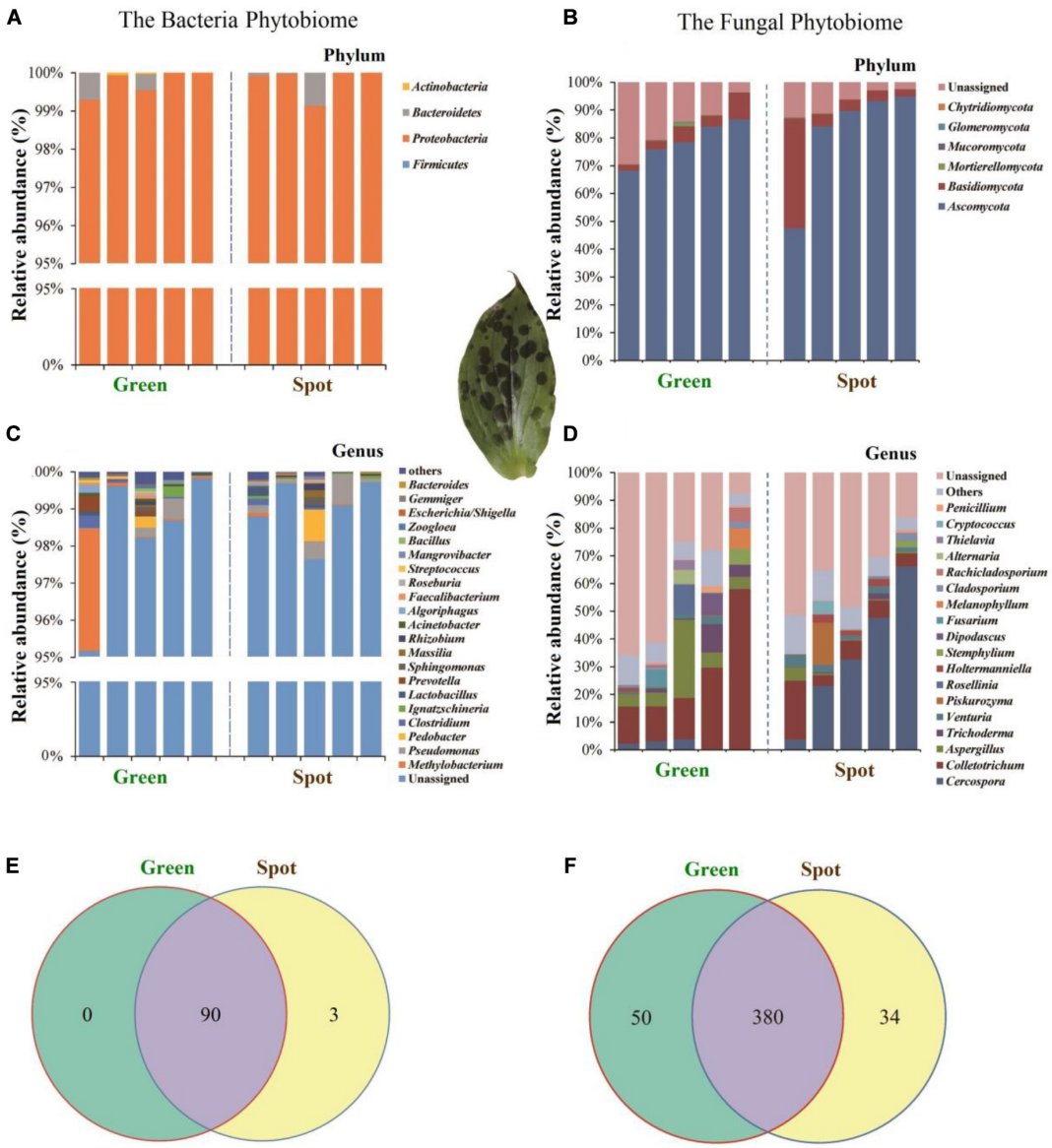

FIGURE 5 | Distribution of species at different classification levels. Selected species with an abundance greater than 0.5% are displayed in the figure and combined other species into "Others"; "Unassigned" represents the species without taxonomic annotation. Different microbial communities are distinguished by different color combinations. The Venn diagram shows the numbers of OTUs in two leaf parts, with the number shown in the overlapping part of the diagrams indicating the total number of OTUs shared between the two leaf parts and the numbers shown in the non-overlapping parts indicating the numbers of unique OTUs in each part. **(A)** Relative abundance of bacteria at the phylum level. **(B)** Relative abundance of fungi at the phylum level. **(C)** Relative abundance of bacteria at the genus level. **(D)** Relative abundance fungi at the genus level. **(E)** Venn diagram of bacteria. **(F)** Venn diagram of fungi.

and green parts of *T. macropoda* leaves. We screened 527 types of metabolites whose VIP value was more than 1 in the OPLS-DA model results as well as 68 kinds of metabolites that were decreased in the spots of leaves. In addition, 70 metabolites were significantly upregulated, including 6 amino acids (including tryptophan), 4 aldehydes, 4 sugars, 16 organic acids, and 5 lipids. Regarding the analysis of plant metabolites, the metabolites of the same cluster were also significantly different between spots and non-spots, and the change in these metabolites may be the cause of the change in leaf color.

To investigate whether the endosphere microbiome is uniformly colonized in leaf spots and non-spots areas, we studied the endosphere microbiome in spotted and non-spotted areas by high-throughput sequencing. As shown in the box plot (**Figure 3**), the results indicated that the α diversity of the endosphere microbiome in the spotted and non-spotted areas was not significantly different. Also, no significant differences in the beta diversity of the bacterial endophytes were found between the two leaf parts. As regards the β diversity of endophytic microorganisms in leaves, there was no significant difference

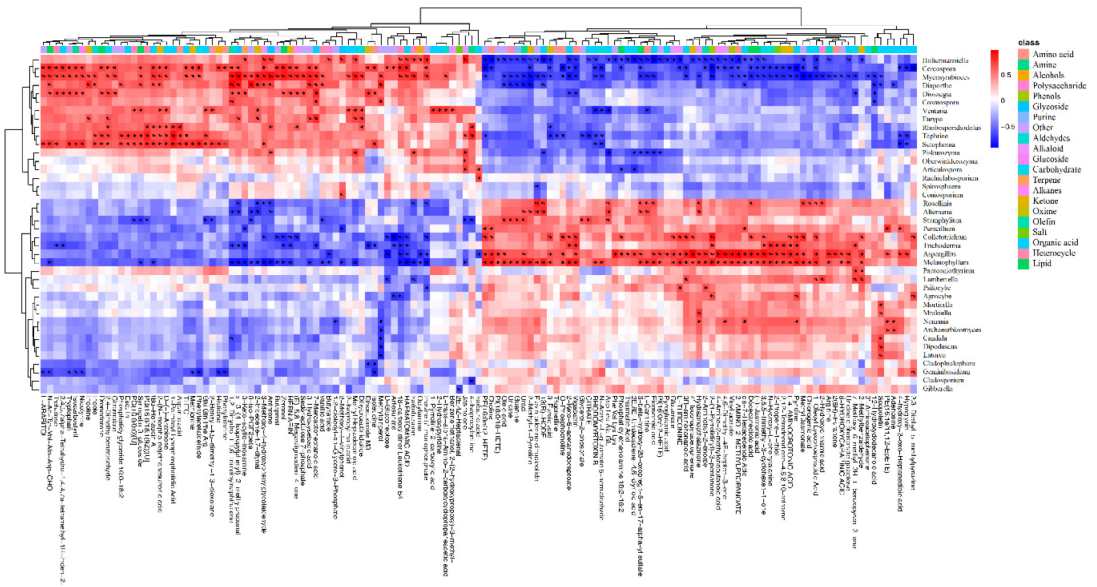

FIGURE 6 | Heat map of the correlations between plant metabolites and the endophyte community. The metabolites were significantly related to 40 genera of endophytic fungi belonging to 3 phyla and 11 classes. Red indicates a positive correlation between these metabolites and the microbes, blue indicates a negative correlation, and white indicates a non-significant correlation (correlation = 0). The data with a correlation *P*-value less than 0.05 are marked with "#." The abscissa shows the metabolites, and the ordinate shows the taxa.

in the β diversity of endophytic bacteria, but in our study, Proteobacteria were significantly enriched in the leaf. This result is different from previous studies. Proteobacteria have different enrichment abundances in different plants, which may be due to the effect of plant genotypes on bacterial colonization (Cregger et al., 2018) or may be caused by primer bias. Further study is required to verify this result. In contrast, the β diversity of endophytic fungi was significantly different, which may be caused by the uneven colonization of endophytic fungi in leaves. In the annotation of microbial species, we found differences in species diversity and abundance between the leaf spots and green parts. At the phylum level, Ascomycota was the main fungal colonizer of leaves, and this phylum is considered to be the most common group of endophytes in plants (Guo, 2016). Ascomycota is also widely found in other plants, such as grasses, flowers, and crops (Ingrid et al., 2007; Joseph et al., 2015; Stephane et al., 2015). At the genus level, *Cercospora*, *Colletotrichum*, and *Aspergillus* in Ascomycota accounted for 40.88% of the endophytic fungi in this study (**Figure 5D**) and differed significantly in abundance between the two leaf parts ($P < 0.05$). *Cercospora* was significantly more abundant in the spots than in the green parts ($P < 0.05$). *Cercospora* is believed to cause the formation of leaf spots in plants and eventually lead to leaf spot disease (Albert and Charles, 1950; Heng et al., 2020). *Cercospora* can cause necrotic damage to the leaves, thus leading to suborbicular, oil-stained brown spots on the leaf surface (Chupp, 1954; Xie et al., 2017), and *Cercospora* can cause frog eye spots in cigar tobacco in Hainan (Zhao et al., 2020). Therefore, we speculate that the formation of leaf spots may be closely related to the colonization of leaves by fungi.

TABLE 1 | Different chlorophyll fluorescence parameters in different leaf regions of *T. macropoda*.

	Green	Spot	*P*-value
F0	599.09 ± 247.02	481.41 ± 203.85	0.001**
Fm	2833.177 ± 987.19	2325.74 ± 934.60	0.001**
Fv	2234.08 ± 740.16	1844.34 ± 775.54	0.001**
Fv/Fm	0.79 ± 0.05	0.79 ± 0.05	0.777
Fv/F0	3.76 ± 0.71	3.82 ± 1.05	0.532
FDI	1.16 ± 0.83	1.27 ± 0.89	0.370

*Spotted and non-spotted parts of T. macropoda leaves subjected to chlorophyll fluorescence experiments. After dark adaptation, the initial fluorescence parameter (F0), maximum fluorescence parameter (Fm), variable fluorescence parameter (Fv), fluorescence decay index (FDI), PSII potential activity (Fv/F0), and maximum photosynthetic efficiency (Fv/Fm) of the two regions were obtained and the t-test method for significant differences was performed between the two regions. Significance levels: **P ≤ 0.001. The results are presented graphically in* ***Supplementary Figure 2****.*

Because microorganisms can regulate plant immunity and affect plant metabolism (Lee and Mazmanian, 2010; Lebeis et al., 2015; Beckers et al., 2017), the relationship between microbes and metabolites was studied. We used a thermograph to show the relationships between microorganisms and metabolites in different leaf regions and calculated the *P*-values of the correlations between endophytic fungi and differential metabolites. The results showed significant correlations of *Cercospora* and *Diaporthe* in Ascomycota and *Holtermanniella* and *Dioszegia* in Basidiomycota with 118 different metabolites. This result was consistent with the different microbes observed between the two parts; therefore, it can be preliminarily inferred that colonization by endophytic fungi may play a role in changes in plant metabolites. To date, Arabidopsis, rice, corn,

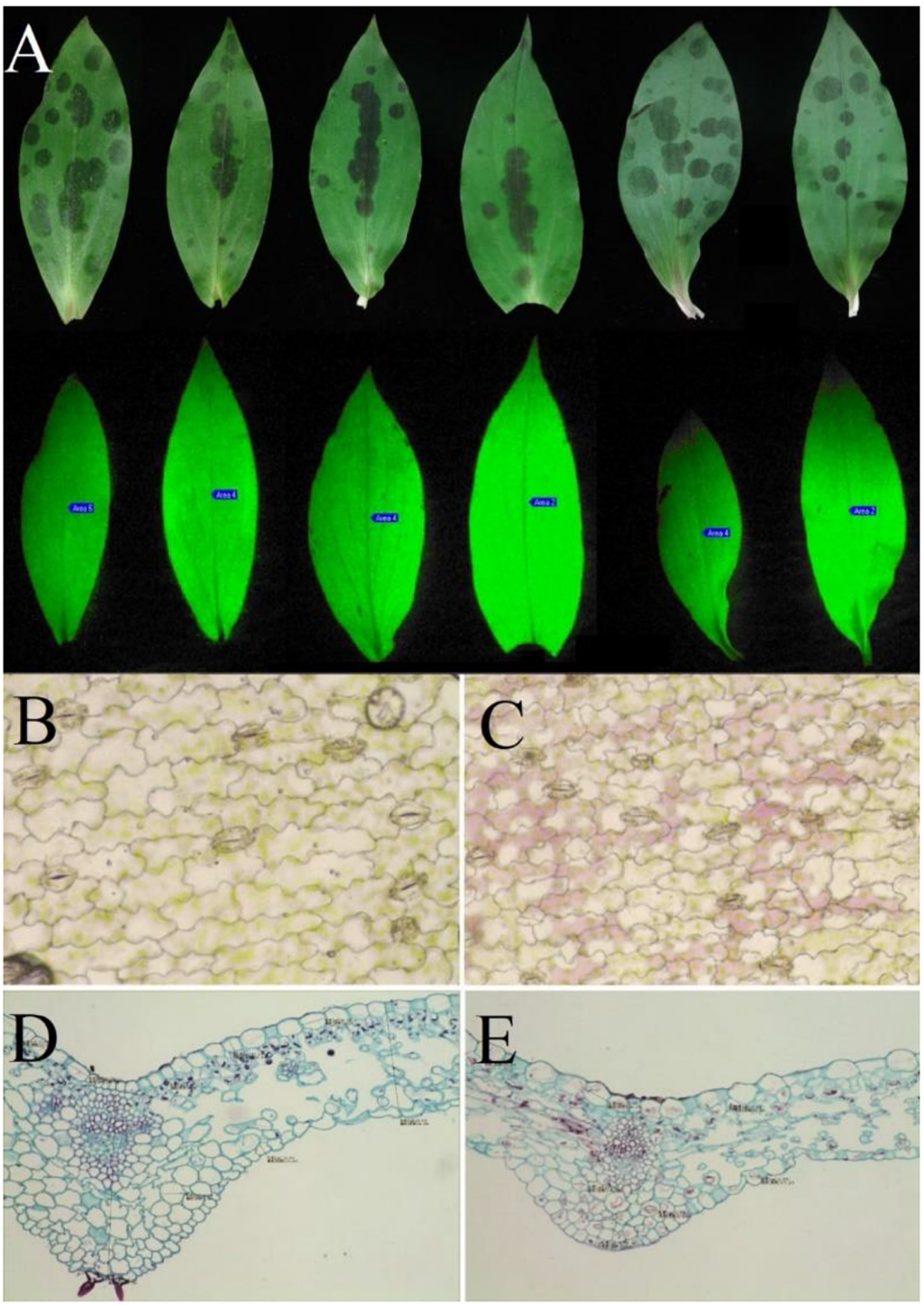

FIGURE 7 | Optical images of the leaves of *T. macropoda*. **(A)** Image of fluorescence on the blade of *T. macropoda*. **(B)** Microscopic image of a cross-section of the green part of the blade, with a magnification of 16 × 10. **(C)** Microscopic image of a cross-section of a spot on the blade, with a magnification of 16 × 10. **(D)** Microscopic image of a longitudinal section of the green part of the blade, with a magnification of 10 × 10. **(E)** Microscopic image of a longitudinal section of a spot on the blade, with a magnification of 10 × 10.

and other model plants have been studied in detail. A study on the Arabidopsis endophytic microbiome described the root and leaf microbial communities and explored the function of the host microbiome. Microbiota specializations have their own functional capabilities to their respective niche (Bai et al., 2015). A study on the functional characteristics of the endophytic

community of rice roots showed that plant endophytes may participate in the metabolic processes of rice (Vain et al., 2014). Recent research has shown that *Salvia miltiorrhiza* has a unique microbial community that is rich in functions related to secondary metabolism. These microorganisms can aid in the metabolic processes encoded by the host plant genome. The interactions between *S. miltiorrhiza* and endophytes can enhance the biomass production of the plant and may also affect the tanshinone pathway (Chen H. H. et al., 2018; Huang et al., 2018). This result suggests that different microbial communities can cause differences in the metabolites in *S. miltiorrhiza*. It can be preliminarily speculated that the uneven colonization of endophytic fungi may have an effect on the changes in plant metabolites and the relationships between metabolites and phenotype may be mediated by changes in the composition of the microbiome. The impact of microorganisms on the metabolic pathways, functions, and dynamics of host plants requires further study.

In addition, to determine whether differential colonization by fungi affects the photosynthesis of plants or causes damage to leaves, we measured the chlorophyll fluorescence parameters of leaves, which can not only characterize photosynthesis but also reflect the intrinsic characteristics of photosynthesis (Wang et al., 2019). For the determination of chlorophyll fluorescence parameters of *T. macropoda* leaves, the values of F0 and Fm were obtained. F0 is the parameter describing the dark adaptation of leaves when their reaction centers are fully open, although it does not characterize the state of photochemical reactions. F0 is related to chlorophyll concentration and indicates the activity of the photosystem II (PSII) center (Lu et al., 1994), and a decrease in the F0 value indicates an increase in the heat dissipation of antenna chlorophyll in the leaf (Nan and Lin, 2019; Zhou et al., 2019). An F0 increase indicates that the PSII reaction center was damaged (Xu et al., 1999). In this study, a decrease in the F0 values indicates an increase in plant heat dissipation or a decrease in chlorophyll concentration, both of which may reduce light absorption in plant leaves. Bauer found that the photosynthetic rate of seedling leaves of ivy was lower than that of mature leaves and that the adaptability of seedlings to strong light was weaker than that of mature leaves (Bauer and Bauer, 1980). *T. macropoda* leaf spots occur in only three to six leaves of the plant after germination, and leaves far above the ground produce a small amount of spots, if any. *T. macropoda* germinate in early spring when trees and other tall shrubs have not yet formed shaded environments. During this period, the leaf spots may increase as a result of plant heat dissipation or a decrease in chlorophyll concentration, which may enable self-protection against damage by strong light (Brugnoli et al., 1998; Wiklund et al., 2008). This protection mechanism will be investigated in the future. Fv/Fm is the maximum quantum efficiency of PSII photochemistry, and it represents the activity of the PSII centers (Wungrampha et al., 2019). The values of Fv/Fm and Fv/Fo vary very little under non-stress conditions (Shirke and Pathre, 2003; Kumud et al., 2011), and the Fv/Fm or Fv/F0 values between spots and non-spots areas were not significantly different (**Table 1**), which also indicated that uneven colonization of leaves by fungi did not cause stress effects on the plants. In addition, the chloroplast structure of the spots was complete. We speculate that the leaf color pattern may enable *T. macropoda* to adapt to the light environment in the initial stage of growth. Additionally, while ensuring normal photosynthesis, the spots may reduce leaf damage caused by strong light by increasing heat dissipation. Hence, the leaf color pattern may help *T. macropoda* to be well adapted to strong-light environments in the seedling stage, which is likely the result of long-term coevolution between plants and microorganisms.

CONCLUSION

A comprehensive study of the microbiomics and metabonomics of the spots and green parts of leaves was performed. The results showed that the bacterial diversity of green leaf parts and spots was not significantly different and that the diversity of endophytic fungi and metabolites was different in spotted and non-spotted areas. The enrichment or depletion of 118 metabolites was correlated with the occurrence and abundance of four fungi in the two leaf parts, and the results showed that some microorganisms were significantly related to certain types of metabolites. The results showed significant correlations of *Cercospora* and *Diaporthe* in Ascomycota and *Holtermanniella* and *Dioszegia* in Basidiomycota with 118 different metabolites. In addition, studies of chlorophyll fluorescence have shown that these leaf spots conduct normal photosynthesis; thus, this leaf color pattern may enable *T. macropoda* to be well adapted to strong-light environments in the seedling stage. Our research provides new insights into the relationship between endophytic microbes and plant phenotypes and emphasizes the effectiveness of comprehensive methods used to understand this process.

DATA AVAILABILITY STATEMENT

The datasets presented in this study can be found in online repositories. The names of the repository/repositories and accession number(s) can be found in the article/**Supplementary Material**.

AUTHOR CONTRIBUTIONS

YaW and MY: conceptualization, writing—review and editing, and funding acquisition. MY: methodology. FC and LZ: software, formal analysis, and visualization. BW, LZ, and YiW: validation. YaW and HC: investigation. FC: the data curation. YaW: writing—original draft preparation. WY: project administration. All authors have read and agreed to the published version of the manuscript.

FUNDING

This research was funded by "the Science and Technology Program of Shaanxi Province, grant number 2019SF-291" and "the Science and Technology Research Project

of Shaanxi Province Academy of Sciences Project, grant number 2018nk-01."

ACKNOWLEDGMENTS

We are grateful to Yafei Qi of the State Key Laboratory of Crop Stress Biology for Arid Areas, Northwest Agriculture and Forestry University, for assisting in the experiment. We thank our talented former undergraduate students Pengpeng Zhao, Danni Zhu, and Zirun Tu for performing sample collection. We are grateful to Liangdong Guo of the Institute of Microbiology, Chinese Academy of Sciences, for manuscript revision.

SUPPLEMENTARY MATERIAL

The Supplementary Material for this article can be found online at: https://www.frontiersin.org/articles/10.3389/fmicb.2020.599829/full#supplementary-material

REFERENCES

Abarenkov, K., Nilsson, R. H., Larsson, K. H., Alexander, I. J., Eberhardt, U., Erland, S., et al. (2010). The UNITE database for molecular identification of fungi –recent updates and future perspectives. *New Phytol.* 186, 281–295. doi: 10.1111/j.1469-8137.2009.03160.x

Albert, S. M., and Charles, C. (1950). *Cercospora* in Guatemala. *Cercospora in Guatemala* Muller and Chupp: 171–177.

Bai, Y., Daniel, B. M., Girish, S., Ruben, G. O., Eva, P., Matthias, R., et al. (2015). Functional overlap of the Arabidopsis leaf and root microbiota. *Nature* 528, 364–382. doi: 10.1038/nature16192

Bauer, H., and Bauer, U. (1980). Photosynthesis in leaves of the juvenile and adult phase of ivy (Hedera helix). *Physiol. Plant.* 49, 366–372. doi: 10.1111/j.1399-3054.1980.tb03318.x

Beckers, B., Beeck, M., Weyens, N., Boerjan, W., and Vangronsveld, J. (2017). Structural variability and niche differentiation in the rhizosphere and endosphere bacterial microbiome of field grown poplar trees. *Microbiome* 5, 25–42. doi: 10.1186/s40168-017-0241-2

Berendsen, R. L., Pieterse, C. M. J., and Bakker, P. A. H. M. (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

Brader, G., Compant, S., Mitter, B., Trognitz, F., and Sessitsch, A. (2014). Metabolic potential of endophytic bacteria. *Curr. Opin. Biotechnol.* 27, 30–37. doi: 10.1016/j.copbio.2013.09.012

Brugnoli, E., Scartazzas, A., Tullio, M. C. D., Monteverdi, M. C., and Augusti, A. (1998). Zeaxanthin and non–photochemical quenching in sun and shade leaves of C3 and C4 plants. *Physiol. Plant.* 104, 727–734. doi: 10.1034/j.1399-3054.1998.1040430.x

Charlotte, F. M., Pai, P., Mikkel, A. G., Jan, K. S., and Peter, S. (2014). Bacterial diversity in Greenlandic soils as affected by potato cropping and inorganic versus organic fertilization. *Polar Biol.* 37, 61–71. doi: 10.1007/s00300-013-1410-9

Chen, H. M., Chen, H. M., Wu, H. X., Yan, B., Zhao, H. G., Liu, F. H., et al. (2018). Core microbiome of medicinal plant *Salvia miltiorrhiza* seed: a rich reservoir of beneficial microbes for secondary metabolism? *Int. J. Mol. Sci.* 19, 672–687. doi: 10.3390/ijms19030672

Chen, K. Y., Li, Z. N., Cheng, M. M., Zhao, Y. H., Zhou, M. B., and Yang, H. Y. (2018). Chloroplast ultrastructure and chlorophyll fluorescence characteristics of three cultivars of *Pseudosasa japonica*. *Chin. Bull. Bot.* 53, 509–518.

Chen, Y. S., Chesson, P., Wu, H. W., Pao, S. H., and Sheue, C. R. (2017). Leaf structure affects a plant's appearance: combined multiple–mechanisms intensify remarkable foliar variegation. *J. Plant Res.* 130, 311–325. doi: 10.1007/s10265-016-0890-4

Cho, K., Cho, K. S., Sohn, H. B., Ha, I. J., Hong, S. Y., Lee, H., et al. (2016). Network analysis of the metabolome and transcriptome reveals novel regulation of potato pigmentation. *J. Exp. Bot.* 67, 1519–1533. doi: 10.1093/jxb/erv549

Chupp, C. (1954). *A Monograph of the Fungus Genus Cercospora*. USA.

Cregger, M. A., Veach, A. M., Yang, Z. K., Crouch, M. J., Vilgalys, R., Tuskan, G. A., et al. (2018). The *Populus holobiont*: dissecting the effects of plant niches and genotype on the microbiome. *Microbiome* 6, 31–45. doi: 10.1186/s40168-018-0413-8

Davies, K., Albert, N., and Schwinn, K. (2012). From landing lights to mimicry: the molecular regulation of flower colouration and mechanisms for pigmentation patterning. *Funct. Plant Biol.* 39, 619–638. doi: 10.1071/fp12195

Doppler, M., Kluger, B., Bueschl, C., Schneider, C., Krska, R., Delcambre, S., et al. (2016). Stable isotope–assisted evaluation of different extraction solvents for untargeted metabolomics of plants. *Int. J. Mol. Sci.* 17:1017. doi: 10.3390/ijms17071017

Du, Q. X., Qing, J., Zhu, J. L., Du, H. Y., and Du, L. Y. (2017). Pigments and active ingredients in leaves of seven *Eucommia ulmoides* variation types. *Bull. Bot. Res.* 37, 468–473.

Du, W. W., Cui, G. F., Wang, J. H., Duan, Q., Ma, L. L., Jia, W. J., et al. (2019). Effect of foliar variegation structure on leaf colour in *Begonia gulinqingensis*. *Guihaia* 39, 812–820.

Edgar, R. C. (2010). Search and clustering orders of magnitude faster than BLAST. *Bioinformatics.* 26, 2460–2471. doi: 10.1093/bioinformatics/btq461

Glover, B. J. (2014). *Understanding Flowers and Flowering: An Integrated Approach*. Oxford: Oxford University Press. doi: 10.1093/acprof:oso/9780199661596.001.0001

Gu, Z. Y., Zhu, J., Hao, Q., Yuan, Y. W., Duan, Y. W., Men, S. Q., et al. (2019). A novel R2R3–MYB transcription factor contributes to petal blotch formation by regulating organ–specific expression of Pschs in tree peony (*Paeonia suffruticosa*). *Plant Cell Physiol.* 60, 599–611. doi: 10.1093/pcp/pcy232

Guo, S. X. (2016). *Endophytic Mycology of Medicinal Plants*. Beijing: Science Press.

Hanbo, C., and Paul, C. B. (2011). Venn Diagram: a package for the generation of highly–customizable Venn and Euler diagrams in R. *BMC Bioinformatics* 12:35. doi: 10.1186/1471-2105-12-35

Hara, N. (1957). Study of the variegated leaves with special reference to those caused by air spaces. *Jpn. J. Bot.* 117, 86–101.

Heng, T., Kaga, A., Chen, X., and Somta, P. (2020). Two tightly linked genes coding for NAD–dependent malic enzyme and dynamin–related protein are associated with resistance to *Cercospora* leaf spot disease in cowpea (*Vigna unguiculata* (L.) Walp.). *Theor. Appl. Genet.* 133, 395–407. doi: 10.1007/s00122-019-03470-6

Huang, W. J., Long, C. L., and Lam, E. (2018). Roles of plant associated microbiota in traditional herbal medicine. *Trends Plant Sci.* 7, 559–562. doi: 10.1016/j.tplants.2018.05.003

Ingrid, K., Ingeborg, H., Sabrina, S., Juan, P. S., Michael, W., Markus, P., et al. (2007). Guilds of mycorrhizal fungi and their relation to trees ericads orchids and liverworts in a neotropical mountain rain forest. *Basic Appl. Ecol.* 9, 13–23. doi: 10.1016/j.baae.2007.03.007

Joseph, E., Cameron, J., Christian, S. M., Eugene, L., Natraj, K. P., Srijak, B., et al. (2015). Structure variation and assembly of the root–associated microbiomes of rice. *Proc. Natl. Acad. Sci. U.S.A.* 112, 911–920. doi: 10.1073/pnas.1414592112

Kang, H. J., Chai, A. L., Shi, Y. X., Xie, X. W., Guo, J. G., and Li, B. J. (2019). Establishment and application of real–time quantitative PCR assay for detection of fungal pathogen *Pseudocercospora fuligena* in tomato. *J. Plant Prot.* 46, 1214–1221.

Khan, A. L., Han, M., Kim, Y. H., Kang, S. M., and Lee, I. J. (2011). Ameliorative symbiosis of endophyte (*Penicillium funiculosum* LHL06) under salt stress elevated plant growth of *Glycine max* L. *Plant Physiol. Biochem.* 9, 852–861. doi: 10.1016/j.plaphy.2011.03.005

Khizar, M., Shi, J. X., Saleem, S., Liaquat, F., Ashraf, M., Latif, S., et al. (2020). Resistance associated metabolite profiling of *Aspergillus* leaf spot in cotton

through non–targeted metabolomics. *PLoS One* 15:e0228675. doi: 10.1371/journal.pone.0228675

Kumud, B. M., Rina, I., Angelo, P., Anamika, M., Nadia, A., Giovanna, L. V., et al. (2011). Engineered drought tolerance in tomato plants is reflected in chlorophyll fluorescence emission. *Plant Sci.* 182, 79–86. doi: 10.1016/j.plantsci.2011.03.022

Lebeis, S. L., Paredes, S. H., Lundberg, D. S., Breakfield, N., Gehring, J., Mcdonald, M., et al. (2015). Salicylic acid modulates colonization of the root microbiome by specific bacterial taxa. *Science* 349, 860–874. doi: 10.1126/science.aaa8764

Lee, Y. K., and Mazmanian, S. K. (2010). Has the microbiota played a critical role in the evolution of the adaptive immune system? *Science* 330, 1768–1773. doi: 10.1126/science.1195568

Li, W. X., Jiang, F., Ma, J. L., Wang, J. Y., Yang, S. B., and Chen, P. (2017). Ultra strucuture and chlorophyll contents of the variegated leaves in *Ginkgo biloba* L. *J. Yangzhou. Univ.* 37, 103–107.

Lin, M., Beatrice, C., Andrea, F., Tan, K. C., James, C., and Morten, L. (2020). Genetic mapping using a wheat multi–founder population reveals a locus on chromosome 2A controlling resistance to both leaf and glume blotch caused by the necrotrophic fungal pathogen *Parastagonospora nodorum*. *Theor. Appl. Genet.* 133, 785–808. doi: 10.1007/s00122-019-03507-w

Liu, L. P., Zhang, L., Qiu, P. L., Wang, Y., Liu, Y. N., Li, Y., et al. (2020). Leaf spot of *Polygonatum odoratum* caused by *Colletotrichum spaethianum*. *J. Gen. Plant Pathol.* 86, 157–161. doi: 10.1007/s10327-019-00903-4

Lozupone, C., Hamady, M., and Knight, R. (2006). UniFrac–An online tool for comparing microbial community diversity in a phylogenetic context. *BMC Bioinformatics* 7:371. doi: 10.1186/1471-2105-7-371

Lu, C. M., Zhang, Q. D., and Kung, T. Y. (1994). The effects of water stress on PhotosystemII in wheat. *Acta Bot. Sin.* 36, 93–98.

Lynch, J. B., and Hsiao, E. Y. (2019). Microbiomes as sources of emergent host phenotypes. *Science* 365, 1405–1409. doi: 10.1126/science.aay0240

Nan, J. B., and Lin, L. (2019). Comparisons of photosynthetic physiological characteristics of three species of *Hippophae* in Tibet. *Non Wood For. Res.* 37, 96–104.

National Pharmacopoeia Commission (2015). *Pharmacopoeia of the People's Republic of China*. Beijing: Medical Science and Technology Press.

Pilar, C. M., Moran, M. S., Papuga, S. A., Thorp, K. R., Alonso, L., Moreno, J., et al. (2016). Plant chlorophyll fluorescence: active and passive measurements at canopy and leaf scales with different nitrogen treatments. *J. Exp. Bot.* 67, 275–286. doi: 10.1093/jxb/erv456

Shasmita, D. M., Pradipta, K., Mohapatra, S. K., Naik, A., and Mukherjee, K. (2019). Priming with salicylic acid induces defense against bacterial blight disease by modulating rice plant photosystem II and antioxidant enzymes activity. *Physiol. Mol. Plant Pathol.* 108:101427. doi: 10.1016/j.pmpp.2019.101427

Sheue, C. R., Pao, S. H., Chien, L. F., Chesson, P., and Peng, C. I. (2012). Natural foliar variegation without costs? the case of *Begonia*. *Ann. Bot.* 109, 1065–1074. doi: 10.1093/aob/mcs025

Shirke, P. A., and Pathre, U. V. (2003). Diurnal and seasonal changes in photosynthesis and photosystemII photochemical efficiency in *Prosopis juliflora* leaves subjected to natural environmental stress. *Photosynthetica* 41, 83–89. doi: 10.1023/a:1025864513663

Shu, J., Yu, Z. H., Sun, W. X., Zhao, J., Li, Q. L., Tang, L. H., et al. (2020). Identification and characterization of *Pestalotioid* fungi causing leaf spots on mango in Southern China. *Plant Dis.* 3:19. doi: 10.1094/PDIS-03-19-0438-RE

Stephane, H., Ruben, G. O., Antonio, G., Stijn, S., Gail, A., Sarah, L., et al. (2015). Microbiota and host nutrition across plant and animal kingdoms. *Cell Host Microbe* 17, 603–616. doi: 10.1016/j.chom.2015.04.009

Sun, Y. H., Wang, J. H., Geng, Q. W., Xing, H., Zhai, H., and Du, Y. P. (2015). Effects of different concentrations of ozone stress on photosynthetic system II in Vitis vinifera cv. 'Cabernet Sauvignon'. *Plant Physiol. J.* 51, 1947–1954.

Vain, T., Crowell, E. F., Timpano, H., Biot, E., Desprez, T., Mansoori, N., et al. (2014). The cellulase KORRIGAN is part of the cellulose synthase complex. *Plant Physiol.* 165, 1521–1532. doi: 10.1104/pp.114.241216

Wang, J. J., Zhang, M. R., Xu, Y., and He, Y. H. (2019). Light response and chlorophyll fluorescence parameters in dicranopteris dichotoma with light intensity and nitrogen treatments. *J. Zhejiang A F Univ.* 36, 1199–1207.

Wang, J. L., Zhang, T., Shen, X. T., Liu, J., Zhao, D., Sun, Y. W., et al. (2016). Serum metabolomics for early diagnosis of esophageal squamous cell carcinoma by UHPLC–QTOF/MS. *Metabolomics* 12, 116–131. doi: 10.1007/s11306-016-1050-5

Wang, Z. X., Cao, J. R., Qin, H. Y., Zhao, Y., Chen, L., Ai, J., et al. (2016). Common effect of pigment content and leaf structure on leaf color in *Actinidia kolomikta*. *Plant Physiol. J.* 52, 1921–1926.

Wiklund, S., Johansson, E., Sjöström, L., Mellerowicz, E. J., Edlund, U., Shockcor, J. P., et al. (2008). Visualization of GC/TOF–MS–based metabolomics data for identification of biochemically interesting compounds using OPLS class models. *Anal. Chem.* 80, 115–122. doi: 10.1021/ac0713510

Wungrampha, S., Joshi, R., Rathore, R. S., Sneh, L. S. P., Govindjee, and Pareek, A. (2019). CO2 uptake and chlorophyll a fluorescence of *Suaeda fruticosa* grown under diurnal rhythm and after transfer to continuous dark. *Photosynth. Res.* 142, 211–227. doi: 10.1007/s11120-019-00659-0

Xie, X. W., Zhao, Q., and Guo, Y. L. (2017). New records of *Cercospora* and *Pseudocercospora* in China. *Mycosystema* 36, 1164–1167.

Xiong, W., Zhao, Q. Y., Xue, C., Xun, W. B., Zhao, J., Wu, H. S., et al. (2016). Comparison of fungal community in black pepper-vanilla and vanilla monoculture systems associated with Vanilla Fusarium wilt disease. *Front. Microbiol.* 7:117. doi: 10.3389/fmicb.2016.00117

Xu, C. C., Li, D. Q., Zou, Q., and Zhang, J. H. (1999). Effect of drought on chlorophyll fluorescence and xanthophyll cycle components in winter wheat leaves with different ages. *Acta Photophysiol. Sin.* 25, 29–37.

Yang, H. Y. (2015). *Study on Mechanism of Spontaneous Leaf Color Variation of Pseudosasa japonica* f. Ph.D. thesis, Beijing Forestry University, Beijing.

Zhang, T. (2019). Comparison of leaf photosynthetic and physiological characteristics of 6 Hedera helix Cultivars. *J. Northwest For. Univ.* 34, 62–67.

Zhao, Q., Chen, X., Liu, D. Y., Xia, C. J., Yang, J. G., Lv, H. K., et al. (2020). First report of *Cercospora nicotianae* causing frog eye spot in Cigar Tobacco in Hainan, China. *Plant Dis.* doi: 10.1094/PDIS-03-20-0540-PDN [Epub ahead of print].

Zhou, Y. X., Ju, T. Z., Wang, Y. D., Wan, Z. D., Yang, Y. L., Gao, T. P. (2019). Diurnal variation of chlorophyll fluorescence parameters of three xerophytes. *J. Arid Land Resour. Environ.* 33, 164–170.

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 Microbiology

ORIGINAL RESEARCH
published: 12 January 2021
doi: 10.3389/fmicb.2020.600393

Whole Genome Sequencing and Root Colonization Studies Reveal Novel Insights in the Biocontrol Potential and Growth Promotion by *Bacillus subtilis* MBI 600 on Cucumber

Anastasios Samaras[1], Marios Nikolaidis[2], Maria Luisa Antequera-Gómez[3], Jesus Cámara-Almirón[3], Diego Romero[3], Thomas Moschakis[4], Grigoris D. Amoutzias[2] and Georgios S. Karaoglanidis[1]*

[1] Laboratory of Plant Pathology, Faculty of Agriculture, Forestry and Natural Environment, School of Agriculture, Aristotle University of Thessaloniki, Thessaloniki, Greece, [2] Bioinformatics Laboratory, Department of Biochemistry and Biotechnology, University of Thessaly, Larisa, Greece, [3] Instituto de Hortofruticultura Subtropical y Mediterránea "La Mayora"—Departamento de Microbiología, Universidad de Málaga, Málaga, Spain, [4] Laboratory of Dairy Science and Technology, Department of Food Science and Technology, Faculty of Agriculture, Forestry and Natural Environment, School of Agriculture, Aristotle University of Thessaloniki, Thessaloniki, Greece

OPEN ACCESS

Edited by:
Christos Zamioudis, Democritus University of Thrace, Greece

Reviewed by:
Silvia Proietti, University of Tuscia, Italy
Antonio Leon-Reyes, Universidad San Francisco de Quito, Ecuador

***Correspondence:**
Anastasios Samaras
samarasanast@gmail.com

Specialty section:
This article was submitted to Microbe and Virus Interactions with Plants, a section of the journal Frontiers in Microbiology

Received: *29 August 2020*
Accepted: *11 December 2020*
Published: *12 January 2021*

Citation:
Samaras A, Nikolaidis M, Antequera-Gómez ML, Cámara-Almirón J, Romero D, Moschakis T, Amoutzias GD and Karaoglanidis GS (2021) Whole Genome Sequencing and Root Colonization Studies Reveal Novel Insights in the Biocontrol Potential and Growth Promotion by Bacillus subtilis MBI 600 on Cucumber. Front. Microbiol. 11:600393. doi: 10.3389/fmicb.2020.600393

Bacillus spp. MBI 600 is a gram-positive bacterium and is characterized as a PGPR strain involved in plant growth promotion and control of various plant pathogens which has recently been introduced into the agricultural practice. In this study we performed a Next Generation Sequencing analysis, to analyze the full genome of this microorganism and to characterize it taxonomically. Results showed that MBI 600 strain was phylogenetically close to other *Bacillus* spp. strains used as biocontrol agents and identified as *B. subtilis*. GOG analysis showed clusters contributed to secondary metabolites production such as fengycin and surfactin. In addition, various genes which annotated according to other plant-associated strains, showed that play a main role in nutrient availability from soil. The root colonization ability of MBI 600 strain was analyzed *in vivo* with a yellow fluorescence protein (*yfp*) tag. Confocal laser scanning microscopy of cucumber roots treated with *yfp*-tagged MBI 600 cells, revealed that the strain exhibits a strong colonization ability of cucumber roots, although it is affected significantly by the growth substrate of the roots. *In vitro* and *in planta* experiments with MBI 600 strain and *F. oxysporum* f.sp. *radicis cucumerinum* and *P. aphanidernatum*, showed a high control ability against these soilborne pathogens. Overall, our study demonstrates the effectiveness of MBI 600 in plant growth promotion and antagonism against different pathogens, highlighting the use of this microorganism as a biocontrol agent.

Keywords: *Bacillus* spp., *Fusarium oxysporum* f.sp. *radicis cucumerinum*, yellow fluorescence protein-tagging, plant growth Promoting bacteria, root colonization, whole genome analysis

INTRODUCTION

During the last decades, the use of beneficial bacteria for the biological control of plant pathogens became a major weapon in the protection of several crops, with a continuously increasing number of them registered throughout the world as biopesticides. Among those beneficial bacteria, species that have the ability to colonize plant roots and support plant growth and/or protection against pathogens are commonly referred to as plant-growth-promoting rhizobacteria (PGPR). The interactions of PGPR with plants and plant pathogens is accomplished through multiple direct and indirect modes of action. Direct mechanisms include nitrogen fixation, siderophore and phytohormone production, competition with microorganisms in rhizosphere or production of secondary metabolites (Shafi et al., 2017; Backer et al., 2018). Indirect mechanisms include the induction of systemic resistance (ISR) and the inhibition of plant ethylene synthesis (Chen et al., 2006; Doornbos et al., 2012). Within the group of PGPR, *Bacillus* spp. has a dominant role, with a continuously increasing number of strains being evaluated and used as biofertilizers or biopesticides in different crops and against a variety of soil-borne and foliar pathogens. The extensive development and registration of *Bacillus*-based products is related to some unique characteristics of the genus that include high replication rate, resistance to adverse environmental conditions, increased efficiency in plant growth promotion and broad spectrum activity (Wang et al., 2013; Magno-Perez-Bryan et al., 2015).

B. subtilis MBI 600 (thereafter MBI 600) is a biological control agent (BCA) commercialized recently by BASF. Currently, published information on its biocontrol and plant growth promotion efficiency is restricted on crops such as rice and tomato. Previous research conducted in our laboratory showed that MBI600 can provide high control efficacy against a major soil-borne fungal pathogen of tomato, *Fusarium oxysporum* f.sp. *radicis lycopersici* (Samaras et al., 2018). In the same host, it has been shown to be effective against important tomato viruses such as TSWV and PVY (Beris et al., 2018). More recently, gene expression studies on MBI 600-treated tomato plants revealed that this protective function against viruses is achieved through eliciting defense responses by activation of salicylic acid (SA)-responsive genes and a synergistic cross-talk between jasmonic acid/ ethylene (JA/ET) and SA-signaling (Dimopoulou et al., 2019). Similarly, on rice, MBI 600 was found to be effective against *Rhizoctonia solani*, an important soil-borne pathogen of rice (Kumar et al., 2012).

Cucumber, (*Cucumis sativus* L.) is one of the most important vegetable crops cultivated throughout the world either in open fields or in greenhouses and suffers attacks from several foliar and soil-borne fungal pathogens (Keinath et al., 2018). Among the soilborne pathogens that attack cucumber plants, *Pythium aphanidermatum* causes damping off disease on young plants, whereas, *Fusarium oxysporum* f. sp. *radicis-cucumerinum* (*Forc*) causes Fusarium crown and root rot (FCRR). In the past, the most popular control methods for those diseases was either the use of fungicides with specific action against Oomycetes including *Pythium* spp. or soil fumigation with methyl bromide, that was effective against both pathogens (Pavlou and Vakalounakis, 2005). However, social concerns for the use of synthetic fungicides along with the removal of methyl bromide from the market led in an increased interest for application of BCAs in cucumber crops. During the last two decades several *Bacillus* spp. strains such as *B. subtilis* ME488, *B. subtilis* SQR-9 and *Paenobacillus polymyxa* SQR-21, have been reported to effectively control *Pythium* spp. and *Forc* in cucumber. In several cases, the control efficacy achieved by *Bacillus* strains was associated to the production of antifungal compounds and volatile organic compounds (VOCs) that induced plant defense reactions (Chung et al., 2008; Cao et al., 2012; Khalaf and Raizada, 2018).

The first whole genome sequence of a *Bacillus* strain became available for *B. subtilis* strain Marbug 168 and since then, about 200 *Bacillus* strains have been sequenced (Kunst et al., 1997; Liu et al., 2012; Franco-Sierra et al., 2020). Whole genome analysis of Bacillus species constitutes the basis to understand the interactions with plants and other microorganisms (Magno-Perez-Bryan et al., 2015; Shaligram et al., 2016). However, the precise taxonomic position of MBI 600 is puzzling since it is referred either as *B. subtilis* or *B. amyloliquefaciens*. *B. amyloliquefaciens* has been delineated from *Bacillus subtilis* (*Bs*) sensu lato based on phylogenetic differences and physiological characteristics associated with antibiotic production and root colonization ability and currently comprises of two subspecies, the plant-associated *B. amyloliquefaciens* subsp. *plantarum* and the non-plant associated *B. amyloliquefaciens* subsp. *amyloliquefaciens* (Borriss et al., 2011; Zhang et al., 2016).

Variations that have been observed in plant growth promoting capacity and biocontrol efficiency of several PGPR strains have been correlated to differences in their root-colonizing ability (Poonguzhali et al., 2008; Cao et al., 2011; Posada et al., 2018). Root colonization ability is a crucial factor for plant-PGPR interactions, determining the success of a PGPR strain in promoting plant growth and providing protection against pathogens (Kamilova et al., 2005; Ugoji et al., 2006). PGPR strains belonging to the *Bacillus* taxa are often formulated in the form of spores that are tolerant to adverse environmental conditions and the first step in growth promotion and biological control processes mediated by Bacillus applications is the spore germination. However, successful colonization of a plant root by a PGPR passes through additional stages that include attraction to the roots and establishment on them (De Souza et al., 2015; Posada et al., 2018). Numerous studies in the past have focused on the investigation of parameters affecting the colonization performance of several *Bacillus* strains (Rudrappa et al., 2008; Cao et al., 2011; Fan et al., 2011; Zhang et al., 2014; Yuan et al., 2015). Establishment of PGPR on plant roots as a stage of the colonization process is mediated through biofilm formation, surfactin production and metabolic enzymes production that are regulated by quorum sensing (Beauregard et al., 2013).

Despite the fact that root colonization ability has been extensively studied in Gram-negative bacterial strains, the number of similar studies for Gram-positive PGPR is limited because of the absence of a reliable and stably expressed

molecular marker (Jansson, 2003; Cao et al., 2011). *In situ* visualization of bacterial cells on the root surface and the rhizosphere using green fluorescent protein (GFP) as a marker became a tool that has revolutionized root colonization studies by PGPR. However, the structural instability of the plasmid-based GFP vectors in Gram-positive strains limited their applications in studies aiming to determine the colonization ability of *Bacillus* spp. (Cao et al., 2011; Posada et al., 2018). Nevertheless, optimization of *Bacillus* transformation protocols along with the use of electron confocal microscopy or fluorescent *in situ* hybridization contributed to a recent increase of studies aiming to determine the root colonizing ability of several *Bacillus* strains in natural environment and different substrates (Romero et al., 2006; Fan et al., 2011; Posada et al., 2018).

Based on the above, the current study was initiated aiming to provide insights: (a) on the whole genome sequence of MBI 600 that will define the precise taxonomic position of MBI 600 and will lead to identification of genes likely to be involved in plant growth promotion and plant defense mechanisms, (b) on the ability of the MBI 600 to colonize cucumber roots using YFP-labeled bacterial cells by combining a natural transformation system and confocal laser scanning microscopy (c) on the MBI 600 ability to colonize cucumber roots grown in different growth substrates by taking advantage of the chloramphenicol resistant cassetteinserted in the *yfp*-plasmid and (d) on the MBI 600 biocontrol efficiency against two major soil-borne pathogens of cucumber, *Forc* and *P. aphanidermatum*.

MATERIALS AND METHODS

Maintenance of MBI 600 and Plant Pathogen Strains

The MBI 600 strain used in the study was isolated from a commercial formulation of the product (Serifel 9.9 WP) kindly provided by BASF Hellas S.A. For the isolation, 1 g of product's powder was diluted in 50 ml of dd H_2O, centrifuged for 5 min at 4,000 rpm followed by discard of the supernatant and resuspension of the pellet in 5 ml of PBS buffer. Then, 10-fold serial dilutions were performed and 100 μl of each dilution were spread on Tryptone Soy Agar medium (TSA, LabM, Hungary) and incubated at 37°C for 24 h. 16S rRNA gene sequence analysis was used to confirm the identity of the isolated bacterial strain by using primers 27F 5′-AGAGTTTGATCMTGGCTCAG-3′) and 511R (5′-GCGGCTGCTGGCACRKAGT-3′) (Liu et al., 2015).

Forc and *P. aphanidermatum* isolates used in the study belong to the fungal collection of the Lab of Plant Pathology, AUTH. Both pathogens were isolated from diseased cucumber plants. The fungal isolates were grown and maintained on Potato Dextrose Agar (PDA, LabM, Hungary) slants at 4°C.

Whole Genome Sequence and Bioinformatics

MBI 600 was cultured in 0.1% Luria Broth (LB) medium under optimal growth conditions (pH 7.0 and 37°C) for 12 h. Bacterial cells were collected by centrifugation and DNA was purified using a Qiagen Dneasy kit according to the manufacturer (Qiagen, Germany). DNA was firstly sequenced in a Pacific Biosciences platform using SMRT cell 8 Pac V3, DNA Polymerase Binding Kit P6 v2. To increase the quality of the sequenced genome. DNA was additionally sequenced with Illumina HiSeq X, using the TrueSeq DNA PCR Free (350) library kit for 150 nt paired-end reads.

The raw PACBIO subreads data files were converted to fastq files and were assembled with Blasr (Chaisson and Tesler, 2012) and Canu V.1.6 (Koren et al., 2017). The resulting assembly comprised of one circular contig. Next, the Illumina reads were assembled into contigs with Spades version 3.12 (Antipov et al., 2016), using the PacBio draft genome as trusted-contig. These contigs were subsequently used as BLASTn query against the PacBio draft genome with *e*-value cutoff of 1e-10. Four contigs were then selected that had a total of 99.2% genome coverage and were used to manually apply corrections in the PacBio genome. As a quality control, the MBI 600 genome was compared with known genomes from NCBI non-redundant nucleotide database with the BlastN web interface. The top hit was *B. subtillis* 3NA (CP010314.1) with a query coverage of 99%. Blast2Seq and the resulting dot plot clearly showed that these two genomes are co-linear except for a segment of 3 NA between positions 2.2–2.3 Mb that is missing in MBI 600. Gene annotation was performed by the NCBI Prokaryotic Genome Annotation Pipeline (Tatusova et al., 2016).

To ensure the exact phylogenetic position of this strain within the *Bacillus* genus, a phylogenomic analysis was performed with 147 *Bacillus subtilis* sensu lato proteomes, including strains from *B. subtilis, B. atrophaeus, B, amyloliquefaciens, B. velezensis, B. licheniformis, and B. paralicheniformis*. The MBI 600 strain was used as a reference point for best reciprocal Blastp hits against the other 147 proteomes (*e*-value cut off 1e-5). This resulted in 2,317 core proteins of the strain MBI 600, that were present in all other 147 proteomes and were used for the phylogenomic analysis. Each of these orthologous groups were aligned with the Muscle software (Edgar, 2004) within Seaview V.4 (Gouy et al., 2010) and were subsequently concatenated to one protein super-alignment. The super-alignment was then filtered with Gblocks default parameters (Castresana, 2000) and the resulting alignment (294,136 variable sites) was used to compute a Neighbor Joining tree with 500 bootstrap replicates and Kimura model which is embedded into the Seaview program (Gouy et al., 2010). The tree was annotated and visualized using the iTOL webserver and Treedyn software (Chevenet et al., 2006; Letunic and Bork, 2016). A second pylogenomic analysis was prepared that included MBI600 and another 69 annotated *B. subtilis* proteomes, resulting in 2,736 core proteins. These core proteins were processed as described earlier resulting in 270,725 variable sites and second phylogenomic tree was also prepared.

COG annotation was performed with the WebMGA server (Wu et al., 2011) for MBI 600, 12 *B. subtilis*, 3 *B. amyloliquefaciens,* and 1 *B. velezensis* proteome of the dataset, that are known to promote plant growth. To access the unique gene content of MBI 600 strain against other plant-associated *Bacillus* strains, BlastN was performed against 18

strains. Prediction of genes involved in secondary metabolites was conducted by antiSMASH software tool (Blin et al., 2019).

MBI 600 Transformation and Confocoal Microscopy

B. subtilis MBI 600 was transformed with the strong constitutive promoter *upp* from the type strain of *Bacillus cereus* ATCC 14,579 (Eijlander and Kuipers, 2013) fused to a yellow fluorescent protein (*yfp*) inserted in the replicative plasmid pHCMC0$_2$ (Nguyen et al., 2005; Caro-Astorga et al., 2020). MBI600 was transformed by natural genetic competence using protocols similar to those used for *Bacillus subtilis* strain 168. Briefly, a MBI 600 culture in 20 ml of LB medium was incubated overnight at 37°C, under continuous shaking, in a 125 ml flask. Ten ml of the overnight culture were transferred into a 15 ml falcon and centrifuged at 7,000 rpm for 7 min. The supernatant was removed and the pellet re-suspended in 10 ml of competence medium: 100 mM potassium phosphate buffer pH 7.2, 2% D-glucose, 0.01% casaminoacids, 0.02% L-glutamate (monopotassium salt), 3 mM sodium citrate and 0.022 mg/ml ferric ammonium citrate, supplemented with 3.33 mM of $MgSO_4$ and 0.05 mg/ml of phenylalanine and tryptophan. The Optical Density (OD) was calculated and measured at ∼5. Cells were transferred in a 125 ml flask and transformation buffer was added until the OD was measured at ∼1. The flask was placed at 37°C into a shaker to an A_{600} 1-1, 5 (5–7 h). One ml of the culture was transferred in a sterile 1.5 ml Eppendorf tube, followed by the addition of 10 μg of the plasmid and incubation at 37°C for 45 min. In LB plates amended with 5 ng ml^{-1} chloramphenicol, 300 μl of the suspension were spread, while the rest of the suspension was centrifuged for 5 min at 4,000 × g. The remaining pellet was re-suspended in 100 μl of CM buffer and spread again in antibiotic-amended LB plates. The dishes were incubated at 37°C, overnight. Positive colonies were checked by fluorescence microscope and by colony PCR. For confocal microscopic observations of colonization ability a slightly modified procedure described previously was followed (Cao et al., 2011). Briefly, cucumber seeds were grown in a hydroponic floating system for 5 days. Then the roots were submerged in a cell suspension (OD∼0.8) of the GFP-tagged MBI 600, for 20 min and placed again in the floating system. For control plants the same procedure was applied, however, MBI 600 wild-type cells were used. Roots of cucumber plants were collected at 4, 24, and 48 h after bacterial application and washed with PBS buffer. Each root was placed on a glass slide with phosphate buffered saline (PBS, pH 7.2) under a coverslip. Observation was performed with a Leica TCS SP5 II confocal laser scanning microscope (CLSM), mounted on a Leica Model DMI 6000B inverted microscope, and operated in the fluorescence mode with a 60 × oil-immersion objective of numerical aperture 1.40. Fluorescence from the sample was excited with the 488 nm of an argon (Ar) laser line and with the 633 nm of a red HeNe laser line. The size of the images was adjusted to 512 × 512 pixels in x-y plane. The signal from the samples was collected and eight (8) scans were averaged during the creation of each image. The temperature during the microscopy tests was kept constant at 20°C.

Colonization Assays in Various Growth Substrates

Colonization patterns of MBI 600 on cucumber roots were tested in four different growth systems: sterile conditions (gnotobiotic system), commercial peat mixture, natural soil suitable for vegetable production (vegetable soil) and hydroponic cubes (Grodan, Netherlands). In all the experimental procedures the chloramphenicol-resistant and *yfp*-labeled strain was used.

Cucumber seeds were sterilized by immersion in 1% (v/v) sodium hypochlorite for 1 min, rinsed five times in sterile water before sowing and then placed under gnotobiotic conditions in glass tubes (200 × 25 mm diameter) filled with 20 cm^3 Perloflor® and 30 g pure sea sand, mixed with 10% (v/v) nutrient solution PNS (plant nutrition solution) (Hoffland et al., 1989). After sterilization each cucumber seed was placed in the substrate. The substrate was then, drenched with 2 ml of bacterial water suspension (OD∼0.8), as described above. A negative control with distilled water was also included in the experimental design. After inoculation, glass tubes were placed in a growth chamber (16 h photoperiod and a light/dark temperature regime of 18:25°C). Roots were collected 5, 15, and 20 days after sowing. Each root was placed into a tube with phosphate buffered saline (PBS) and transferred in Elmasonic S30 to detach bacterial cells from the roots using ultrasonic waves at a frequency of 37 kHz. After appropriate dilution, the suspensions were plated onto LB plates amended with 5 ng ml^{-1} chloramphenicol. After 24 h of incubation at 37°C, colonies were counted and the concentration was calculated as cfu/ml.

For root colonization in soil environments, 14 days-old cucumber seedlings were used. Seedlings were inoculated by soaking their roots for 20 min in a suspension containing 10^7 cfu ml^{-1} of chloramphenicol-resistant/*YFP*-tagged MBI 600. Roots treated with ddH_2O were used as control. Plants were transferred to pots with 250 gr of 2 different types of soil, a commercial peat mixture and a vegetable soil. The commercial peat mixture contained peat moss (60%), vermiculite (15%), perlite (10%), geolite (5%), guano (2%), and humic acid (1%). In the natural vegetable soil, an analysis was conducted revealing that it was a loamy sand (pH 6–4, 0–4% organic matter (OM), 84% sand (S), 8% silt (Si), and 8% clay (C). Plants grown in these soil substrates were kept in greenhouse conditions for 20 days. For root colonization in hydroponic systems, the same procedure for the bacterial application was followed. Plants were transferred to hydroponic cubes and placed in floating systems with the appropriate nutrient solution. Samplings were conducted at three time points 5, 15, and 20 days after the application. Bacterial colonies were measured as described previously.

Plants Growth Promotion Assays

The effect of MBI 600 on cucumber plants (cv. Bamboo) growth was estimated by measuring the following growth parameters: shoot height, root length, shoot fresh weight, and root fresh weight. Cucumber seeds were individually

sown in plastic pots containing 80 cm^3 of a 5:1 mixture of peat and perlite. Bacterial cultures were prepared in LB medium containing flasks and shaking overnight at 37°C. The suspension was then centrifuged at 4,000 × g for 5 min and the pellet was re-suspended in dd H_20, until the OD (measured at 600 nm) of the culture reached values of 0.8. Ten milliliter of the bacterial suspension were applied in each pot, just after sowing, by soil drenching, while the application was repeated 20 days after sowing. In addition to MBI 600 the commercially available *Bacillus amyloliquefaciens* QST713 strain (Serenade ASO, 1.34SC, BAYER CropScience), was included in the experimental design as a reference biological treatment. Seedlings were kept under greenhouse conditions. Plants were irrigated regularly but did not receive any fertilization, and watered every 2 days. Measurements were conducted 30 days after sowing. In total, there were five replicates of 10 plants each, in a complete randomized block design.

In vitro Assays for the Antagonistic Activity of MBI 600 Against *Forc* and *P. aphanidermatum*

MBI 600 was tested for its ability to inhibit the growth of *Forc* and *P. aphanidermatum* in dual-cultures (Romero et al., 2004). Dual cultures consisted of the bacterial isolate and each of the 2 fungal isolates inoculated on opposite sides of the plate at approximately 10 mm distance from the margins of the plate. The bacterial cells were streaked as a straight line onto PDA medium in 9-cm diameter Petri dishes and the plates were inoculated with a 6 mm-diameter plug of mycelium taken from the colony margins of actively growing 7 day-old cultures. Plates were incubated for 7 days at 25°C and antagonistic activity was evaluated by measuring the diameter of the fungal colonies and the length of the inhibition zones (mm). Five replicate dishes were prepared per treatment and the experiment was repeated 3 times.

Assays for the MBI600 Biocontrol Efficiency of Against *Forc* and *P. aphanidermatum* on Cucumber Plants

Eleven days-old cucumber plants (cv. Bamboo) were inoculated with *Forc* and *P. aphanidermatum*. For the production of *Forc* inoculum, mycelium was placed on PDA in 9 cm Petri dishes and incubated at 25°C for 7 days in darkness. Four mycelial plugs, taken from 7 day-old cultures, were transferred into 250 mL Czapek-Dox broth (CDB; Duchefa, Haarlem, The Netherlands) in 500 mL Erlenmeyer flasks and incubated for 3 days at 28°C in a rotary shaker at 150 rpm. After filtration through 4 layers of cheesecloth, the concentration of the resulting spore suspension was estimated by using a haemocytometer under light microscopy and adjusted to 5×10^5 conidia ml^{-1}. For *P. aphanidernatum,* inoculum was prepared in V-8 liquid medium (200 ml of vegetable juice V8 and 3 g of $CaCO_3$ per L of deionized water). Flasks were incubated for 10 days at 25°C in the dark without shaking, Then, mycelial mats were washed two times with tap water and blended for 30 s at high speed in a blender (Waring, New Hartford, United States). Oospores were counted with a haemocytometer and their concentration was adjusted at 7×10^3 ml^{-1}.

For the artificial inoculation of the plants, each pot was drenched with 10 ml of the inoculum suspensions. Control plants were drenched with sterile distilled water. The application of MBI 600 was conducted by drenching each pot with 15 ml of bacterial suspension (OD ∼0.8), 24 h before the inoculation with the pathogens. In addition to MBI 600, in the experimental design a standard chemical and a standard biological reference treatment were included. *Ba* QST713, (Serenade ASO, 1.34SC, BAYER CropScience, Greece) was the biological reference treatment applied at the commercially recommended dose of 16 ml L^{-1}, f. p., 24 h before the inoculation of the plants with the pathogens. 8-hydroxyquinoline (Beltanol 37.5 SL, Agrology SA, Greece) was the chemical reference treatment applied at the commercially recommended dose of 0.53 ml L^{-1}, f.p. 24 h before the inoculation of the plants.

FCRR symptoms were assessed 10 days after the inoculation of the plants using a 0–4 disease scale (Chen et al., 2010). Damping off symptoms caused by *P. aphanidernatum* were assessed using a 0–1 disease index scale, 7 days after the inoculation of the plants. Fifty cucumber plants per treatment were inoculated and the experiment was repeated 3 times.

Statistical Analyses

Data of the independent replications on plant growth parameters, disease incidence/severity and bacterial cell enumeration in colonization experiments, were combined after testing for homogeneity of variance using Levene's test. The combined data were then, subjected to one-way analysis of variance (ANOVA). Duncan's Multiple Range Test was used for comparison of means. The statistical analysis was supported by SPSS 21.0 (SPSS, Chicago, IL, United States).

RESULTS

Phylogenomic Analysis of *B. subtilis* MBI 600

To define the MBI 600 taxonomy, gene analysis of the 16sRNA was initially performed. BLAST analysis showed that the bacterial strain under investigation belongs to the *B. subtilis* species with a high coverage (100%) and identity (99%) score. To further reveal the evolutionary relationship of MBI 600 with other *Bacillus* spp. strains, a phylogenomic analysis was performed including 2,317 and 2,736 core proteins of 2 different sets of organisms, *B subtilis sensu lato* and *B. subtilis* species, respectively. The first phylogenomic tree clearly showed that MBI 600 is a member of the *Bacillus subtilis* subsp. *subtilis* evolutionary group and not a member of the *B. amyloliquefaciens* evolutionary group (**Supplementary Figure 1**). The second phylogenomic tree revealed the position of MBI 600 within the *B. subtilis* species (**Figure 1**). Compared to the closest plant associated genome of *B. subtilis* str. 168, which is already characterized as a biocontrol

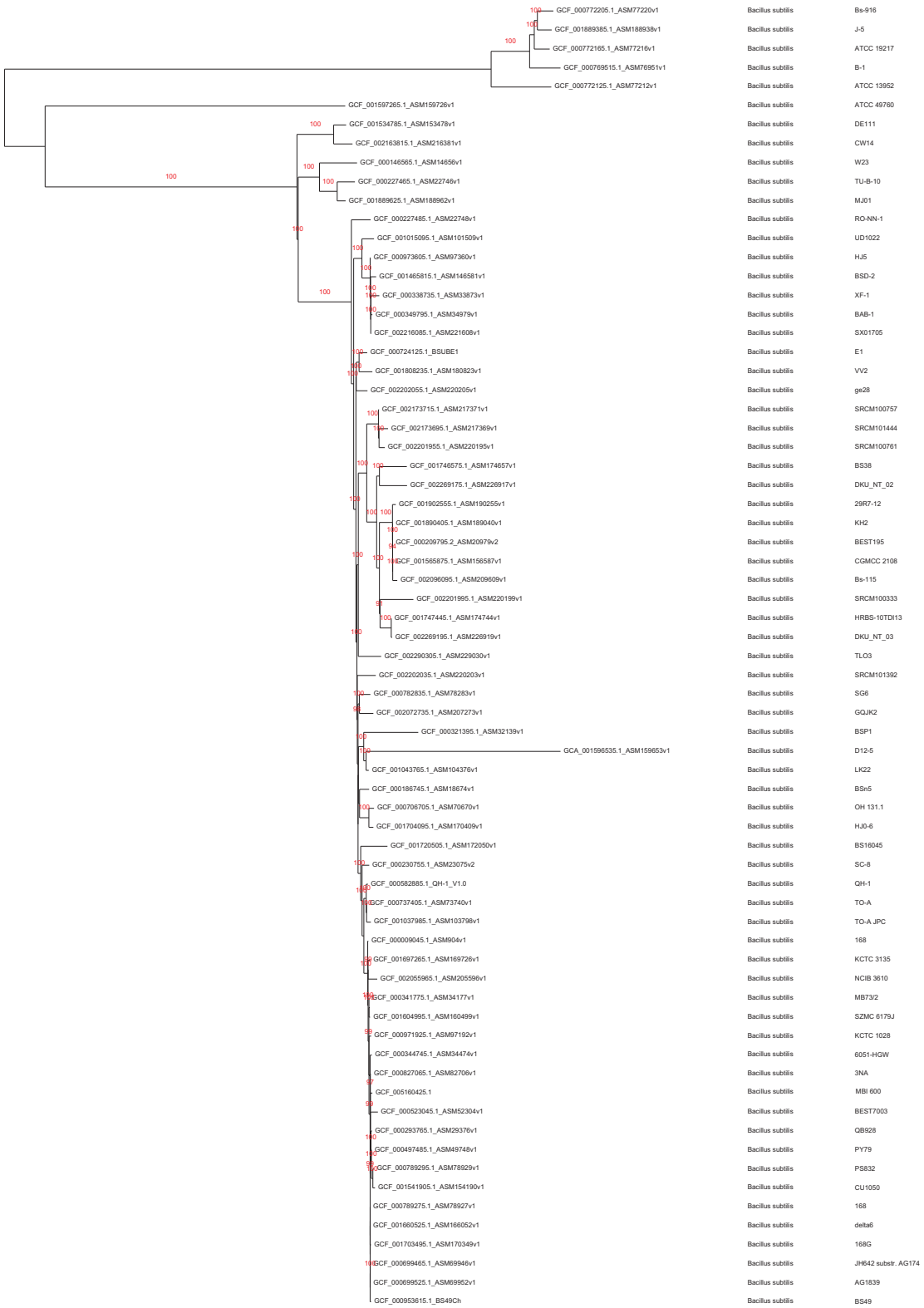

FIGURE 1 | Phylogenomic Neighbor Joining tree (with Kimura two parameter model and 500 bootstraps) of 70 *Bacillus subtilis* proteomes based on 2,736 core protein orthologous groups.

agent, the genome of MBI 600 was found to share identity higher than 95% (**Figure 2**).

Genome Analysis of *B. subtilis* MBI 600

Upon genome sequencing and assembly, MBI 600 was given as circular chromosome of 4,076,736 bp with a GC content of 43.84%, a size similar to that of other *Bacillus* genomes and was deposited in Genbank (Accession Number CP033205.1). The genome annotation report by NCBI revealed that the genome constituted of a total of 4,259 genes, with 4,076 coding CDS, 121 RNA genes, 86 tRNAs (**Figure 2**). After annotation, 2,850 CDSs were assigned to putative biological functions whereas 1,229 CDSs were characterized as hypothetical proteins with unknown function. Using Gene Ontology Consortium (GOC) analysis, 25 functional classes were identified with some related to biocontrol activity, such as production of secondary metabolites. In addition, some of the functional classes identified by the GOC analysis were related to biofertilizing, such as inorganic ion transport and metabolism (**Figure 3**).

A targeted GOC analysis was further conducted to compare the MBI 600 genome to the genomes of 18 different plant-associated strains belonging to the *Bacillus* cluster. This analysis revealed that the number of MBI 600 annotated genes was almost equal to the other strains for the majority of the functional categories. Further molecular evidence for the PGPR properties of the MBI 600 strain was provided by the GOC comparison with above mentioned PGPR *Bacillus* strains which revealed that the GOC content of MBI 600 was remarkably similar to that of the 18 reference PGPR isolates (**Figure 4**). A comparative analysis was further performed by focusing on 3 well-known biocontrol agents, *B. subtilis* 168, *B. amyloliquefaciens* FZB42 and *B. amyloliquefaciens* UMAF 6,639. Most genes related to plant growth promotion and plant protection were detected in all PGPR strains including MBI 600 (**Table 1**).

(i) Growth promotion

Beneficial bacteria contribute to plant growth promotion by involving into nutrients uptake and different unique genes are related with each of the specific nutrient elements. Two gene clusters, *nas*A-*nas*B-*nas*C and *nar* (H-Z-J-I-G) were found and annotated as nitrate transporter and as nitrate reductase, respectively, and predicted to be involved in nitrate transport and reduction. Two genes for magnesium transportation, *mgt*E and *yqx*L, were predicted to a double function, uptake of nutrients

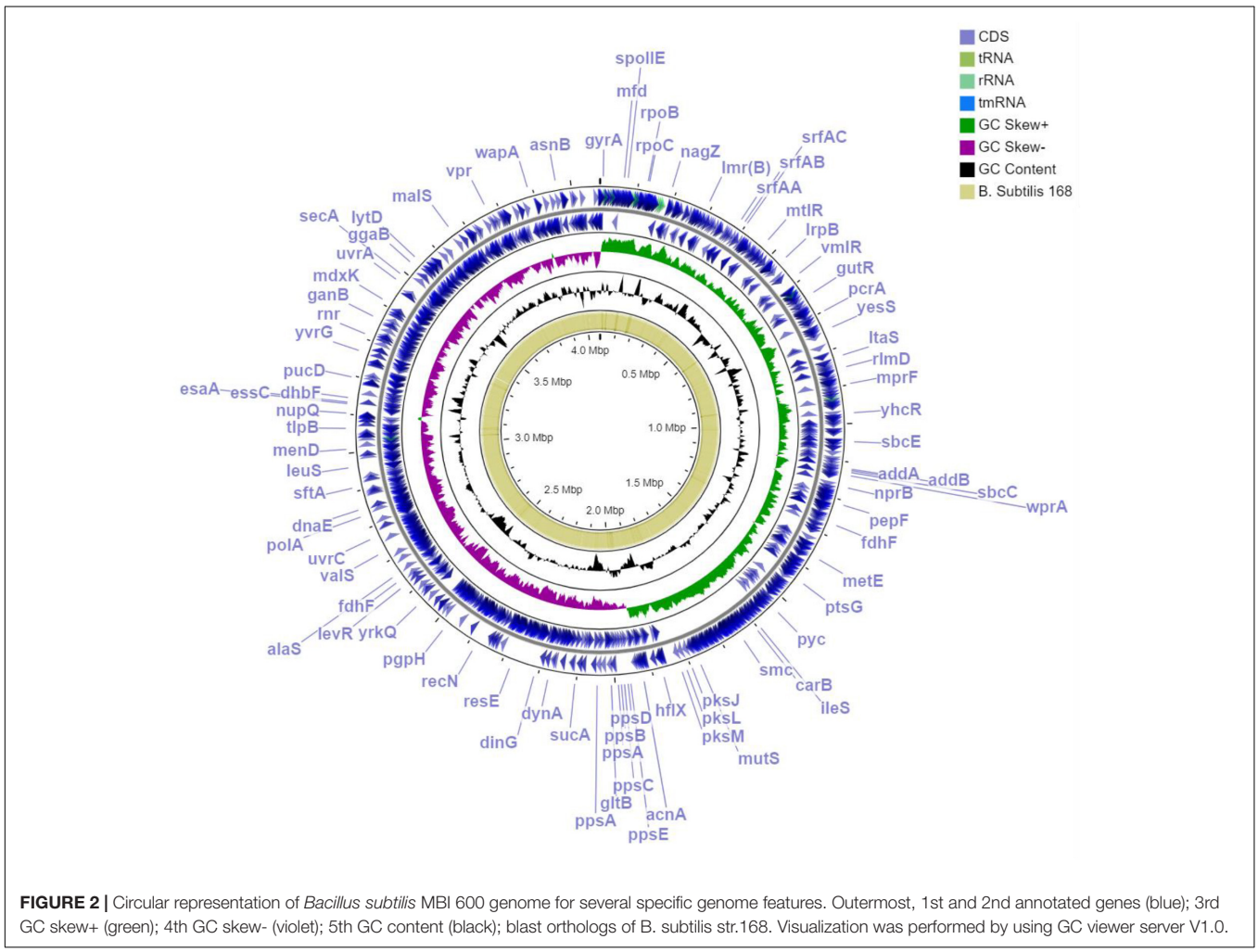

FIGURE 2 | Circular representation of *Bacillus subtilis* MBI 600 genome for several specific genome features. Outermost, 1st and 2nd annotated genes (blue); 3rd GC skew+ (green); 4th GC skew- (violet); 5th GC content (black); blast orthologs of B. subtilis str.168. Visualization was performed by using GC viewer server V1.0.

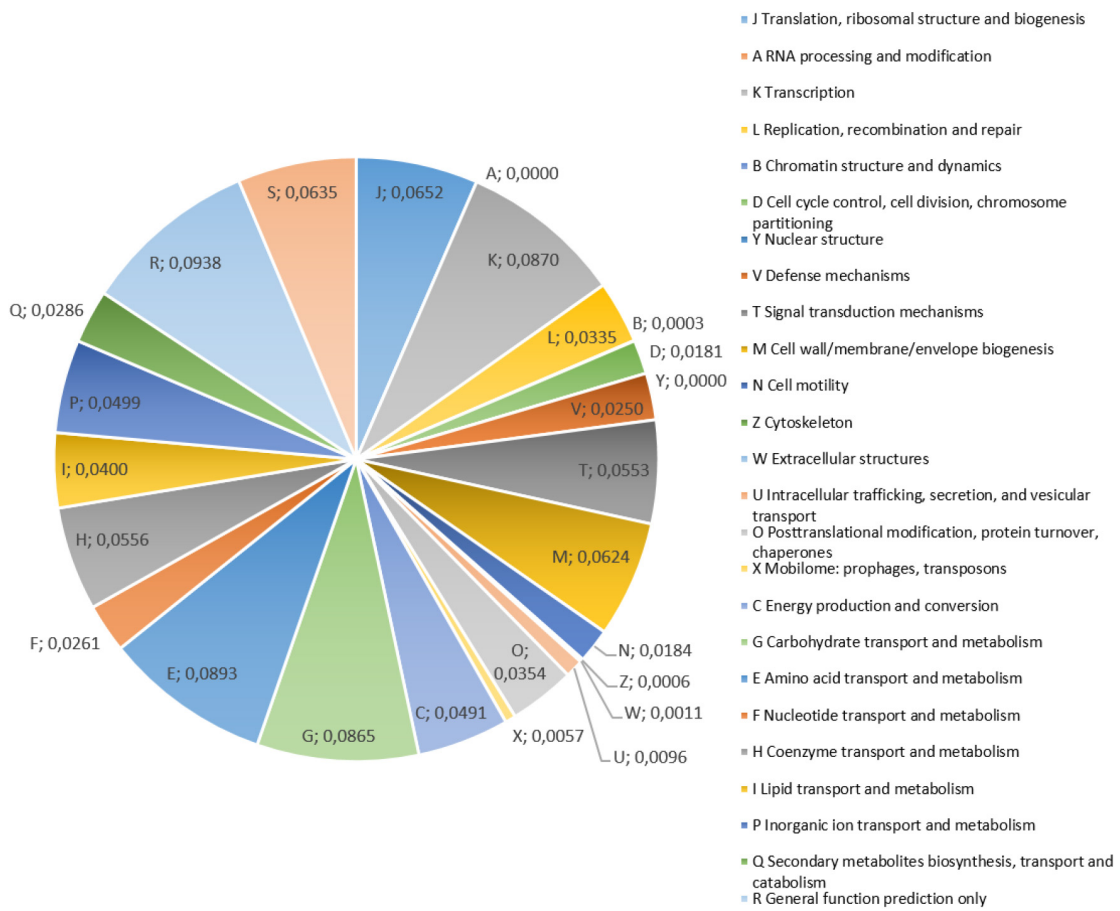

FIGURE 3 | Pie chart of GO (Gene Ontology) analysis summarized *Bacillus subtilis* MBI 600 genes, according to molecular function.

and detoxification of heavy metals ions for the host plant and the bacteria. In addition a gene cluster consisting from 4 genes *ktr* (A–D) was found in MBI 600 genome and is predicted to be involved in potassium uptake (**Table 1**).

(ii) Root colonization

Flagellar proteins play major role in the colonization ability of PGPR strains. In the MBI 600 genome we found in total 36 genes involved in flagellar protein coding. The majority of these genes was localized in 2 clusters, the flg cluster consisted of 5 genes (*flg*B—*flg*C—*flg*E—*flg*K—*flg*M) and a larger cluster with 16 genes (*fli*D- *fli*E- *fli*F- *fli*G- *fli*H- *fli*IJ- *fli*K- *fli*L- *fli*M- *fli*P- *fli*Q- *fli*S- *fli*T- *fli*Y- *fli*Z) (**Table 1**).

(iii) Direct inhibition–antibiotic production

PGPR strains produce a variety of antibiotics involved in the direct inhibition of plant pathogens in the root vicinity. Sequence of *srfA*, *srfB,* and *srfC* genes, that are likely involved in surfactin synthesis, the respective sequences of the FZB42 was highly similar to the MBI600 strain. In addition, the presence of the fengycin biosynthesis associated gene cluster consisting of 5 NRPs (ppsA-ppsE), was also found in MBI 600, with a sequence highly similar to that of the respective genes of FZB24. In addition to the above mentioned genes that identified with high similarity to genes from other PGPR strains, the prediction analysis with antiSMASH software revealed the existence in MBI 600 genome of additional regions that, according to the prediction, were involved in other antibiotic biosynthesis including compounds such as bacillaene, bacillibactin, subtilosin A and basilysin (**Table 2**).

Root Colonization Ability of YFP-Tagged MBI 600

In order to realize the colonization patterns of MBI in the cucumber root surface, cucumber roots were inoculated with YFP-tagged bacterial cells and observed by confocal microscopy. In addition, the required colonization time was investigated. The MBI 600 YFP-tagged cells emitted a constant fluorescence allowing to easily distinguish them from the background root auto-fluorescence (**Figure 5**). In contrast, no fluorescent cells were observed in the roots of plant treated with the non YFP-tagged bacterial strain. The confocal microscopy images showed that colonization of the root surface initiated 24 h after the

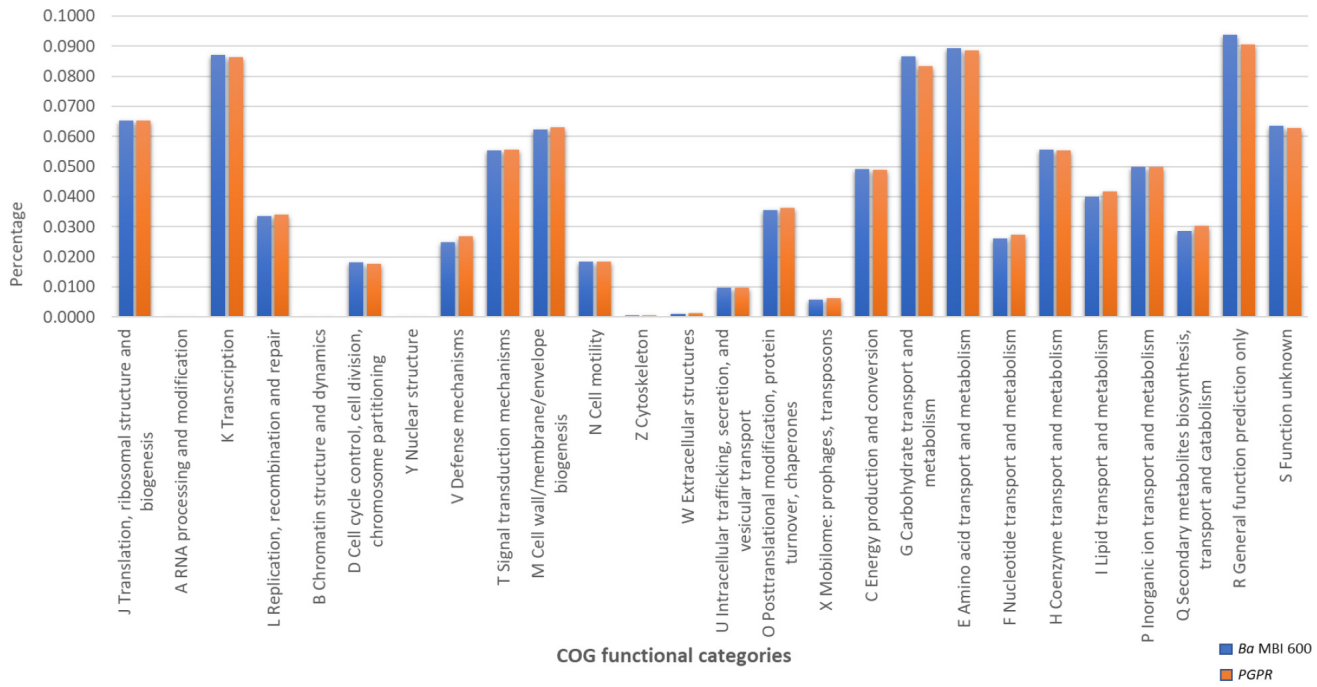

FIGURE 4 | GOC annotation differences between *Bacillus subtilis* MBI 600 and 16 additional Plant Growth Promoting *Bacillus* sp. strains.

TABLE 1 | Presence (+) or absence (–) of selected genes associated with plant growth promotion and plant protection against pathogens in biocontrol agents belonging in the Bacillus family.

Gene	Annotation	Function	PGPR species[a]			
			Bs MBI 600	***Bs 168***	***Ba FZB 42***	***Ba UMAF 6,639***
yvra	Iron ABC transporter ATP-binding protein	Putative iron availability	+	+	+	+
yvrb	Corrinoid ABC transporter permease	Putative iron availability	+	+	+	+
yvrc	Corrinoid ABC transporter permease	Putative iron availability	+	+	+	+
Nar	Nitrate trasporter	Nitrate transporter	+	+	+	+
Nas	Nitrate trasporter	Nitrate transporter	+	+	+	+
ktrA	Potassium transporter	Potassium transporter	+	+	+	+
yugO	Potassium channel	Potassium transporter	+	+	+	+
mgtE	Magnesium transporter	Magnesium transporter	+	–	+	–
Ktr	Potassium uptake	Potassium transporter	+	+	+	–
srfa	Surfactin synthase subunit 1	Secondary metabolite production	+	+	+	+
srfb	Surfactin synthase subunit 2	Secondary metabolite production	+	+	+	+
srfc	Surfactin synthase subunit 3	Secondary metabolite production	+	+	+	+
sfp	Fengysin production	Secondary metabolite production	+	–	+	–
npr	Bacillolysin	Secondary metabolite production	+	+	–	–
sbo-alb	Antilisterial bacteriocin subtilosin biosynthesis protein AlbB	Secondary metabolite production	+	+	–	–
flgB	Flagellar coding protein	Cells active movevent	+	–	+	+
fliD	*Flagellar coding protein*	*Cells active movevent*	+	–	+	+

[a] *Bs, Bacillus subtilis; Ba, Bacillus amyloliquefaciens.*

immersion. After 48 h, bacterial cells were clearly visible along the entire length of the root segment. Laser scanning in different internal root layers did not show any presence of YFP-tagged bacterial cells (data not shown). Cells were observed on root surface as single cells or clustered in microcolonies (**Figure 5**). Transformed cells of MBI 600 were tested and showed that the *yfp* gene didn't affect growth parameters (**Supplementary Figure 3**).

In order to determine the ability of MBI 600 to colonize cucumber roots growing in different substrates, to determine the application availability of the strain in various cultivated

TABLE 2 | Prediction of clusters in *Bacillus subtilis* MBI 600 genome involved in secondary metabolites production, using AntiSMASH tool.

Regions (bp)	Type	Compound	Similarity%
Region 1 (204,359–226,256)[a]	Sactipeptide	Sporulation killing factor	100
Region 2 (358,311–421,751)	NRPs	Surfactin	82
Region 3 (1,761,171–1,866,418)	TransAT-PKs	Bacillaene	100
Region 4 (1,932,857–2,015,146)	NRPs	Fengycin	100
Region 5 (3,119,403–3,169,144)	NRPs	Bacillibactin	100
Region 6 (3,686,669–3,708,280)	Sactipeptide	Subtilosin A	100
Region 7 (3,711,289–3,752,707)	Other	Basilysin	100

[a] *The specific nucleotide position of its region, in the full genome of Bs MBI 600, according to AntiSMASH tool.*

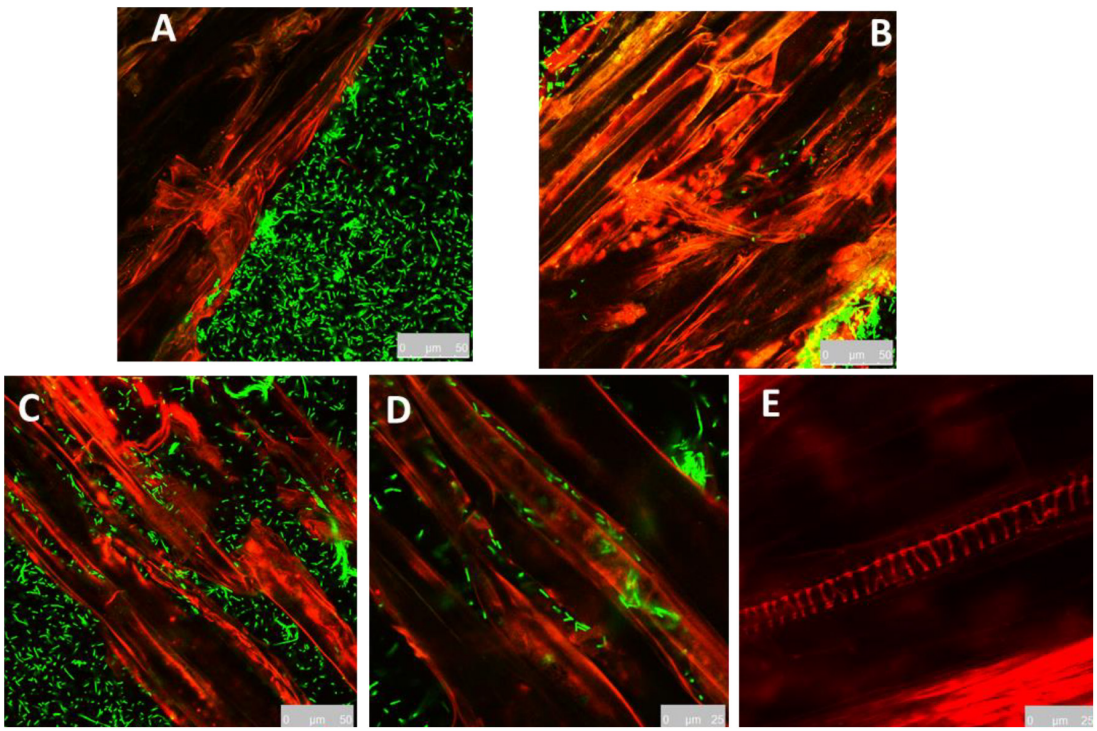

FIGURE 5 | Root colonization of YFP–tagged *Bacillus subtilis* MBI 600, visualized under confocal scanning laser microscope. **(A)** 4 h post inoculation, **(B)** 24 h post inoculation, **(C,D)** 48 h post inoculation, bacterial cells in the elongation zone of cucumber root. **(E)** Cucumber root inoculated with non YFP-tagged bacterial cells.

methods. To achieve that we successfully constructed a yfp-labeled mutant of the strain. Bacterial suspensions obtained from 1 cm homogenized root tissues were spread on TSA plates supplemented with 10 μg ml^{-1} of chloramphenicol, in which only transformed cells and no wild type cells of MBI 600 showed growth. The counts of bacterial cells on the chloramphenicol-amended medium showed that MBI 600 strain was able to successfully colonize cucumber roots in all the 4 different growth substrates tested, although with ranging effectiveness between these different systems. In all the 4 different growth substrates tested the higher levels of bacterial presence on cucumber roots was observed at 1st sampling, 5 days post application. At this time point the higher counts of bacterial cells were measured in the gnotobiotic system and in the commercial peat mixture with values of 3×10^8 cfu cm^{-1} and 3.2×10^7 cfu cm^{-1}, respectively (**Table 3**). The colonization pattern remained the same up to the 10th day, although a decrease in bacterial population was observed 15 and 20 days after inoculation in all 4 growth substrates (**Table 3**). Nevertheless, the gnotobiotic and the commercial peat mixture systems still recorded the greatest colonization levels. The hydroponic system proved to be the least effective concerning bacterial colonization, showing the lowest amount of bacterial population in every single time period.

Growth Promotion of Cucumber Plants

Pot experiments with applications of MBI 600 in cucumber plants allowed to investigate the effect in plant growth promotion. Measurements of the growth parameters on cucumber plants after 35 days under greenhouse conditions revealed that applications of MBI 600 resulted in a significant ($P < 0.05$) increase in shoot height, root length and shoot fresh weight compared to that of the untreated control plants (**Table 4**).

Differences in root length and shoot height of untreated control plants and plants treated with MBI 600 are evident in **Supplementary Figure 2**. Similarly, applications of the reference BCA product *Ba* QST 713 resulted in an increase ($P < 0.05$) of root length and shoot fresh weight compared to that of control treatment, while, no difference ($P > 0.05$) was observed between the control treatment and *Ba* QST713 regarding shoot height. In contrast, no significant differences were observed ($P > 0.05$) among control and biological treatments in root fresh weight (**Table 4**).

In vitro Antagonistic Activity of MBI 600 Against *Forc* and *P. aphanidermatum*

The *in vitro* antagonistic activity of MBI 600 was tested on PDA, a nutrient medium suitable for the growth of all the 3 microorganisms used in the study. After 7 days of dual culturing with the plant pathogens, MBI 600 reduced significantly the mycelial growth of both *Forc* and *P. aphanidermatum*. More specifically, the relative inhibition of mycelial growth for *Forc* and *P. aphanidernatum* in the presence of MBI 600 was 26 and 33%, respectively. In addition, formation of inhibition zone of mycelial growth was observed in the dual cultures with both pathogens (**Table 5** and **Figure 6**).

Biocontrol Activity of MBI 600 Against *Forc* and *P. aphanidermatum*

To determine the ability of MBI 600 to control plant pathogens, we selected 2 main soil-borne pathogens of cucumber, *Pythium aphanidermatum* and *Fusarium oxysporum* f.sp. *radicis-cucumerinum*. MBI 600 significantly inhibited both *Fusarium* crown and root rot and *Pythium* damping-off on cucumber plants in greenhouse pot experiments. The applications of MBI 600 resulted in a significant reduction of disease severity compared to that observed in the untreated control treatment both for FCRR and *Pythium* damping off (**Table 6**). The observed control efficacy reached values of 80 and 85% for FCRR and *Pythium* damping off, respectively (**Table 6**). The observed control efficacy values achieved by MBI 600 applications were similar ($P < 0.05$) to that achieved by reference chemical treatment (8-hydroxyquinoline)

TABLE 3 | Counts (cfu cm^{-1}) of chloramphenicol-resistant/YFP-tagged *Bacillus subtilis* MBI 600 strain on cucumber roots grown in 4 different growth substrates.

Growing system[a]	Days after inoculation		
	5	15	20
Gnotobiotic system	3×10^8 a[b]	3.2×10^6 a	3.2×10^5 a
Commercial Peat mixture	3.2×10^7 a	4×10^5 ab	2.5×10^4 a
Vegetable soil	2×10^5 ab	2×10^4 b	1.5×10^4 a
Hydroponic cubes	4×10^4 b	2×10^2 c	1.7×10^2 b

[a] *Initial application rate of Bs MBI 600 was* 2×10^{10} *cfu* ml^{-1}.
[b] *Mean values followed by different letters in the column indicate significant differences among treatments according to Duncan's Multiple Range Test ($P \leq 0.05$).*

TABLE 4 | Effect of *Bacillus subtilis* MBI 600 applications on cucumber plants growth parameters compared to the growth of untreated control plants and *Bacillus amyloliquefaciens* QST713-treated plants (reference biological treatment).

Treatment	Growth parameter			
	Shoot height (cm)	Root length (cm)	Shoot fresh weight (gr)	Root fresh weight (gr)
Control	13.09 a*	30.10 a	15.97 a	2.78 a
Bs MBI 600 (OD = 0.7)	17.23 b	40.40 b	16.42 b	2.14 a
Ba QST 713 (OD = 0.7)	15.35 ab	48.50 c	15.94 a	2.17 a

**Mean values followed by different letters in the column indicate significant differences among treatments according to Duncan's Multiple Range Test ($P < 0.05$).*

TABLE 5 | Effect of *Bacillus subtilis* MBI 600 on the *in vitro* mycelial growth of the cucumber pathogens *Fusarium oxysporum* f.sp. *radicis-cucumerinum* and *Pythium aphanidermatum* after 7 days in dual culture.

Treatment	Pathogen					
	Fusarium oxysporum f.sp. *radicis cucumerinum*			*Pythium aphanidermatum*		
	Colony diameter (mm)	Relative inhibition	Inhibition zone[a]	Colony diameter (mm)	Relative inhibition	Inhibition zone
Control (Pathogen)	46b[b]	0b	–	60b	0b	–
MBI 600 + Pathogen	25a	26a	+	45a	33a	+

[a] *Diameter (mm) of inhibition zone between pathogens and biocontrol agent on PDA plates: – no inhibition, +, inhibition zone of < 10 mm; ++, inhibition zone of > 10 mm.*
[b] *Mean values followed by different letters in the column indicate significant differences among treatments according to Duncan's Multiple Range Test ($P \leq 0.05$).*

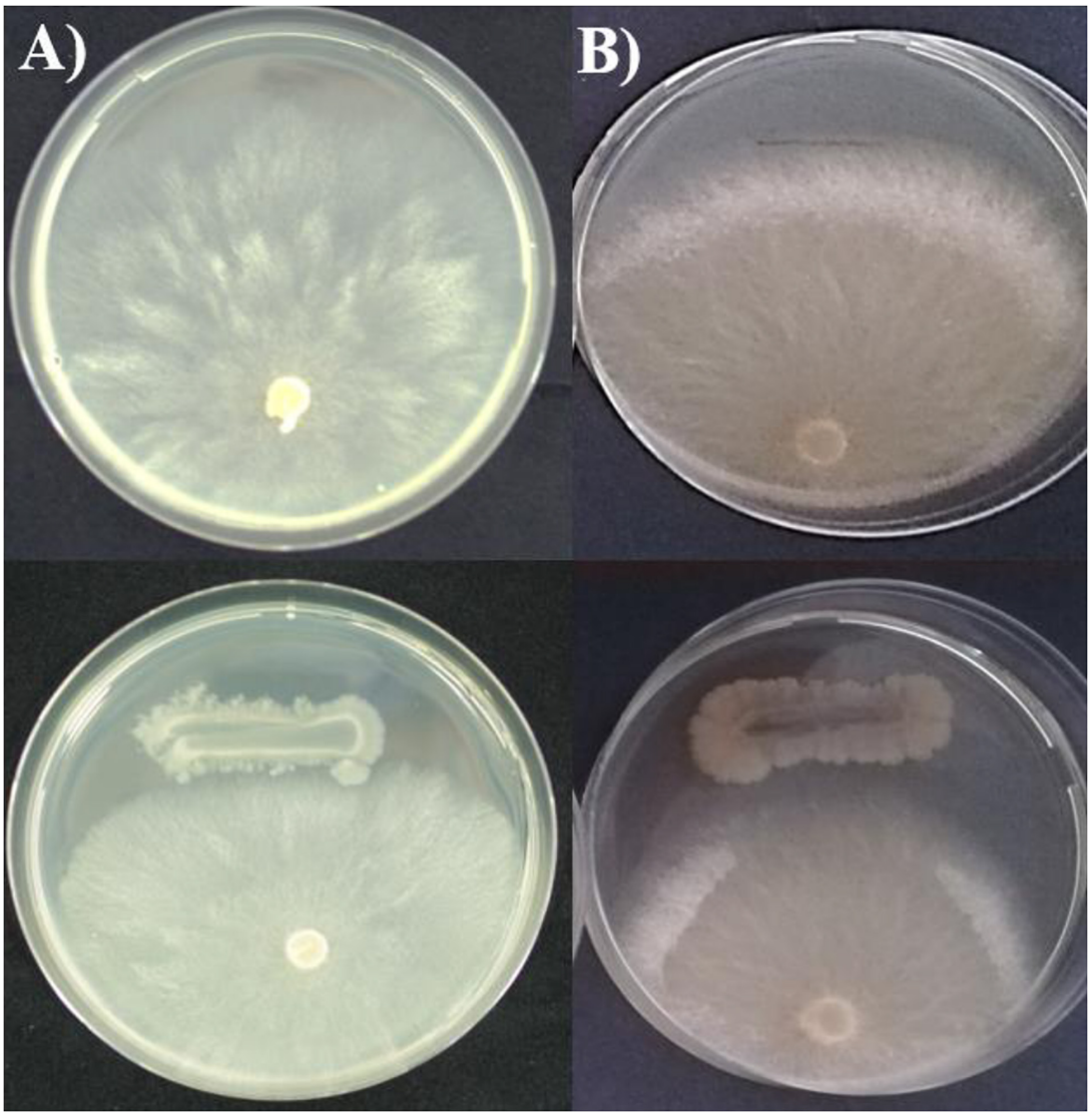

FIGURE 6 | Mycelial growth of **(A)** *Pythium aphanidermatum* and **(B)** *Fusarium oxysporum* f.sp. *radicis-cucumerinum* in the absence (up) and in the presence (down) of *Bacillu subtilis* MBI 600.

TABLE 6 | Biocontrol efficacy of *Bacillus subtilis* MBI 600 against Fusarium crown and root rot and *Pythium* damping off diseases on cucumber plants in pot experiments.

Treatment	Application rate	Pathogen			
		Fusarium oxysporum* f.sp. *radicis-cucumerinum		***Pythium aphanidermatum***	
		Disease severity[a]	**Control efficacy (%)**	**Disease severity**	**Control efficacy (%)**
Ba MBI600	15 ml L^{-1} (OD~0.8)	0.09 a[b]	80 b[c]	0.02a	85 b
Ba QST713[d]	15 ml L^{-1} (OD~0.8)	0.25 ab	73 a	0.11ab	68 a
8-Hydroxyquinoline[d]	0.53 ml L^{-1}, f.p.	0.06 a	90 b	0.06a	90 b
Control	–	1.09 b	–	0.54b	–

[a] *Disease severity values for Forc and P. aphanidermatum were measured based on 0–4 and a 0–1 disease scale, respectively. Measurements were conducted 10 days after artificial inoculation of the plants.*
[b] *Values followed by different letter in the column are significantly different at P = 0.05, according to Duncan's Multiple Range Test.*
[c] *Values followed by different letter in the column are significantly different at P = 0.05, according to X^2-test.*
[d] *Bacillus amyloliquefaciens QST713 (Ba QST713) and 8-hydroxyquinoline were the commercial biological and chemical reference treatments, respectively.*

and higher ($P > 0.05$) to that achieved by the reference biological treatment (*Ba* QST713) (**Table 6**).

DISCUSSION

In the current study we employed a multitasking strategy to understand the beneficial role of a recently commercialized biopesticide, *B. subtilis* MBI 600 on cucumber plants. The strain was already known for its biocontrol ability against soilborne pathogens such as *Fusarium oxysporum* f.sp. *radicis-lycopersici* on tomato plants and *Pythium* spp. on sugar beet seedlings (Schmidt et al., 2004; Samaras et al., 2018), while more recently was shown that it can exhibit antiviral action against viruses infecting tomato (Beris et al., 2018). This antiviral activity on tomato was mediated by the activation of SA-responsive genes and a synergistic cross-talk between JA/ET- and SA-signaling that triggered defense responses (Dimopoulou et al., 2019).

The whole genome sequencing and annotation of MBI 600 conducted in this study was selected as a powerful tool to determine the taxonomic position of the strain and to further study the molecular basis of mechanisms involved in plant growth promotion, root colonization and plant protection against fungal pathogens. Genomic assembly of MBI 600 was found to be similar to that of other *B. subtillis* genomes (Liu et al., 2018; Rahimi et al., 2018). The taxonomy identification between the Bacillus species is very difficult and is not clear with strict lines were each isolate belongs. In order to be more accurate, we conducted phylogenetic analysis with the 2 main species *Bacillus subtillis* and *Bacillus amyloliquefaciens.* The taxonomy identification was performed by a phylogenetic analysis that included several other plant-associated or non-associated *Bacillus* sp. Strain MBI 600 was classified in the *Bacillus subtilis* subsp. *subtilis* group, in the same branch with other plant-associated strains such as 168 and XF-1 (Guo et al., 2015).

Previous studies that compared the genome of plant- and non-plant-associated *Bacillus* spp. showed that various genes involved in biosynthesis of secondary metabolites were more abundant in plant-associated strains (Zhang et al., 2016). The GOC analysis conducted in our study to compare the genome of MBI 600 to the genomes of 18 different plant-associated strains belonging to *Bacillus* complex showed that the number of annotated genes from MBI 600 was almost equal, compared to the other strains in the majority of function categories. Such comparison provides an indirect evidence for the ability of MBI 600 to be a powerful agent of plant growth promotion and successful biological control of plant pathogens.

It is well established that enhancement of plant hormone biosynthesis that is closely related to nutrient uptake availability, mediates plant growth promotion and yield (Chen et al., 2007). Pot experiments with applications of MBI 600 in cucumber plants revealed an increase in shoot height and root length. In a previous study of our group, a similar growth pattern had been observed in tomato plants treated with MBI 600 (Samaras et al., 2018). The MBI 600 genome annotation conducted in the current study demonstrated that a large number of MBI 600 genes were involved in plant growth by enhancing nutrient up-take and availability. In detail, in MBI 600 genome, the nitrate transporters *nark* and *nas* clusters (A-B-C), the nitrate reductase narH-narZ-narJ-narI-narG and their putative regulator gene *arf*M were found. These gene clusters are predicted to be involved in nitrate transport and reduction (Wray et al., 1994). In addition to genes involved in nitrate transport, the existence of genes involved in potassium transport were identified in MBI 600 genome. Potassium is one of the most important elements in plant nutrition and PGPR play the main role for plant up taking from the soil (Hayat et al., 2010). Two genes, *ktr*A and *yug*O that had been identified and characterized as K transporters in *B. subtilis* (Holtmann et al., 2003) were also found in MBI 600 genome. In a recent full genome analysis of *B. subtillis* XF-1, some genes were found and predicted to be involved in magnesium uptake and de-toxification of heavy metal ions in host plants (Guo et al., 2015). In MBI 600 *mgt*E and *yqx*L were found and probably play the same role. Iron is an important micronutrient, which acts as a co-factor in more than 120 enzymatic activities, including chlorophyll biosynthesis and is thus, related to plant growth (Brittenham, 1994; Miller et al., 1995). The role of PGPR in iron availability is very crucial and succeeded by the siderophore production involved in the process of chelating ferric iron from the soil (Schalk et al., 2011). Strain MBI 600 is able to produce siderophores (data not shown). In addition, a cluster consisting of 3 genes (*yvr*A-*yvr*B-*yvr*C), was found to the MBI 600 genome and predicted on putative iron availability. The same cluster was reported in 2 *B. amyloliquefaciens* strains, CECT 8,237 and 8,238 (Magno-Perez-Bryan et al., 2015). In addition to the previously mentioned genes identified by the whole genome sequencing, antiSmash analysis of MBI 600 genome detected a domain/region with a very high query of bacillibactin synthesis. Bacillibactin is involved in the uptake of iron ions from the natural environment under iron limitation (Chen et al., 2009). However, the presence of this specific cluster doesn't guarantee the production of the bacillibactin by MBI 600 as has been observed in other *Bacillus* strains such as *B. subtillis* 168 (May et al., 2001). A MALDI-TOF analysis could provide a clear evidence related to the production of bacillabactin by MBI 600. To determine the ability of MBI 600 to control plant pathogens, we selected 2 main soil-borne pathogens of cucumber, *Pythium aphanidermatum* and *Fusarium oxysporum* f.sp. *radicis-cucumerinum*. Assessment of the antagonistic activity of MBI 600 against the 2 pathogens in dual cultures *in vitro* revealed a significant reduction of mycelial growth of both pathogens. Previous studies with other *Bacillus* strains such as B068150 or SQR -9 revealed variable results against *Forc* when their antagonistic activity was tested *in vitro* (Cao et al., 2011; Li et al., 2012). Such variability is most probably related to the BCA ability to produce antifungal compounds. A lot of *Bacillus* strains are able to synthesize enzymes and non-ribosomal peptide synthetases, which are composed of multi-modulary arranged catalytic domains, catalyzing peptide formation (Stein et al., 2005). Amongst them, surfactin, fengycin, bacillomycin D, and bacillicin are the most important, indicating hemolytic, antimicrobial and antiviral activities (Chen et al., 2009). The

whole genome sequence conducted in our study revealed the presence of genes encoding several of these metabolites. For instance, the gene cluster *srf* (A-B-C) that is involved in surfactin production was found in MBI 600 genome. Fengycin, a cyclic lipodecapeptide, that is highly active against filamentous fungi, was firstly identified by Nishikori et al. (1986). It is biosynthesized by a gene cluster (*pps*A-*pps*E) that was detected in MBI 600 and showed a high similarity to FZB42 cluster. In addition, the AntiSmash tool predicted a region (6) that is associated to Suntilosin A production, while two additional genes, *npr* and *sbo-alb*, found in the genome of MBI 600 are involved in the production of bacillolysin and subtolisin, respectively. These antibiotics are already known for their activity against fungal and bacterial pathogens (Halimi et al., 2010; Goswami et al., 2016). Studies on the isolation and characterization of lipopeptides with antimicrobial activity produced by MBI 600 are now being carried out in our laboratory.

In our experiments, strain MBI 600 was able to control *Fusarium* crown and root rot and *Pythium* damping off. Two drenching applications in cucumber seedlings was found to increase the control efficacy in levels equal to that of chemical treatment. Several *Bacillus* strains were reported to control these pathogens in different conditions and environments, and confirm our results (Raza et al., 2017). Our experiments underline that biocontrol agents might be an effective solution against soilborne pathogens of cucumber seedlings. Nevertheless, field experiments are necessary to verify the efficacy of MBI 600 against these pathogens and to determine the effects of natural interactions and soil conditions on its performance.

It is well established that root colonization ability plays a crucial role in the interaction between plants and PGPR (Fan et al., 2011; Vacheron et al., 2013). PGPR that are successful colonizers of plant roots reach the surface of the roots in 2 different ways, either by passive movement in water fluxes or by active flagella- propelled swimming. The active movement is determined by a special genetic motif that has been identified in most *Bacillus* sp. This motif consists of one flagella biosynthesis operon (*flalche*) and two stator elements *mot*AB and *mot*PS (Werhane et al., 2004). Two gene clusters related to flagellar motion, *flg* with 5 genes and *fli* with 16 genes were found in MBI 600 genome. Genes from these clusters were found in several *Bacillus* strains genomes and are associated with root colonization ability (Guo et al., 2015; Magno-Perez-Bryan et al., 2015).

Another major objective of our study was to determine the ability of MBI 600 to colonize cucumber roots growing in different substrates. To achieve that we successfully constructed a yfp-labeled mutant of the strain. To accomplish high stability and to avoid genetic burden, we chose to integrate a single copy of the *yfp* gene by using natural DNA transformation and take advantage of a functional homologous recombination system, that reported in a previous study for *B. amyloliquefaciens* FZB42 (Koumoutsi et al., 2004). Transformed cells of MBI 600 were tested and showed that the *yfp* gene didn't affect growth parameters and seems to be suitable for long term studies, carried out in natural environments As we expected, the *yfp*-labeled cells were more brightly fluorescent when growing in LB media compared to cells grown on plant roots. Other studies with other *gfp*-labeled from *Bacillus* sp. showed an opposite effect suggesting that the expression levels of fluorescence protein are strain-dependent, while, in addition the root exudates of each host consisting of different metabolites may affect fluorescence level (Fan et al., 2011). The results of our study showed that the *yfp*-labeled cells of MBI 600 needed at least 24 h to colonize the primary root of cucumber seedlings. Confocal microscopy showed that bacterial cells colonize the surface of the primary root and mainly the lower rhizoplane part. This observation could be explained by the precise localization of root exudates. Previous studies with FZB42 showed that rhizobacteria colonized only a small part of rhizoplane, mainly in the region between epidermal cells and areas where lateral roots arise (Timmusk et al., 2005; Cao et al., 2011; Fan et al., 2011). Unfortunately, we couldn't proceed to more observations and comparisons of colonization ability in more root parts since the experiment was conducted in roots of very young seedlings. Further research on roots of older plants will aid toward a more detailed localization of MBI 600 growth on plant roots. Nevertheless, images from confocal microscope showed that MBI 600 cells were localized only in rhizoplane. This pattern suggests that MBI 600 is a true epiphyte as has been previously observed for other *Bacillus* spp. commercialized as BCAs such as *B. amyloliquefaciens* FZB42 on different plant species and *B. subtilis* SQR9 on cucumber roots (Cao et al., 2011; Fan et al., 2011).

The colonization ability of MBI 600 was tested on cucumber roots grown in 4 different substrates and plate counts showed that MBI 600 had the ability to colonize the roots in all the 4 different substrates, although with marked differences in colonization efficiency. In all the 4 substrates the higher densities of the introduced strains were recovered from the rhizoplane 5 days after the introduction, while densities remained high until 15 days after BCA application. Comparisons among the 4 substrates showed the higher population densities were observed in roots grown in the gnotobiotic system and in the peat mixture. The microbial community in these 2 substrates was probably more "poor" than in the remaining 2 substrates, so the antagonism for space and nutrients was low. In addition, there are a lot of biotic and abiotic factors such as root exudates, chemical signaling between microorganisms, acidification and high molar C/N ratios that may affect colonization of roots grown in a gnotobiotic system (Dutta and Podile, 2010). The lower bacterial densities were counted in cucumber roots grown in the grodan cubes. Such low colonization ability of MBI 600 in the grodan cubes is probably related to toxicity of the mineral solution used for the nutrition of the plants. *In vitro* bioassays conducted aiming to determine the effect of the mineral solution on the growth of MBI 600 confirmed this hypothesis (data not shown). Such a toxic effect of mineral solutions used for plant nutrition on the bacterial growth has been previously observed in PGPR strains (Lee and Lee, 2015). In vegetable soil, the number of recovered bacterial cells was lower than that from roots grown in the peat mixture or the gnotobiotic system. Such finding can be explained by the ecology of bacteria competition and many mechanisms

involved in that. As has been shown by studies conducted in our lab MBI 600 is a strain able to produce siderophores (unpublished data). However, production of siderophores is energy costly and therefore, siderophore-producing populations are "available" to social cheating by individuals that lose this ability but maintain the capacity to take them up (West and Buckling, 2003; Steinauer et al., 2016). This mechanism creates an energy imbalance between the microbial populations (Hibbing et al., 2010) and possibly contributes to the lower recovery of MBI 600 in the vegetable soil.

In conclusion, this is the first study that provides information on the whole genome sequence of a novel biological control agent commercialized recently in Europe and US. Using this approach, we unraveled its taxonomy as a strain of *B. subtilis*, while, in addition, we identified in the genome of MBI 600 a series of several genes that may play a crucial role in plant growth promotion, root colonization ability and biological control of plant pathogens. However, further research is required to confirm, by chemical analytical methods, the production of metabolites encoded by these genes, that are implicated to growth promotion and/or biological control of pathogens. Furthermore, by taking advantage of a functional homologous recombination system we successfully obtained a *yfp*-labeled MBI 600 mutant enabling us to localize the growth patterns of bacterial cells on cucumber roots using confocal microscopy. By using this tool we showed that MBI 600 exhibits only epiphytic growth, while bacterial cells maybe persistent on the root surface at least 20 days post inoculation. *In vitro* measurements of antagonistic effects and pot experiments showed that MBI 600 can effectively control 2 major pathogens of cucumber, *P. aphanidermatum* and *Forc* that is hard to be managed successfully with conventional chemicals. However, further research is required to optimize the use of MBI 600 under field/greenhouse conditions, while additional studies on the effect of MBI 600 against foliar pathogens of cucumber or against pathogens in other crops may contribute to the expansion of its use in agricultural crops.

DATA AVAILABILITY STATEMENT

The datasets presented in this study can be found in online repositories. The names of the repository/repositories and accession number(s) can be found in the article/ **Supplementary Material.**

AUTHOR CONTRIBUTIONS

AS was the principal investigator, conceived and designed the experiments, and contributed to the writing of the manuscript. MN conducted the bioinformatic analysis. TM was responsible for confocal microscopy studies. GA conducted the bioinformatic analysis and wrote parts of the manuscript. MA-G and JC-A contributed to the transformation procedure of MBI 600. DR supervised the Bacillus transformation procedure and wrote the related part of the manuscript. GK supervised the study, conceived, and designed the experiments in collaboration with AS and wrote part of the manuscript. All authors read and approved the final manuscript.

FUNDING

This research has been co-financed by the European Union and Greek national funds through the Operational Program Competitiveness, Entrepreneurship and Innovation, under the call RESEARCH—CREATE—INNOVATE (project code: T1EDK- 01442).

ACKNOWLEDGMENTS

We would like to thank Mr. A. Chaliotis for initial genome assembly and bioinformatics analyses. MN would like to thank the Bodossakis foundation for financial support (MSc studentship: BDA-394).

SUPPLEMENTARY MATERIAL

The Supplementary Material for this article can be found online at: https://www.frontiersin.org/articles/10.3389/fmicb.2020.600393/full#supplementary-material

Supplementary Figure 1 | A phylogenetic tree showing relationships between a few closely related plant-associated Bacillus species strains, including *B. subtillis*, *B. atrophaeus*, *B. amyloliquefaciens*, *B. velezensis*. *B. licheniformis*—B. paralicheniformis was used as the outlier.

Supplementary Figure 2 | Growth promotion in 35 days-old cucumber plants treated with MBI 600, compared to non-treated control plants.

Supplementary Figure 3 | Growth curves studies of yfp- and non-yfp-tagged bacterial cells of *Bacillus subtilis* MBI 600 strain.

REFERENCES

Antipov, D., Hartwick, N., Shen, M., Raiko, M., Lapidus, A., and Pevzner, P. A. (2016). PlasmidSPAdes: assembling plasmids from whole genome sequencing data. *Bioinformatics* 32, 3380–3387.

Backer, R., Rokem, J. S., Ilangumaran, G., Lamont, J., Praslickova, D., Ricci, E., et al. (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

Beauregard, P. B., Chai, Y. R., Vlamakis, H., Losick, R., and Kolter, R. (2013). Bacillus subtilis biofilm induction by plant polysaccharides. *Proc. Natl Acad. Sci. U.S.A.* 110, 1621–1630.

Beris, D., Theologidis, I., Skandalis, N., and Vassilakos, N. (2018). *Bacillus amyloliquefaciens* strain MBI600 induces salicylic acid dependent resistance in tomato plants against tomato spotted wilt virus and Potato virus Y. *Sci. Rep.* 8:10320. doi: 10.1038/s41598-018-28677-3

Blin, K., Shaw, S., Steinke, K., Villebro, R., Ziemert, N., Lee, S. Y., et al. (2019). antiSMASH 5.0: updates to the secondary metabolite genome mining pipeline. *Nucleic Acids Res.* 47, W81–W87.

Borriss, R., Chen, X. H., Rueckert, C., Blom, J., Becker, A., Baumgarth, B., et al. (2011). Relationship of *Bacillus amyloliquefaciens* clades associated with strains DSM 7T and FZB42T: a proposal for *Bacillus amyloliquefaciens* subsp. *amyloliquefaciens* subsp. nov. and *Bacillus amyloliquefaciens* subsp. *plantarum* subsp. nov. based on complete genome sequence comparisons. *Int. J. Syst. Evol. Micr.* 61, 1786–1801. doi: 10.1099/ijs.0.023267-0

Brittenham, G. (1994). New advances in iron metabolism, iron deficiency, and iron overload. *Curr. Opin. Hematol.* 1, 101–106.

Cao, Y., Xu, Z., Ling, N., Yuan, Y., Yang, X., Chen, L., et al. (2012). Isolation and identification of lipopeptides produced by *B. subtilis SQR* 9 for suppressing *Fusarium* wilt of cucumber. *Hortic. Sci.* 135, 32–39. doi: 10.1016/j.scienta.2011.12.002

Cao, Y., Zhang, Z., Ling, N., Yuan, Y., Zheng, X., Shen, B., et al. (2011). *Bacillus subtilis* SQR 9 can control *Fusarium* wilt in cucumber by colonizing plant roots. *Biol. Fertil. Soils* 47, 495–506. doi: 10.1007/s00374-011-0556-2

Caro-Astorga, J., Álvarez-Mena, A., Hierrezuelo, J., Guadix, J. A., Heredia-Ponce, Z., Arboleda-Estudillo, Y., et al. (2020). Two genomic regions encoding exopolysaccharide production systems have complementary functions in *B. cereus* multicellularity and host interaction. *Sci. Rep.* 10:1000.

Castresana, J. (2000). Selection of conserved blocks from multiple alignments for their use in phylogenetic analysis. *Mol. Biol. Evol.* 17, 540–552. doi: 10.1093/oxfordjournals.molbev.a026334

Chaisson, M. J., and Tesler, G. (2012). Mapping single molecule sequencing reads using basic local alignment with successive refinement (BLASR): application and theory. *BMC Bioinform.* 13:238. doi: 10.1186/1471-2105-13-238

Chen, F., Wang, M., Zheng, Y., Luo, J., Yang, X., and Wang, X. (2010). Quantitative changes of plant defense enzymes and phytohormone in biocontrol of cucumber *Fusarium* wilt by *Bacillus subtilis* B579. *World J. Microbiol. Biotechnol.* 26, 675–684. doi: 10.1007/s11274-009-0222-0

Chen, X. H., Koumoutsi, A., Scholz, R., Eisenreich, A., Schneider, K., Heinemeyer, I., et al. (2007). Comparative analysis of the complete genome sequence of the plant growth–promoting bacterium *Bacillus amyloliquefaciens* FZB42. *Nat. Biotechnol.* 25, 1007–1014. doi: 10.1038/nbt1325

Chen, X. H., Koumoutsi, A., Scholz, R., Schneider, K., Vater, J., Süssmuth, R., et al. (2009). Genome analysis of *Bacillus amyloliquefaciens* FZB42 reveals its potential for biocontrol of plant pathogens. *J. Biotechnol.* 140, 27–37. doi: 10.1016/j.jbiotec.2008.10.011

Chen, X. H., Vater, J., Piel, J., Franke, P., Scholz, R., Schneider, K., et al. (2006). Structural and functional characterization of three polyketide synthase gene clusters in *Bacillus amyloliquefaciens* FZB 42. *J. Bacteriol.* 188, 4024–4036. doi: 10.1128/jb.00052-06

Chevenet, F., Brun, C., Bañuls, A. L., Jacq, B., and Christen, R. (2006). TreeDyn: towards dynamic graphics and annotations for analyses of trees. *BMC Bioinform.* 7:439. doi: 10.1186/1471-2105-7-439

Chung, S., Kong, H., Buyer, J. S., Lakshman, D. K., Lydon, J., Kim, S. D., et al. (2008). Isolation and partial characterization of *Bacillus subtilis* ME488 for suppression of soilborne pathogens of cucumber and pepper. *Appl. Microbiol. Biotechnol.* 80, 115–123. doi: 10.1007/s00253-008-1520-4

De Souza, R., Ambrosini, A., and Passaglia, L. (2015). Plant growth promoting-bacteria as inoculants in agricultural soils. *Genet. Mol. Biol.* 38, 401–419. doi: 10.1590/s1415-475738420150053

Dimopoulou, A., Theologidis, I., Liebmann, B., Kalantidis, K., Vassilakos, N., and Skandalis, N. (2019). *Bacillus amyloliquefaciens* MBI600 differentially induces tomato defense signaling pathways depending on plant part and dose of application. *Sci. Rep.* 9:19120. doi: 10.1038/s41598-019-55645-2

Doornbos, R. F., van Loon, L. C., and Bakker, P. A. H. M. (2012). Impact of root exudates and plant defense signaling on bacterial communities in the rhizosphere. A review. *Agron. Sustain. Dev.* 32, 227–243. doi: 10.1007/s13593-011-0028-y

Dutta, S., and Podile, A. (2010). Plant growth promoting Rhizobacteria (PGPR): the bugs to debug the root zone. *Crit. Rev. Microbiol.* 36, 232–244. doi: 10.3109/10408411003766806

Edgar, R. C. (2004). MUSCLE: multiple sequence alignment with high accuracy and high throughput. *Nucleic Acids Res.* 32, 1792–1797. doi: 10.1093/nar/gkh340

Eijlander, R. T., and Kuipers, O. P. (2013). Live-cell imaging tool optimization to study gene expression levels and dynamics in single cells of *Bacillus cereus*. *Appl. Environ. Microbiol.* 79, 5643–5651. doi: 10.1128/aem.01347-13

Fan, B., Chen, X. H., Budiharjo, A., Bleiss, W., Vater, J., and Borriss, R. (2011). Efficient colonization of plant roots by the plant growth promoting bacterium *Bacillus amyloliquefaciens* FZB42, engineered to express green fluorescent protein. *J. Biotech.* 151, 303–311. doi: 10.1016/j.jbiotec.2010.12.022

Franco-Sierra, N. D., Posada, L. F., Santa-Maria, G., Romero-Tabarez, M., Villegas-Escobar, V., and Alvarez, J. C. (2020). *Bacillus subtilis* EA-CB0575 genome reveals clues for plant growth promotion and potential for sustainable agriculture. *Funct. Integr. Genomics* 20, 575–589. doi: 10.1007/s10142-020-00736-x

Goswami, D., Thakker, J. N., and Dhandhukia, P. C. (2016). Portraying mechanics of plant growth promoting rhizobacteria (PGPR): a review. *Cogent Food Agric.* 2:1127500.

Gouy, M., Guindon, S., and Gascuel, O. (2010). SeaView version 4: a multiplatform graphical user interface for sequence alignment and phylogenetic tree building. *Mol. Biol. Evol.* 27, 221–224. doi: 10.1093/molbev/msp259

Guo, S., Li, X., He, P., Ho, H., Wu, Y., and He, Y. (2015). Whole-genome sequencing of *Bacillus subtilis* XF-1 reveals mechanisms for biological control and multiple beneficial properties in plants. *J. Ind. Microbiol. Biotechnol.* 42, 925–937. doi: 10.1007/s10295-015-1612-y

Halimi, B., Dortu, C., Arguelles-Arias, A., Thonart, P., Joris, B., and Fickers, P. (2010). Antilisterial activity on poultry meat of amylolysin, a bacteriocin from *Bacillus amyloliquefaciens* GA1. *Probiot. Antimicrob. Proteins* 2, 120–125. doi: 10.1007/s12602-010-9040-9

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

Hibbing, M. E., Fuqua, C., Parsek, M. R., and Peterson, S. B. (2010). Bacterial competition: surviving and thriving in the microbial jungle. *Nat. Rev. Microbiol.* 8, 15–25. doi: 10.1038/nrmicro2259

Hoffland, E., Findenegg, G. R., and Nelemans, J. A. (1989). Solubilization of rock phosphate by rape. *Plant Soil* 113, 161–165.

Holtmann, G., Bakker, E. P., Uozumi, N., and Bremer, E. (2003). KtrAB and KtrCD: two K+ uptake systems in *Bacillus subtilis* and their role in adaptation to hypertonicity. *J. Bacteriol.* 185, 1289–1298. doi: 10.1128/jb.185.4.1289-1298.2003

Jansson, J. K. (2003). Marker reporter genes: illuminating tools for environmental microbiologists. *Curr. Opin. Microbiol.* 6, 310–316. doi: 10.1016/s1369-5274(03)00057-2

Kamilova, F., Validov, S., Azarova, T., Mulders, I., and Lugtenberg, B. (2005). Enrichment for enhanced competitive plant root tip colonizers selects for a new class of biocontrol bacteria. *Environ. Microbiol.* 7, 1809–1817. doi: 10.1111/j.1462-2920.2005.00889.x

Keinath, A. P., Wintermantel, W. M., and Zitter, T. A. (2018). *Compendium of Cucurbit Diseases and Pests*. St. Paul, MN: APS Press, 24–159.

Khalaf, E. M., and Raizada, M. N. (2018). Bacterial seed endophytes of domesticated cucurbits antagonize fungal and oomycete pathogens including powdery mildew. *Front. Microbiol.* 9:42. doi: 10.3389/fmicb.2018.00042

Koren, S., Walenz, B. P., Berlin, K., Miller, J. R., Bergman, N. H., and Phillippy, A. M. (2017). Canu: scalable and accurate long-read assembly via adaptive k-mer weighting and repeat separation. *Genet. Res. Int.* 27, 722–736. doi: 10.1101/gr.215087.116

Koumoutsi, A., Chen, X. H., Henne, A., Liesegang, H., Hitzeroth, G., Franke, P., et al. (2004). Structural and functional characterization of gene clusters directing nonribosomal synthesis of bioactive cyclic lipopeptides in *Bacillus amyloliquefaciens* strain FZB42. *J. Bacteriol.* 186, 1084–1096. doi: 10.1128/jb.186.4.1084-1096.2004

Kumar, K. V. K., Yellareddygari, S. K., Reddy, M. S., Kloepper, J. W., Lawrence, K. S., Zhou, X. G., et al. (2012). Efficacy of *Bacillus subtilis* MBI 600 against sheath blight caused by *Rhizoctonia solani* and on growth and yield of rice. *Rice Sci.* 19, 55–63. doi: 10.1016/s1672-6308(12)60021-3

Kunst, F., Ogasawara, N., and Moszer, I. (1997). The complete genome sequence of the Gram-positive bacterium *Bacillus subtilis*. *Nature* 390, 249–256.

Lee, S., and Lee, J. (2015). Beneficial bacteria and fungi in hydroponic systems: types and characteristics of hydroponic food production methods. *Sci. Hortic.* 195, 206–215. doi: 10.1016/j.scienta.2015.09.011

Letunic, I., and Bork, P. (2016). Interactive tree of life (iTOL) v3: an online tool for the display and annotation of phylogenetic and other trees. *Nucleic Acids Res.* 44, W242–W245.

Li, L., Ma, J., Li, Y., Wang, Z., Gao, T., and Wang, Q. (2012). Screening and partial characterization of *Bacillus* with potential applications in biocontrol of cucumber *Fusarium* wilt. *Crop Prot.* 35, 29–35. doi: 10.1016/j.cropro.2011.12.004

Liu, A. C., Chou, C. Y., Chen, L. L., and Kuo, C. H. (2015). Bacterial community dynamics in a swine wastewater anaerobic reactor revealed by 16S rDNA sequence analysis. *J. Biotechol.* 194, 124–131. doi: 10.1016/j.jbiotec.2014.11.026

Liu, L., Li, Y., Li, S., Hu, N., He, Y., Pong, R., et al. (2012). Comparison of next-generation sequencing systems. *BioMed Res. Int.* 2012:251364.

Liu, Y., Lai, Q., and Shao, Z. (2018). Genome analysis-based reclassification of *Bacillus weihenstephanensis* as a later heterotypic synonym of *Bacillus mycoides*. *Int. J. Syst. Evol. Microbiol.* 68, 106–112. doi: 10.1099/ijsem.0.002466

Magno-Perez-Bryan, M. C., Martinez-Garcia, P. M., Hierrezuelo, J., Rodriguez-Palenzuela, P., Arrebola, E., Ramos, C., et al. (2015). Comparative genomics within *Bacillus* genus reveal the singularities of two robust *Bacillus amyloliquefaciens* biocontrol strains. *Mol. Plant Microbe Interact.* 28, 1102–1116. doi: 10.1094/mpmi-02-15-0023-r

May, J. J., Wendrich, T. M., and Marahiel, M. A. (2001). The dhb operon of *Bacillus subtilis* encodes the biosynthetic template for the catecholic siderophore 2, 3-dihydroxybenzoate-glycine-threonine trimeric ester bacillibactin. *J. Biol. Chem.* 276, 7209–7217. doi: 10.1074/jbc.m009140200

Miller, G., Huang, I. J., Welkie, G., and Pushnik, J. (1995). "Function of iron in plants with special emphasison chloroplasts and photosynthetic activity," in *Iron Nutrition in Soils and Plants*, ed. J. Abadia (Dordrecht: Springer), 19–28. doi: 10.1007/978-94-011-0503-3_4

Nguyen, H. D., Nguyen, Q. A., Ferreira, R. C., Ferreira, L. C., Tran, L. T., and Schumann, W. (2005). Construction of plasmid-based expression vectors for *Bacillus subtilis* exhibiting full structural stability. *Plasmid* 54, 241–248. doi: 10.1016/j.plasmid.2005.05.001

Nishikori, T., Naganawa, H., Muraoka, Y., Aoyagi, T., and Umezawa, H. (1986). Plispastins; new inhibitors of phospholopase A2, produced by *Bacillus cereus* BMG302-fF67. III. Structural elucidation of plispastins. *J. Antibiot.* 39, 755–761.

Pavlou, G. C., and Vakalounakis, D. J. (2005). Biological control of root and stem rot of greenhouse cucumber, caused by *Fusarium oxysporum* f. sp. radicis-cucumerinum, by lettuce soil amendment. *Crop Prot.* 24, 135–140. doi: 10.1016/j.cropro.2004.07.003

Poonguzhali, S., Madhaiyanm, M., Yim, W., Kim, K., and Sa, T. (2008). Colonization pattern of plant root and leaf surfaces visualized by use of green-fluorescent-marked strain of *Methylobacterium suomiense* and its persistence in rhizosphere. *Appl. Microbiol. Biotechnol.* 78, 1033–1043. doi: 10.1007/s00253-008-1398-1

Posada, L. F., Alvarez, J. C., Romero-Tabarez, M., de-Bashan, L., and Villegas-Escobar, V. (2018). Enhanced molecular visualization of root colonization and growth promotion by *Bacillus subtilis* EA-CB0575 in different growth systems. *Int. J. Microbiol. Res.* 217, 69–80. doi: 10.1016/j.micres.2018.08.017

Rahimi, T., Niazi, A., Deihimi, T., Taghavi, S. M., Ayatollahi, S., and Ebrahimie, E. (2018). Genome annotation and comparative genomic analysis of *Bacillus subtilis* MJ01, a new bio-degradation strain isolated from oil-contaminated soil. *Funct. Integr. Genomics* 18, 533–543. doi: 10.1007/s10142-018-0604-1

Raza, W., Ling, N., Zhang, R., Huang, Q., Xu, Y., and Shen, Q. (2017). Success evaluation of the biological control of *Fusarium* wilts of cucumber, banana, and tomato since 2000 and future research strategies. *Crit. Rev. Biotechnol.* 37, 202–212. doi: 10.3109/07388551.2015.1130683

Romero, D., Pérez-García, A., Rivera, M. E., and Cazorla, F. M. (2004). Isolation and evaluation of antagonistic bacteria towards the cucurbit powdery mildew fungus *Podosphaera fusca*. *Appl. Microbiol. Biotechnol.* 64, 263–269. doi: 10.1007/s00253-003-1439-8

Romero, D., Perez-Garcia, A., Veening, J. W., and Kuipers, O. P. (2006). Transformation of undomesticated strains of *Bacillus subtilis* by protoplast electroporation. *J. Microbiol. Methods* 66, 556–559. doi: 10.1016/j.mimet.2006.01.005

Rudrappa, T., Czymmek, K. J., Pare, P. W., and Bais, H. P. (2008). Root-secreted malic acid recruits beneficial soil bacteria. *Plant Physiol.* 148, 1547–1566. doi: 10.1104/pp.108.127613

Samaras, A., Efthimiou, K., Roumeliotis, E., and Karaoglanidis, G. S. (2018). Biocontrol potential and plant-growth-promoting effects of *Bacillus amyloliquefaciens* MBI 600 against *Fusarium oxysporum* f. sp. radicis-lycopersici on tomato. *Acta Hort.* 1207, 139–145. doi: 10.17660/actahortic.2018.1207.18

Schalk, I. J., Hannauer, M., and Braud, A. (2011). New roles for bacterial siderophores in metal transport and tolerance. *Environ. Microbiol.* 13, 2844–2854. doi: 10.1111/j.1462-2920.2011.02556.x

Schmidt, C. S., Agostini, F., Leifert, C., Killham, K., and Mullins, C. E. (2004). Influence of soil temperature and matric potential on sugar beet seedling colonization and suppression of Pythium damping-off by the antagonistic bacteria *Pseudomonas fluorescens* and *Bacillus subtilis*. *Phytopathology* 94, 351–363. doi: 10.1094/phyto.2004.94.4.351

Shafi, J., Tian, H., and Ji, M. (2017). Bacillus species as versatile weapons for plant pathogens: a review. *Biotechnol. Bioeng. Equip.* 31, 446–459. doi: 10.1080/13102818.2017.1286950

Shaligram, S., Kumbhare, S. V., Dhotre, D. P., Muddeshwar, M. G., Kapley, A., Joseph, N., et al. (2016). Genomic and functional features of the biosurfactant producing Bacillus sp. *AM*13. *Funct. Integr. Genomics* 16, 557–566. doi: 10.1007/s10142-016-0506-z

Stein, D. B., Linne, U., and Marahiel, M. A. (2005). Utility of epimerization domains for the redesign of nonribosomal peptide synthetases. *FEBS J.* 272, 4506–4520. doi: 10.1111/j.1742-4658.2005.04871.x

Steinauer, K., Jensen, B., Strecker, T., De Luca, E., Scheu, S., and Eisenhauer, N. (2016). Convergence of soil microbial properties after plant colonization of an experimental plant diversity gradient. *BMC Ecol.* 16:19. doi: 10.1186/s12898-016-0073-0

Tatusova, T., DiCuccio, M., Badretdin, A., Chetvernin, V., Nawrocki, E. P., Zaslavsky, L., et al. (2016). NCBI prokaryotic genome annotation pipeline. *Nucleic. Acids Res.* 44, 6614–6624. doi: 10.1093/nar/gkw569

Timmusk, S., Grantcharova, N., and Wagner, E. G. H. (2005). *Paenibacillus polymyxa* invades plant roots and forms biofilms. *Appl. Environ. Microbiol.* 71, 7292–7300. doi: 10.1128/aem.71.11.7292-7300.2005

Ugoji, E. O., Laing, M. D., and Hunter, C. H. (2006). Colonization of *Bacillus* spp. on seeds and in plant rhizoplane. *J. Environ. Biol.* 263, 459–466.

Vacheron, J., Desbrosses, G., Boufaud, 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

Wang, B., Yuan, J., Zhang, J., Shen, Z., Zhang, M., Li, R., et al. (2013). Effects of novel bioorganic fertilizer produced by *Bacillus amyloliquefaciens* W19 on antagonism of *Fusarium* wilt of banana. *Biol. Fert. Soils* 49, 435–446. doi: 10.1007/s00374-012-0739-5

Werhane, H., Lopez, P., Mendel, M., Zimmer, M., Ordal, G. W., and Márquez-Magaña, L. M. (2004). The last gene of the fla/che operon in *Bacillus subtilis*, ylxL, is required for maximal sD function. *J. Bacteriol.* 186, 4025–4029. doi: 10.1128/jb.186.12.4025-4029.2004

West, S. A., and Buckling, A. (2003). Cooperation, virulence and siderophore production in bacterial parasites. *Proc. R. Soc. Lond. Ser. B Biol. Sci.* 270, 37–44. doi: 10.1098/rspb.2002.2209

Wray, L. V., Atkinson, M. R., and Fisher, S. H. (1994). The nitrogen-regulated *Bacillus subtilis* nrgAB operon encodes a membrane protein and a protein highly similar to the *Escherichia coli* glnB-encoded PII protein. *J. Bacteriol.* 176, 108–114. doi: 10.1128/jb.176.1.108-114.1994

Wu, S., Zhu, Z., Fu, L., Niu, B., and Li, W. (2011). WebMGA: a customizable web server for fast metagenomic sequence analysis. *BMC Genomics* 12:444. doi: 10.1186/1471-2164-12-444

Yuan, J., Zhang, N., Huang, Q., Raza, W., Li, R., Vivanco, J. M., et al. (2015). Organic acids from root exudates of banana help root colonization of PGPR strain *Bacillus amyloliquefaciens* NJN-6. *Sci. Rep.* 5:13438.

Zhang, N., Wang, D., Liu, Y., Li, S., Shen, Q., and Zhang, R. (2014). Effects of different plant root exudates and their organic acid components on chemotaxis, biofilm formation and colonization by beneficial rhizosphere –associated bacterial strains. *Plant Soil* 374, 689–700. doi: 10.1007/s11104-013-1915-6

Zhang, N., Yang, D., Kendall, J. R. A., Borriss, R., Druzhinina, I. S., Kubicek, C. P., et al. (2016). Comparative genomic analysis of *Bacillus amyloliquefaciens* and *Bacillus subtilis* reveals evolutional traits for adaptation to plant-associated habitats. *Front. Microbiol.* 7:2039. doi: 10.3389/fmicb.2016.02039

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: 04 February 2021
doi: 10.3389/fpls.2021.599742

Algae as New Kids in the Beneficial Plant Microbiome

Sang-Moo Lee[1,2,3] *and Choong-Min Ryu*[1,2]*

[1] Molecular Phytobacteriology Laboratory, Infectious Disease Research Center, KRIBB, Daejeon, South Korea, [2] Department of Biosystems and Bioengineering, KRIBB School of Biotechnology, University of Science and Technology, Daejeon, South Korea, [3] Department of Applied Bioscience, Dong-A University, Busan, South Korea

Previously, algae were recognized as small prokaryotic and eukaryotic organisms found only in aquatic habitats. However, according to a recent paradigm shift, algae are considered ubiquitous organisms, occurring in plant tissues as well as in soil. Accumulating evidence suggests that algae represent a member of the plant microbiome. New results indicate that plants respond to algae and activate related downstream signaling pathways. Application of algae has beneficial effects on plant health, such as plant growth promotion and disease control. Although accumulating evidence suggests that secreted compounds and cell wall components of algae induce physiological and structural changes in plants that protect against biotic and abiotic stresses, knowledge of the underlying mechanisms and algal determinants is limited. In this review, we discuss recent studies on this topic, and highlight the bioprotectant and biostimulant roles of algae as a new member of the plant beneficial microbiome for crop improvement.

Keywords: microalgae, microbiome, *Chlorella*, cyanobacteria, plant immunity, plant growth promotion (PGP), biological control

OPEN ACCESS

Edited by:
Ioannis Stringlis,
Utrecht University, Netherlands

Reviewed by:
Ke Yu,
Henan University, China
Wu Xiong,
Utrecht University, Netherlands

***Correspondence:**
Choong-Min Ryu
cmryu@kribb.re.kr

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

Received: *28 August 2020*
Accepted: *13 January 2021*
Published: *04 February 2021*

Citation:
Lee S-M and Ryu C-M (2021) Algae as New Kids in the Beneficial Plant Microbiome. Front. Plant Sci. 12:599742. doi: 10.3389/fpls.2021.599742

INTRODUCTION

Algae is a group of ancient photosynthetic organisms ranging from prokaryotic cyanobacteria to eukaryotic microalgae (Parker et al., 2008). Generally, algae are classified mainly depending on their color, shape, and life cycle (Blaby-Haas and Merchant, 2019). Out of more than 800,000 species of algae that exist in nature, only 5,000 have been characterized to date. Out of 5,000 species, only small number of the algae species have been selected to determine their potential applications in plant growth under defined growth conditions. Algae are broadly classified as micro- and macroalgae based on size. Macroalgae indicates large aquatic photosynthetic plants that can be seen without the aid of a microscope and can generally be divided into three groups: Green (Chlorophyta), Red (Rhodophyta), and Brown-Kelps (Phaeophyta—related to Chromista). Microalgae comprise representative genera, including *Arthrospira*, *Chlorella*, *Dunaliella, Nostoc*, and *Aphanizomenon* (Elster, 2002). Prokaryotic microalgae, namely, cyanobacteria, play a critical role in the natural ecosystem, particularly in plant–microbe interactions. However, the idea that algae are a member of the plant-associated microbial community has long been debated (Berg et al., 2020).

DEFINITION AND MEMBERSHIP OF THE MICROBIOME

It is important to understand the definition of the microbiome before discussing algae as a new member of the plant microbiome, since microbe and microbiome are distinct terminologies. Most scientists follow the definition of microbiome first provided by Whipps et al. (1988), according to which a microbiome "may be defined as a characteristic microbial community occupying a reasonably well defined habitat which has distinct physio-chemical properties. This term not only refers to the microorganisms involved but also encompasses their theater of activity" (Whipps et al., 1988). However, the definition of microbiome has been revised several times in the last 20+ years to meet the technological and conceptual advances. "The microbiome is defined as a characteristic microbial community occupying a reasonable well-defined habitat which has distinct physio-chemical properties. The microbiome not only refers to the microorganisms involved but also encompass their theater of activity, which results in the formation of specific ecological niches. The microbiome, which forms a dynamic and interactive micro-ecosystem prone to change in time and scale, is integrated in macro-ecosystems including eukaryotic hosts, and here crucial for their functioning and health" (Berg et al., 2020).

Many microbiologists less considered algae and protists as members of the plant-associated microbiome (Longford et al., 2019; Wilpiszeski et al., 2019). However, most microbiologists agree that algae, except some macroalgae, are microorganisms based on their size and characteristics. In this review, we discuss only microalgae species, including both prokaryotic and eukaryotic organisms. The ecological niche of algae had also been debated. Here, we focus on algae as a member of the microbiome and their beneficial effects on plant fitness. To meet the minimum conceptual role, algae must exist on or around the plant surface and inside plant tissues.

ALGAE AS MEMBERS OF THE SOIL MICROBIOME

Because fresh and seawater were previously recognized as the habitat of algae, most microbiologists did not consider that algae could thrive in soil or on plant surfaces. However, more than 30 years ago, scientists investigated the distribution of algae in soil (Davey, 1989, 1991; Davey and Clarke, 1991). Early studies were conducted to identify cryptogrammic flora on the Antarctic fellfield soil based on their chlorophyll contents and microscopic observations. These studies revealed that Oscillatoriaceae was the dominant family in the soil, up to a depth of 8 cm below the soil surface (Davey and Clarke, 1991). Limitations of the classification on algal species based on conventional microbiological approaches, including isolation and *in vitro* culture on artificial media, led to the development of molecular techniques, including PCR-based 18S rDNA sequencing of the algae community in the soil (Bérard et al., 2005; Bradley et al., 2016; Khaw et al., 2020). In areas with harsh climatic conditions, such as semi-arid steppes, warm deserts, and polar regions, the algal community forms a biological soil crust along with other microorganisms to protect against abiotic and biotic stresses (Zhang et al., 2011; Pushkareva et al., 2016; Krug et al., 2020). Algae were also identified as active microbes in agricultural fields by 18S rDNA sequencing (Bérard et al., 2005). For instance, four classes of algae were identified in soil samples collected from a vegetable field (depth: 0–15 cm) in Nigeria: Chlorophyceae, Cyanophyceae, Bacillariophyceae, and Euglenophyceae (Adesalu and Olugbemi, 2015). Collectively, these studies suggest that algae are distributed across diverse environments, ranging from polar areas to agricultural fields. However, the interaction between land plants and algae has not been studied intensively. To utilize algae as plant health-promoting factors, it is important to understand the ecological niche of algae.

ECOLOGICAL NICHE

Previously, freshwater and seawater were considered as the ecological niches of algae, as described above. Considering algae as a member of the plant microbiome (phytobiome) has been debated because algae could not be isolated from the rhizosphere, phyllosphere, or endosphere (Gantar and Elhai, 1999; Gantar, 2000; Treves et al., 2016; Zhu et al., 2018). Moreover, the role of algae in plant fitness has not been evaluated extensively by biochemical and molecular analyses. Only recent studies demonstrate that algae are a member of the phytobiome. For instance, *Chlorella* species are found in the soil and on the plant leaf surface (Liu and Chen, 2016; Treves et al., 2016; Zhu et al., 2018), and cyanobacteria, such as *Nostoc* and *Anabaena* spp., were identified on the plant root surface (Gantar et al., 1991, 1995; Spiller et al., 1993; Gantar and Elhai, 1999; Gantar, 2000). However, recent microbiome analysis using the DNA sequence-based metagenome technology revealed that microalgae, including eukaryotic and prokaryotic (cyanobacteria) species, must be considered as members of the microbiome (Mendes et al., 2013; Xu et al., 2018). Microalgae have also been identified in the soil and in plant tissues (Leach et al., 2017). Previous studies on plant–algae interactions did not demonstrate the beneficial effects of algae on plant growth and defense. In this review, we focus on algae as a member of the beneficial microbiome and on their beneficial effects on plant health. Since the concept of 'beneficial microbiome' has not been defined clearly (Berg et al., 2020), beneficial algae could be categorized as having direct and indirect beneficial effects on plant, similarly to other beneficial microbes (e.g., PGPR). The bacterial and fungal inoculants on seeds, seedlings, and propagating plant materials secrete growth-enhancing compounds directly, which mimic plant hormones and promote increased plant growth and yield (Lugtenberg and Kamilova, 2009). The inoculants also promote plant growth by inhibiting pathogenic and deleterious plant-associated microbes and by activating plant innate immunity against plant pathogens; the latter represents an indirect effect of beneficial bacteria and fungi on plants. Another indirect effect of such inoculants is modulation of the microbiome, referred to as microbiome engineering (Dessaux et al., 2016). The direct and indirect effects

of bacteria and fungi on plants are well known, but those of algae are a new emerging concept. Here, we summarize the beneficial effects of algae on crop plants in the greenhouse and field.

PLANT ROOT COLONIZATION AND PARA-NODULE FORMATION

Many species, ranging from moss to angiosperms, exhibit symbiotic interactions with algae (Meeks and Elhai, 2002; Santi et al., 2013). To interact with plants, algae must colonize the plant surface and cells within plant tissues, similar to other microbial organisms involved in symbiotic and mutualistic interactions with plants (**Figure 1**). Most examples of plant–algae interactions involve prokaryotic algae, i.e., cyanobacteria (Gantar and Elhai, 1999; Gantar, 2000; Treves et al., 2016; Zhu et al., 2018). Cyanobacteria can enter the plant through the stomata and colonize the intercellular space, forming loops and intracellular coils (Krings et al., 2009) (**Figure 1**). *Anabaena* spp. colonize the roots of wheat and cotton plants (Karthikeyan et al., 2009; Babu et al., 2015; Bidyarani et al., 2015) (**Figure 1**). *Calothrix* sp. was also found on the root system of wheat (Babu et al., 2015; Bidyarani et al., 2015). Beyond colonization of the root surface, *Tolypothvix* sp. and *Leptolyghya* sp. were detected in the intercellular space in Cycads plants (Cuddy et al., 2012) (**Figure 1**). Thus, the algae–plant interactions represent another example of a symbiotic relationship between the two organisms. A good example of this relationship is colonization of monocots, such as wheat and rice, by *Nostoc* spp. (Gantar et al., 1991; Ahmed et al., 2010; Hussain et al., 2013, 2015). Gantar et al. (1991) isolated diverse heterocystous nitrogen-fixing cyanobacteria, including *Nostoc*, *Anabaena*, and *Cylindrospermum,* from plant root and soil. Assessment of wheat seedling roots revealed two types of association patterns: loose colonization of root hair by *Anabaena* and tight colonization of the root surface within a restricted zone by *Nostoc* (Gantar et al., 1991) (**Figure 1**).

In addition to the free-living lifestyle on the plant root surface, *Nostoc* species also exist as endosymbionts in the flowering land plant (angiosperm) *Gunnera* (Silverster and Smith, 1969; Silvester, 1976; Lindblad et al., 1990). Cyanobacteria also form symbiotic relationships with other plant species, including bryophytes (e.g., *Anthoceros*), gymnosperms (e.g., Cycads), and water fern (*Azolla*) (Braun-Howland and Nierzwicki-Bauer, 1990; Lindblad et al., 1990; Meeks and Rai, 1990). Among these four multicellular plants, *Gunnera* L. develops well-organized, unique organs named glands through symbiosis with *Nostoc* (Bergman et al., 1992). Intriguingly, the glands of *Gunnera* are morphologically similar to crown galls formed by *Agrobacterium tumefaciens*. The detailed mechanisms by which the following processes happen, have been elucidated as gland morphology, cell penetration, intracellular colonization, hormogonium formation, gland development, and host specificity. During symbiosis, cyanobacteria influence nitrogen fixation and release, heterocyst development, and consistence of symbiosis. Glands formed by *Nostoc* on the stem of *Gunnera* plants are similar to nodules formed by *Rhizobium* spp. and crown gall formed by *A. tumefaciens* (Rasmussen et al., 1996). The *Nostoc* genome does not contain homologs of the two *Agrobacterium* genes required for T-DNA transfer-induced crown gall formation in plants, indicating that the mechanism of gland formation is distinct from that of gall formation. By contrast, the genome of *Nostoc* harbors homologs of the *Rhizobium* nod-box genes including *nodEF*, *nodMN*, and *enoY*. However, the induction of other critical nod genes, including *nodABC*, *nodD1*, and *nodD2*, and nod protein, could not be detected in *Nostoc* when treated with acidic mucilage secreted by stem glands (Rasmussen et al., 1996). These data indicate that *Rhizobium*–legume symbiosis is distinct from *Gunnera–Nostoc* symbiosis.

Scientists have attempted to form nodule-like structures and to functionally fix nitrogen in non-legume plants. Tchan and Kennedy (1989) succeeded in developing nodule-like structures, named para-nodules, using 2,4-dichlorophenoxy acetic acid (2,4-D), a synthetic compound that mimics auxin, but they failed to fix nitrogen using nitrogenase-containing bacteria. Inoculation of *Nostoc* sp. strain 2S9B into the 2,4-D led para-nodule increased the acetylene reduction capacity by more than threefold compared with that of the untreated control (Gantar and Elhai, 1999) (**Figure 1**). In the absence of supplemental nitrogen, wheat shoot growth could be increased by co-inoculation with 2,4-D and *Nostoc* sp. strain 2S9B (Gantar and Elhai, 1999; Gantar, 2000). Similarly, para-nodule formation and nitrogen fixation could also be induced in rice seedlings by treatment with 2,4-D and *Nostoc* spp. (Nilsson et al., 2002). Two possibilities could explain why para-nodules do not occur naturally in land plants such as wheat and rice: (i) below-threshold levels of auxin, and (ii) lack of *Nostoc* spp. colonization on the wheat and rice tissues as the ecological niche (**Figure 1**). This can be used to identify specific *Nostoc* spp. that elicit para-nodule formation in land plants in near future. Intriguingly, unlike prokaryotic algae, it is not reported that eukaryotic algae colonize on plant tissues.

ALGAE AS A NEW MEMBER OF THE BENEFICIAL PLANT MICROBIOME

Biological Control of Plant Pathogens

Algal species have been used intensively for biological control of fungal pathogens (**Figure 2** and **Table 1**). In tomato and cotton, root-drench application of prokaryotic *Anabaena variabilis*, *Anabaena torulosa*, *Anabaena laxa*, and *Calothrix* sp. reduced damping-off symptoms caused by *Pythium debaryanum*, *Fusarium oxysporum*, *F. moniliforme*, and *Rhizoctonia solani* (Prasanna et al., 2008, 2013; Chaudhary et al., 2012) (**Figure 2** and **Table 1**). Additionally, the eukaryotic *Chlorella fusca* protects host plant against pathogenic fungi *Colletotrichum orbiculare* and *Botrytis squamosa* in cucumber and Chinese chive (Lee et al., 2016, 2017; Kim et al., 2018a). The cell extract or filtered supernatant of cyanobacteria and *Chlorella* species also exhibits biological control activity against *F. oxysporum*, *P. aphanidermatum*, and *Sclerotinia sclerotiorum* in tomato, pepper, and brinjal (Biondi et al., 2004; Kim and Kim, 2008; Manjunath et al., 2010). Algae can suppress fungal disease via two putative mechanisms. First mechanism involves inhibition of fungal pathogen growth (**Figure 2** and **Table 1**). For

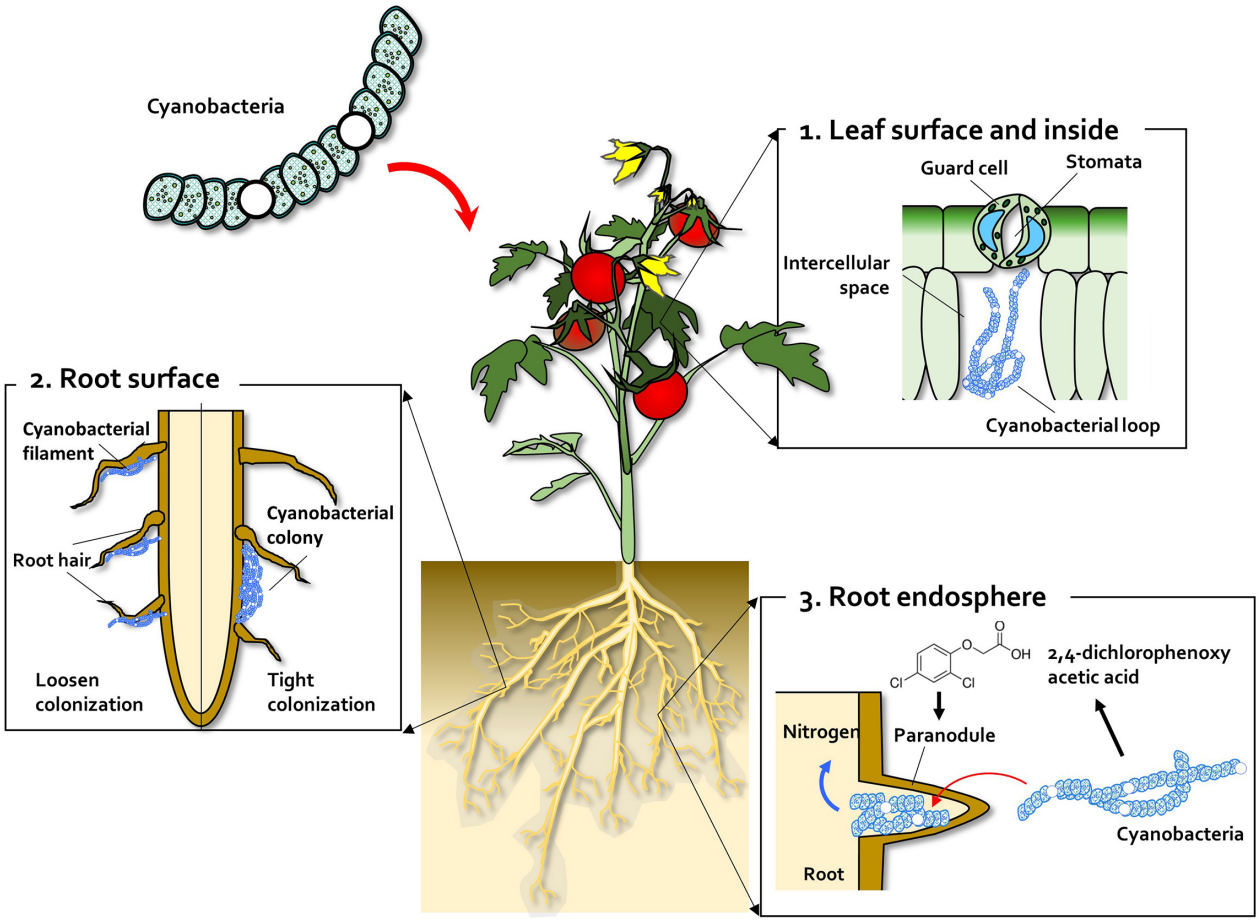

FIGURE 1 | Leaf and root colonization by cyanobacteria. **(1)** Cyanobacteria enter the leaf tissue through the stomata and colonize the intercellular space, forming a cyanobacterial loop. **(2)** On the root surface, cyanobacteria exhibit two types of colonization pattern; in the root hair, filaments of *Anabaena* and *Nostoc* species form loose colonies, and in the restricted zone on the root surface, specific *Nostoc* species form cyanobacterial colonies. **(3)** Co-inoculation with 2,4-dichlorophenoxy acetic acid (2,4-D) (synthetic auxin) and *Nostoc* spp. increases *para*-nodule formation and nitrogen fixation. A large number of *Nostoc* spp. isolates colonize the root endosphere and form *para*-nodules to fix nitrogen.

example, cyanobacteria *Anabaena* and *Calothrix* species showed antagonistic activity against *Fusarium* spp., *Pythium* spp., and *Rhizotoctonia* spp. *in vitro* (Chaudhary et al., 2012; Prasanna et al., 2013, 2016), and eukaryotic *C. fusca* also inhibited the growth of *C. orbiculare* hyphae *in vitro* and suppressed the formation of appressorium on cucumber leaves (Lee et al., 2016, 2017). The second mechanism involves activation of plant immune responses. *C. fusca* treatment showed antagonistic activity against *C. orbiculare* as well as the induction of defense-related structural modifications such as cell wall thickness, vesicle accumulation, and sheath formation, in cucumber leaves (Kim et al., 2018b).

Microalgae species have also been used to control pathogenic nematodes and insect pests (Sathiyamoorthy and Shanmugasundaram, 1996; Choleva et al., 2005; Khan et al., 2005, 2007; Bileva, 2013; Hamouda and El-Ansary, 2013) (**Figure 2** and **Table 1**). Root treatment of tomato with cyanobacteria such as *Microcoleus vaginatus*, *Oscillatoria chlorine*, *Aphanocapsa albida*, *Anabaena oryzae*, *Nostoc muscorum*, and *Calothrix marchica* reduced gall formation caused by *Meloidogyne arenaria* and *M. incognita* (Khan et al., 2005, 2007; Hamouda and El-Ansary, 2017). Soil-drench application of *Chlorella vulgaris* extract (1 g per pot) reduced infestation of grapevine roots by *Xiphinema index* by 2–3-fold compared with the untreated control (Choleva et al., 2005, 2007; Bileva, 2013). Foliar application of 0.01–0.1% peptides extracted from the cyanobacterium *Scytonema* MKU 106 reduced the feeding frequency of a chewing insect, *Sylepta derogata*, in cotton plants (Sathiyamoorthy and Shanmugasundaram, 1996). The algae species can protect host plant against pathogenic nematode and insect pests by nematocidal or repellent activity rather than as plant immune activation (Sathiyamoorthy and Shanmugasundaram, 1996; Choleva et al., 2007).

Compared with fungal pathogens and insect pests, biological control of bacterial pathogens using algae has remained largely unknown until 2020, when we reported for the first time the biological control of the bacterial pathogen *Pseudomonas*

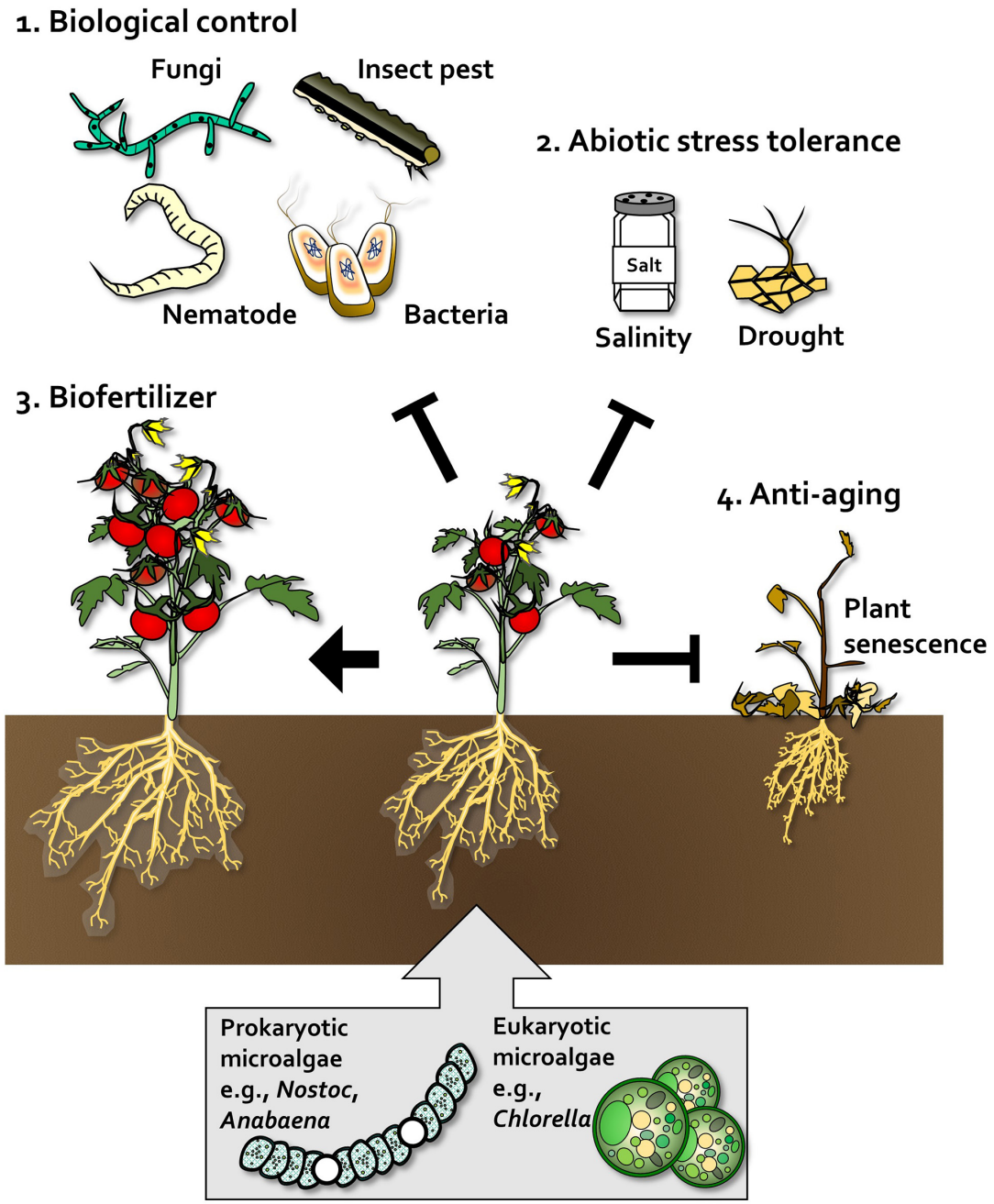

FIGURE 2 | Beneficial effects of algae on plants. In plants, prokaryotic microalgae such as *Nostoc* and *Anabaena*, and eukaryotic microalgae such as *Chlorella*, act as biological control agents **(1)**, abiotic stress tolerance enhancers **(2)**, biofertilizers that promote plant growth and crop yield **(3)**, and anti-aging agents that delay senescence and enhance plant robustness **(4)**.

syringae pv. *tomato* (*Pto*) by *C. fusca* in the model plant, *Arabidopsis thaliana* (Lee et al., 2020a, **Figure 2** and **Table 1**). Foliar application of *C. fusca* culture (10^7 cells/ml) reduced the population of *Pto* in *Arabidopsis* leaves by 10-fold. Further investigation revealed that *C. fusca* and its determinant D-lactic acid prime plant innate immunity against *Pto* (Lee et al., 2020a). To the best of our knowledge, there have been no reports on the biocontrol activity of algae against phytopathogenic viruses. Therefore, testing the potential application of algae against plant viruses is important.

Induced Tolerance Against Abiotic Stresses

The application of algae-derived substances could also increase tolerance against abiotic stresses (**Figure 2** and **Table 2**). In

TABLE 1 | Biological control of plant pathogens and insects using algal species.

Group	Algae species/consortia	Pathogen/insect type	Pathogen/insect name	Host plant	Treatment method	Product applied	References
Prokaryotic cyanobacteria	*Anabaena variabilis* RPAN59, *Anabaena laxa* RPAN8	Fungal pathogen	*Pythium debaryanum, Fusarium oxysporum* f. sp. *lycopersici, Fusarium moniliforme*, and *Rhizoctonia solani*	Tomato	Soil application	Cell culture and filtered supernatant	Chaudhary et al., 2012; Prasanna et al., 2013
	Anabaena torulosa, A. laxa, Calothrix sp.	Fungal pathogen	*R. solani*	Cotton	Soil application	Cell culture	Prasanna et al., 2016
	Nostoc commune FA-103	Fungal pathogen	*F. oxysporum* f. sp. *lycopersici*	Tomato	Seed coating	Cell extract	Kim and Kim, 2008
	Calothrix elenkenii	Fungal pathogen	*Pythium aphanidermatum*	Tomato, chili, and brinjal	Seed soaking	Filtered supernatant	Manjunath et al., 2010
	Nostoc strain ATCC 53789	Fungal pathogen	*Sclerotinia sclerotiorum*	Tomato	Seed soaking	Cell biomass and methanolic extract	Biondi et al., 2004
	Microcoleus vaginatus	Pathogenic nematode	*Meloidogyne arenaria*	Tomato	Root dipping	Filtered supernatant	Khan et al., 2005
	Oscillatoria chlorina	Pathogenic nematode	*M. arenaria*	Tomato	Soil application	Dried cell suspension	Khan et al., 2007
	Aphanocapsa albida, Anabaena oryzae, Nostoc muscorum, and *Calothrix marchica*	Pathogenic nematode	*Meloidogyne incognita*	Tomato	Soil application	Aqueous extract	Hamouda and El-Ansary, 2013
	Scytonema MKU 106	Chewing insect	*Helicoverpa armigera, Heliothis* larvae, and *Sylepta derogata*	Cotton	Foliar application	Peptide extract	Sathiyamoorthy and Shanmugasundaram, 1996
Eukaryotic green algae	*Chlorella vulgaris*	Pathogenic nematode	*M. arenaria* and *Xiphinema indexin*	Tomato, grape	Soil application	Dried cell extract	Choleva et al., 2005; Bileva, 2013
	Chlorella fusca	Fungal pathogen	*Colletotrichum orbiculare*	Cucumber	Foliar application	Cell culture	Lee et al., 2016, 2017; Kim et al., 2018a
	C. fusca	Fungal pathogen	*Botrytis squamosa*	Chinese chives	Foliar or soil application	Cell culture	Kim et al., 2018b
	C. fusca	Bacterial pathogen	*Pseudomonas syringae* pv. *tomato*	Arabidopsis	Foliar application	Cell culture/cell-free supernatant	Lee et al., 2020a
Cyanobacteria–bacteria consortia	*Anabaena oscillarioides* and *Bacillus subtilis*	Fungal pathogen	*F. oxysporum, P. debaryanum, P. aphanidermatum*, and *R. solani*	Tomato	Soil application	Cell culture	Dukare et al., 2011

TABLE 2 | Enhancement of abiotic stress tolerance and anti-aging capacity of plants after application of algae.

Objective	Group	Algae species	Plant	Treatment	Product applied	Plant response	References
Abiotic stress tolerance	Cyanobacteria	*Scytonema hofmanni*	Rice	Soil application	Extracellular products	Salt stress tolerance	Rodríguez et al., 2006
	Eukaryotic microalgae	*Chlorella ellipsoida*	Wheat	Soil application	Water soluble extract	Enhanced salt tolerance and antioxidant capacity	Abd El-Baky et al., 2010
		Chlorella vulgaris	*Vicia faba* L.	Foliar application	Cell culture	Enhanced reactive oxygen species (ROS) production and more effective stomatal closure and water use efficiency	Li et al., 2014
		Dunaliella salina	Tomato	Foliar application	Polysaccharides	Salt stress tolerance	Arroussi et al., 2018
Anti-aging	Eukaryotic microalgae	*C. vulgaris*	Strawberry, lettuce, beet, and kale	Foliar or soil application	Cell culture	Improved shelf-life	Kim et al., 2014
		Chlorella fusca and *Chlorella* strains ABC001 and HS2	*Erinus alpinus* L.	Soil application	Cell-free supernatant	Delayed plant senescence	Lee et al., 2020b

rice, extracellular products of the cyanobacterium *Scytonema hofmanni* nullified the effects of salt stress (<5 g/ml NaCl) on dry weight and length of shoot (Rodríguez et al., 2006). Under high salt stress, tomato plants treated with 1% *Dunaliella salina* hydrolyzate via spray application showed higher shoot dry weight, root dry weight, and chlorophyll a and b content than untreated plants (Arroussi et al., 2018), and soil treatment with water-soluble extracts of *Chlorella ellipsoida* and *Spirulina maxima* increased the total protein content of wheat grain by 1.4-fold compared with the control (Abd El-Baky et al., 2010). Moreover, treatment of fava bean plants with *C. vulgaris* culture induced drought tolerance (Li et al., 2014). Abiotic stress tolerance triggered by microalgae treatment is mostly linked to production of reactive oxygen species (ROS) and antioxidant activity in plants (Li et al., 2014; Arroussi et al., 2018). In tomato and bean, foliar application of *D. salina* extracts and *C. vulgaris* activated antioxidant enzymes such as peroxidase (POD), superoxide dismutase (SOD), catalase (CAT), and ascorbate peroxidase (APX) (Li et al., 2014; Arroussi et al., 2018). Similarly, in bean plants, treatment with *C. vulgaris* culture increased stomata closure frequency and water use efficiency, thereby reducing transpiration and increasing drought tolerance (Li et al., 2014). However, further investigation of exact molecular mechanism and algal determinant for improving abiotic stress tolerance in plant will be required.

Algae as Biofertilizers

Prokaryotic cyanobacteria have been applied to monocots and dicots as biofertilizers to increase plant growth and crop yield (**Figure 2** and **Table 3**). For example, rice plants treated with *A. variabilis* and *Nostoc* sp. VICCRI via root-drench application showed greater plant height, leaf length, and grain yield than inorganic fertilizer (Singh and Datta, 2007; Innok et al., 2009). Inoculation with *A. laxa* and *Calothrix elenkinii* increased the germination of coriander seeds and promoted root and shoot growth in coriander, cumin, and fennel (Kumar et al., 2013). Soaking of seeds in a solution of *Spirulina platensis* (2×10^4 cells/ml) increased the fresh and dry weight, height, and root length of crop plants, including rocket, Bayam red, and Pak choi, by 1.2–3-fold compared with the untreated control (Wuang et al., 2016). In addition, filtrated supernatant of cyanobacteria *Calothrix* sp., *Hapalosiphon* sp., *Nostoc* sp., and *Westiellopsis* sp., increased coleoptile and radicle length and seed germination in wheat by 2. 7-, 2. 1-, and 1.1-fold, respectively, compared with the sterile water control (Karthikeyan et al., 2009). Interestingly, treatment with multiple species of nitrogen-fixing cyanobacteria has a greater impact on plant growth, probably via synergistic effects on nutrient production (Karthikeyan et al., 2007; Paudel et al., 2012).

Similar to cyanobacteria, eukaryotic *Chlorella* spp. increased the growth of *Perilla*, onion, lettuce, Chinese cabbage, radish, turnip, and spinach plants when applied to roots and leaves (Kim et al., 2012, 2018a) (**Figure 2** and **Table 3**). Seed treatment with *C. vulgaris* promoted germination and shoot and root weights in lettuce, tomato, and cucumber (Faheed and Fattah, 2008; Bumandalai and Tserennadmid, 2019). In the field, root-drench application of *Chlorella pyrenoidosa* increased the

TABLE 3 | Plant growth promotion following algal treatment.

Group	Algae species	Host plant	Treatment	Product applied	References
Prokaryotic cyanobacteria	*Calothrix ghosei, Hapalosiphon intricatus, Nostoc muscorum, Westiellopsis prolifica, Calothrix membranacea*	Wheat	Seed soaking	Filtrated supernatant	Karthikeyan et al., 2009
	Anabaena laxa and Calothrix elenkinii	Coriander, cumin, and fennel	Soil application	Cell culture	Kumar et al., 2013
	Nostoc sp. VICCRI	Rice	Soil application	Cell culture	Innok et al., 2009
	Anabaena variabilis	Rice	Soil application	cell culture	Singh and Datta, 2007
Eukaryotic green algae	*Chlorella vulgaris*	Wheat	Foliar application	Water soluble extract	Shaaban, 2001a
	C. vulgaris	Maize	Soil application	Water soluble extract	Shaaban, 2001b
	C. vulgaris	Lettuce	Soil application	Dried cell extract	Faheed and Fattah, 2008
	C. vulgaris, Scenedesmus quadricauda	Tomato	Hydroponic system	Co-cultivation with plant	Barone et al., 2019
	C. vulgaris, S. quadricauda	Sugar beet	Hydroponic system	Dried cell extract	Barone et al., 2018
	C. vulgaris	Tomato and cucumber	Seed soaking	Cell culture	Bumandalai and Tserennadmid, 2019
	Chlorella fusca	Barely, wheat, lettuce, pepper, melon, cucumber, perilla, onion, radish, and turnip	Soil application	Cell culture	Kim et al., 2012
	C. fusca	Spinach	Foliar or soil application	Cell culture	Kim et al., 2018b
	Chlorella pyrenoidosa	Soybean	Soil application	Cell culture	Dubey and Dubey, 2010
	Chlorococcum infusionum	Tomato	Hydroponic system	Co-cultivation with plant	Zhang et al., 2017
	Nannochloropsis oculata	Tomato	Soil application	Dried cell extract	Coppens et al., 2016
Microalgae consortia	*Chlorella, Scenedesmus, Chlorococcum, Chroococcus, Phormidium, Anabaena, Westiellopsis, Fischerella,* and *Spirogyra*	Wheat	Soil application	Cell culture	Renuka et al., 2016
	C. ghosei, H. intricatus, and *Nostoc* sp.	Wheat	Soil application		Karthikeyan et al., 2007
	Nostoc, Anabaena, Westiellopsis, Aulosira, and *Scytonema*	Rice	Soil application	Cell culture	Paudel et al., 2012
Cyanobacteria–other microbe consortia	Unidentified cyanobacteria and rhizobacteria	Wheat	Soil application	Cell culture	Nain et al., 2010
	Anabaena oscillarioides CR3, *Brevundimonas diminuta* PR7, and *Ochrobactrum anthropi* PR10	Rice	Soil application	Cell culture	Rana et al., 2015
	A. torulosa and *Trichoderma viride*	Maize	Soil application	Extracted biofilms	Sharma et al., 2020

shoot weight and grain yield of soybean plants by 70 and 53%, respectively, compared with control plants (Dubey and Dubey, 2010). Cell extracts of *C. vulgaris* and dried biomass suspension of *Nannochloropsis oculata* showed plant growth-promoting activity in wheat, maize, tomato, and sugar beet (Shaaban, 2001a,b; Coppens et al., 2016; Barone et al., 2018). Interestingly, recent studies show that co-cultivation of sugar beet and tomato plants with *C. vulgaris*, *Chlorococcum infusionum*, and *Scenedesmus quadricauda* using the hydroponic system simultaneously increases the biomass of both the host plant and eukaryotic algal species (Zhang et al., 2017; Barone et al., 2018, 2019).

Plant Anti-aging Agents

Treatment with algae or algal solution also affect plant senescence (**Figure 2** and **Table 2**). The ability to prolong plant development and delay the onset of age-related phenotypes is economically important in crop science and critical for fundamental plant research (Lim et al., 2007; Shahri and Tahir, 2014). During

initial development of this anti-aging method, treatment with beneficial bacteria such as *Pseudomonas* spp. and *Bacillus* spp. was used to delay plant senescence (Ali et al., 2012; Carlson et al., 2015; Kuan et al., 2016; Naing et al., 2017). Interestingly, spray and irrigation application of *C. vulgaris* culture prolonged the shelf-life of strawberry, lettuce, beet, and kale (Kim et al., 2014). In addition, we reported that root-drench application of the cell-free supernatant of *C. fusca*, *Chlorella* sp. HS2 and *Chlorella* sp. ABC001, delayed shoot and flower senescence by up to 4 weeks in the ornamental flowering plant *Erinus alpinus* (Lee et al., 2020b). Given that other beneficial bacteria modulate ethylene signaling in plants (Ali et al., 2012; Carlson et al., 2015; Kuan et al., 2016; Naing et al., 2017), it is possible that microalgae suppress the ethylene signaling or biosynthesis pathway in plants. The detailed mechanism by which *Chlorella* mediates anti-aging effects in plants is, however, largely unknown.

ALGAL DETERMINANTS OF PLANT HEALTH

Inhibitory Compounds Effective Against Pathogenic Microbes and Insect Pests

Like classic bacterial biocontrol agents, beneficial algae produce antimicrobial compounds that suppress bacterial and fungal plant pathogen (**Figure 3**). For example, 4,4′-dihydroxybiphenyl, norharmane prokaryotic algae *Nodularia* spp. *and Nostoc* spp. and *Nostoc insulare* produces 4,4′-dihydroxybiphenyl, norharmane, and diterpenoids, which exhibit antibacterial activity against *Escherichia coli* and *Pseudomonas aeruginosa, Bacillus subtilis, B. cereus*, *Staphylococcus epidermdis* (Jaki et al., 2000; Volk and Furkert, 2006). In addition, cyanobacteria *Anabaena* spp., *Chlorella* spp., and *Scenedesmus* spp., produced siderophore as micronutrient ferric and copper ion chelators (McKnight and Morel, 1980; Goldman et al., 1983; Benderliev, 1999; Benderliev et al., 2003). Siderophores produced by microbes, especially such as *Pseudomonas* spp., were known as antimicrobial compounds and biological control agents in plants via chelating ferric iron, which can compete with bacterial pathogens for iron ions (Kloepper et al., 1980; Duijff et al., 1993; Lemanceau and Alabouvette, 1993). On the other hand, prokaryotic algae cyanobacteria can produce fungal cell wall-degrading enzymes including chitosanase, β-1,4-glucanase, β-1,3-glucanase, and benzoic acid, which can suppress growth of *Fusarium* sp., *Penicillium* sp., and *Candida* sp. (López et al., 2002; Chaudhary et al., 2012; Natarajan et al., 2012; Prasanna et al., 2013, 2016). Thus, further identification of microalgal antimicrobial compounds, and their biological control activity, is needed.

In addition to antimicrobial substances, cyanobacteria also produce pesticidal and nematocidal secondary metabolites, referred to as cyanotoxins (Hamouda and El-Ansary, 2017) (**Figure 3**). Cyanotoxins function as neurotoxins and hepatotoxins in animals (Sathiyamoorthy and Shanmugasundaram, 1996; Holajjer et al., 2013). The neurotransmitter-mimicking cyanotoxin, anatoxin-a, binds to eukaryotic nematode receptors and triggers continuous muscle contraction, causing muscle fatigue, and immobility (Carmichael, 1994; Dow and Swoboda, 2000; Mankiewicz et al., 2003). Moreover, cyanobacteria *Microcystis* spp. produce hepatotoxins including microcystins and nodularin (Holajjer et al., 2013), which inhibit the host metabolic system; for example, nodularin produced by *Nodularia spumigena* inhibit protein phosphatase activity in animal cells (Ohta et al., 1994). Additionally, cyanobacteria also produce peptide toxins that act as repellents (Sathiyamoorthy and Shanmugasundaram, 1996); for example, *Anabaena* and *Scytonema* species produce a low molecular weight (<12 kDa) peptide toxin (Konst et al., 1965; Sathiyamoorthy and Shanmugasundaram, 1996). Interestingly, *Scytonema*-derived peptide toxin acts as a repellent due to its strong smell, and reduces the population size of chewing insects *Helicoverpa armigera* and *Stylepta derogate* on cotton leaves (Sathiyamoorthy and Shanmugasundaram, 1996). Collectively, these reports suggest that algal substances can inhibit phytopathogenic bacteria, fungi, pests, and nematodes directly. However, most of these algal compounds exhibit antagonistic activity against phytopathogens only *in vitro*. Thus, it is important to verify the activity of purified algal compounds *in planta*.

Plant Hormone-Mimicking Compounds

The plant growth-promoting microalgae, including prokaryotic cyanobacteria and eukaryotic microalgae, produce phytohormones such as auxin and cytokinin, which affect plant growth and development (Werner et al., 2001; Benjamins and Scheres, 2008).

Auxin regulates plant developmental processes including gametogenesis, embryogenesis, seedling growth, vascular patterning, and flower development (Hamann et al., 2002; Dimitrov and Zucker, 2006; Pagnussat et al., 2009). Auxins, including indole-3-acetic acid (IAA), indole-3-butyric acid (IBA), indole-3-propionic acid (IPA), and 3-methylindole, have been detected in diverse microalgae species (Misra and Kaushik, 1989; Mazur et al., 2001; Stirk et al., 2002, 2013; Karthikeyan et al., 2009; Hashtroudi et al., 2013). Interestingly, algal auxin seems to positively regulate plant–algae interactions (**Figure 3**). IAA produced by *Nostoc* species promotes plant growth in wheat and rice; a *Nostoc* mutant lacking the IAA biosynthesis gene, which encodes indole pyruvate decarboxylase, failed to promote plant growth (Hussain et al., 2013, 2015). In addition to plant growth promotion, algal auxin is also tightly linked with the ability of microalgae to colonize host roots (Ahmed et al., 2010; Hussain et al., 2013, 2015). Auxin production in *Leptolyngbya* sp. MMG-1, *Chroococcidiopsis* sp. MMG-5, and *Synechocystis* sp. MMG-8, was increased during their colonization of plant roots (Ahmed et al., 2010). Strikingly, the lack of indole pyruvate decarboxylase significantly reduced colonization of rice and wheat roots by *Nostoc* species (Hussain et al., 2013, 2015). Collectively, algal auxin might act as a putative signaling molecule that mediates plant–microalgae interactions.

Cytokinin promote division and differentiation of plant cells, especially in apical and axillary meristems, and development of

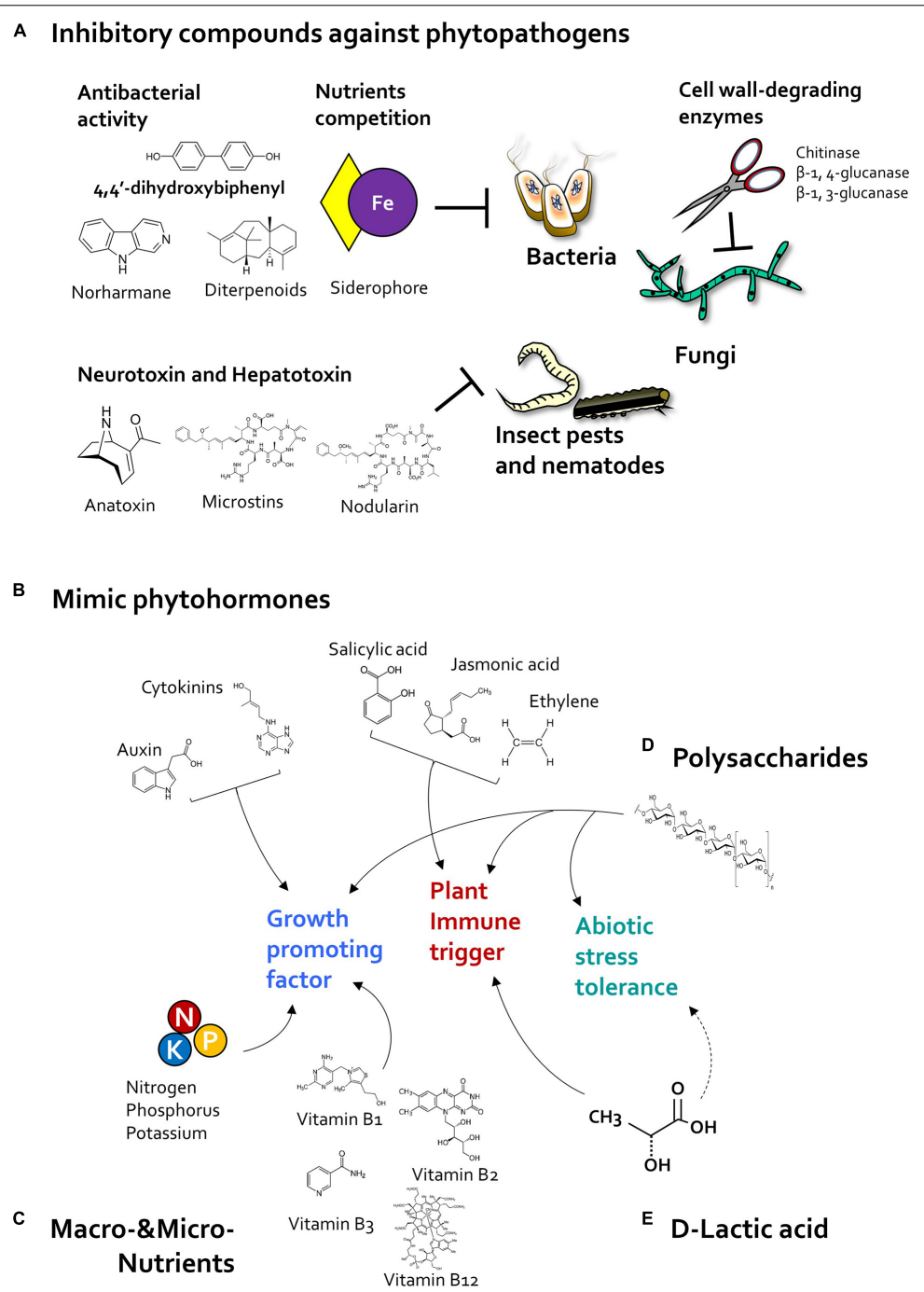

FIGURE 3 | Algal determinants that act as plant protectants and stimulants. **(A)** Inhibitory compounds. Cyanobacteria reduce the population of pathogenic bacteria, fungi, and insect pests by producing antibiotic and pesticidal compounds. Cyanobacteria-derived 4,4′-dihydroxybiphenyl, norharmane, and diterpenoids exhibit antibacterial activity, and microalgal siderophores inhibit bacterial growth through iron (Fe) competition. In addition, cyanobacterial cell wall-degrading enzymes such as chitosanase, β-1,4-glucanase, and β-1,3-glucanase reduce fungal infection. Cyanotoxins such as anatoxin, microcystin, and nodularin can protect the host plant against insect pests. **(B)** Phytohormones. Microalgae-derived phytohormone-mimicking compounds modulate plant growth, immunity, and abiotic stress tolerance. Plant growth regulators such as auxin and cytokinin increase plant growth and development as well as crop yield. Algae species also produce jasmonic acid (JA), salicylic acid (SA), and ethylene (ET), which act as major defense-related hormones in land plants. In addition, microalgae also produce abscisic acid (ABA), a central regulator of abiotic stress tolerance. **(C)** Nutrition. Nitrogen-fixing cyanobacteria promote plant growth by supplying macronutrients such as nitrogen, phosphorus, and potassium. Additionally, microalgae-derived vitamins, including vitamins B1, B2, B3, and B12, elicit plant immune response against phytopathogens. **(D)** Polysaccharides. Polysaccharides extracted from cyanobacteria and eukaryotic microalgae increase immunity and abiotic stress tolerance of the host plant. **(E)** D-lactic acid. Exogenous application of D-lactic acid produced by *Chlorella* elicits plant immunity via activation of D-lactate metabolism and production of mitochondrial reactive oxygen species (ROS). Algal D-lactic acid might also enhance abiotic stress tolerance in host plant by regulating ROS production.

gynoecium, and female gametophyte (Marsch-Martínez et al., 2012; Cheng et al., 2013; Schaller et al., 2014). Cytokinin compounds, including *trans*-zeatin, *cis*-zeatin, zeatin riboside, dihydrozeatin riboside, topolin, and zeatin-*o*-glucoside, were produced by many microalgae species (Stirk et al., 2002, 2013; Tsavkelova et al., 2006; Hussain et al., 2010; Hussain and Hasnain, 2011). Similar with auxin, algal cytokinin also positively regulated plant growth promotion and root colonization (**Figure 3**). Knockout mutant of the cytokinin biosynthesis gene, which encodes isopentenyl transferase, in the plant growth-promoting cyanobacterium *Nostoc* AHM-12 failed to increase plant growth, and significantly reduced root colonization in rice and wheat (Hussain et al., 2013). Thus, in addition to auxin, understanding the molecular basis of how algal-derived cytokinin influence plant–algae interactions will be an interesting topic for future research.

In addition to growth-promoting phytohormone, defense-related hormones such as jasmonic acid (JA), salicylic acid (SA), and ethylene (ET) are produced by algae species (Rodgers et al., 1979; Kreslavsky et al., 1997; Tsavkelova et al., 2006; Natarajan et al., 2012). Plant immunity can be activated systemically by PGPR, depending on JA, SA, and ET signaling (Pieterse et al., 1998, 2014; De Meyer et al., 1999; Kloepper et al., 2004; van Loon et al., 2006) (**Figure 3**). In addition, algae treatment can also induce activation of defense hormone signaling in host plant. Foliar application of the supernatant of *C. fusca* activates SA and JA signaling upon pathogen inoculation in *Arabidopsis* (Lee et al., 2020a). Similarly, treatment with liquid extracts of eukaryotic *Tetraselmis* sp., *D. salina*, *N. gaditana*, *Aphanothece* sp., and *A. maxima* induce the accumulation of the JA precursor, linolenic acid, in tomato (Mutale-joan et al., 2020). Thus, plant immunity triggered by algae is tightly involved in activation of defense-related hormonal signaling.

Polysaccharides

Algae produce diverse polysaccharides as cell wall components. Given their medical and cosmetic applications, algal polysaccharides are recognized as important substances (**Figure 3**). To utilize polysaccharides to improve plant health, studies have been conducted to gain molecular insight into the role of agal polysaccharides in plant protection (Arroussi et al., 2018; Farid et al., 2019). Bacterial and fungal polysaccharides such as lipopolysaccharides (LPSs) and EPSs are plant immune elicitors (Erbs and Newman, 2003; Park et al., 2008). Sulfated EPSs produced by *D. salina* increase salt stress tolerance, expression of genes encoding antioxidant enzymes (CAT, POD, and SOD), and accumulation of JA precursor in tomato (Arroussi et al., 2018). Crude polysaccharides extracted from *Chlorella vulgaris*, *Chlorella sorokiniana*, and *Chlamydomonas reinhardtii* increase expression of *PR* genes and genes encoding antioxidant enzymes such as β-1,3-glucanase, APX, and POD in tomato plants (Farid et al., 2019).

In addition to immune activation, algal polysaccharides can also improve the growth and abiotic stress tolerance of host plants. The application of algal polysaccharides extracted from cyanobacterium *S. platensis* and *A. platensis,* and eukaryotic *D. salina* and *Porphorydium* sp. promoted shoot and root growth in in tomato and pepper (Elarroussia et al., 2016; Rachidi et al., 2020). Moreover, spray treatment of polysaccharides extracted from *D. salina* increased the shoot dry weight, and root dry weight of tomato plants by 1.8- and 5. 5-, respectively, under high salt stress compared with untreated plants (Arroussi et al., 2018), implying that algal polysaccharides enhance salt tolerance. Compared with microalgae polysaccharides, macroalgal polysaccharides such as carrageenans and beta-glucans (laminarin, ulvan, and fucan) mainly function as biostimulants and bioprotectants (Mercier et al., 2001; Sangha et al., 2010, 2015; Vera et al., 2012; Ghannam et al., 2013; Shukla et al., 2016; Pettongkhao et al., 2019; Zou et al., 2019). However, the structure of microalgal polysaccharides is largely unknown. Thus, to elucidate the mode of action of microalgal polysaccharides in plants, it is important to identify the main determinant(s) in crude polysaccharide algal extracts.

D-lactic Acid

D-lactic acid is a major compound produced by *Chlorella* species (Gruber et al., 1974; Lee et al., 2020a). Recently, D-lactic acid in the supernatant of *C. fusca* was identified as a determinant of plant immunity against *Pseudomonas syringae* pv. tomato DC3000 in *Arabidopsis* (Lee et al., 2020a) (**Figure 3**). Especially, foliar application of D-lactic acid primed production of ROS after flagellin 22 (flg22) treatment in *Arabidopsis* (Lee et al., 2020a). Primed ROS production by D-lactic acid might be correlated with D-lactate oxidation and mitochondrial ROS (mtROS) production. D-lactic acid is metabolized by the D-lactate dehydrogenase (D-LDH), which localizes to the intermembrane space of mitochondria (Atlante et al., 2005; Welchen et al., 2016). Activation of D-LDH correlates strongly with activation of mitochondrial antioxidant enzyme (Husic and Tolbert, 1987). In *Arabidopsis*, exogenous application of D-lactic acid increases expression of D-*LDH*, *cytochrome c oxidase subunit 2* (*COX2*), and *alternative oxidase 1* (*AOX1*) in flg22-treated *Arabidopsis* (Lee et al., 2020a). These mitochondrial antioxidant enzymes might be activated to catalyze mtROS produced by D-lactic acid. Thus, microalgal-derived D-lactic acid enhances plant innate immunity and production of mtROS in plant.

The activation of D-LDH by D-lactic acid can also affect abiotic stress tolerance via methylglyoxal (MG) detoxification (**Figure 3**). MG is a cytotoxic compound generated as a byproduct of glycolysis, which accumulates under abiotic stress conditions (Maurino and Engqvist, 2015). To detoxify the accumulated MG, plants activate the expression of D-*LDH*, which encodes the last enzyme in the MG detoxification pathway (Maurino and Engqvist, 2015). Recently, studies showed that D-LDH-mediated MG detoxification correlates with abiotic stress tolerance in yeast, sorghum, and rice (An et al., 2017; Jain et al., 2018, 2020; Bhowal et al., 2020). In sorghum, the expression of D-*LDH1–4* genes was activated under heat, cold, salt, and drought stress conditions (Bhowal et al., 2020). In rice, D-*LDH* RNA interference (RNAi) plants were more sensitive to salt stress (200 mM NaCl) than wild-type plants (An et al., 2017). However, overexpression of D-*LDH2* conferred tolerance to multiple abiotic stresses, including salt stress, oxidative stress, osmotic stress, and heat stress in rice plants (Jain et al., 2020). Thus, microalgae-derived

D-lactic acid might alleviate abiotic stress tolerance in plants via D-LDH-mediated MG detoxification.

Plant Macro- and Micronutrients

Algae have been utilized as a source of macro- and micronutrients for plants (**Figure 3**). Microalgae cyanobacteria possess specialized cells called heterocysts, which can fix atmospheric nitrogen (Singh and Bisoyi, 1989; Gantar et al., 1993; Karthikeyan et al., 2007; Babu et al., 2015). Thus, inoculation of soil with nitrogen-fixing cyanobacteria enhanced plant growth by increasing the availability of nitrogen, carbon, and vitamins (Tripathi et al., 2008; Prasanna et al., 2009; Renuka et al., 2016). In addition, application of microalgae consortium comprising *Chlorella*, *Scenedesmus*, *Chlorococcum*, *Chroococcus*, *Phormidium*, *Anabaena*, *Westiellopsis*, *Nostoc*, *Aulosira*, and *Scytonema* to soil enhanced the content of available nitrogen, phosphorus, and potassium (Paudel et al., 2012; Renuka et al., 2016).

Algae also secrete vitamins, which promote plant growth and plant immunity (Havaux et al., 2009; Goyer, 2010) (**Figure 3**). Previously studies show that bacteria-derived vitamins B1, B2, and K3, act as elicitors of plant immunity against pathogenic fungi, bacteria, and viruses, and that biotin, thiamine, cobalamin, pantothenic acid, and niacin produced by bacteria enhance plant growth (Strzelczyk et al., 1991; Ahn et al., 2005; Taheri and Hofte, 2007; Liu et al., 2010; Taheri and Tarighi, 2010; Song et al., 2013). Cyanobacteria such as *Spirulina*, *Anabaena*, *Microcystis*, *Nostoc*, *Phormidium*, *Oscillatoria*, *Chroococcus*, and eukaryotic algae such as *Euglrena*, also produce thiamine (vitamin B1), riboflavin (vitamin B2), folic acid, ascorbic acid, nicotinic acid (vitamin B3), cyanocobalamin (vitamin B12), and vitamin E (Robbins et al., 1951; Koptera, 1970; Aaronson et al., 1977; Shah and Vaidya, 1977; Gupta et al., 2013). In addition, the extract of *N. muscorum* and *Hapalosiphon* containing vitamin B-complex (including cyanocobalamin, niacin, pantothenic acid, and folic acid) increases coleoptile length and leaf length in rice (Misra and Kaushik, 1989). Since land plants lack vitamin B12, their growth is supported by beneficial microbes containing vitamin B12 (Watanabe and Bito, 2018). Similarly, as beneficial microbes, microalgal species can also alleviate vitamin B deficiency in host plants. Further investigation of the effects of algae-derived macro- and micronutrients in plants is needed.

INTERACTION BETWEEN MICROALGAE AND OTHER MICROBES IN THE PLANT MICROBIOME

Algae benefit plants through several mechanisms. In order to consider microalgae as part of the plant microbiome, it is necessary to understand the interactions between microalgae and other plant microbiota. Interestingly, previous reports showed the synergism between algae and bacteria during co-inoculation of plants. A mixture of cyanobacteria and plant-associated eubacteria or fungi additively or synergistically improves the growth and health of diverse crop plants (**Tables 1**, **3**) (Nain et al., 2010; Dukare et al., 2011; Rana et al., 2015; Sharma et al., 2020). Soil inoculation with a mixture containing the cyanobacterium *Anabaena oscillarioides* and plant growth-promoting bacteria *Brevundimonas diminuta* and *Ochrobactrum anthropi* improved rice yield by 1.2-fold compared with the control (Rana et al., 2015). Treatment with a biofilm comprising *A. torulosa* and the plant growth-promoting fungus *Trichoderma viride* increased the seed germination rate and radicle length in maize (Sharma et al., 2020). In addition, the combined application of *Anabaena* spp. and *B. subtilis* reduced the severity of fungal disease caused by *Fusarium*, *Pythium*, and *Rhizoctonia* by twofold compared with the control (Dukare et al., 2011).

The interaction between microalgae and other microorganisms might be governed by interspecific exchange of metabolites (Gonzalez and Bashan, 2000; Kazamia et al., 2012; Kim et al., 2014). Plant-associated rhizobacteria or fungi support the growth and root colonization of microalgae species by providing secondary metabolites such as vitamin B12, siderophores, volatile compounds, *N*-acylhomoserine lactone, and EPSs (Gobler et al., 2007; Choix et al., 2012; Kazamia et al., 2012; Santos and Reis, 2014; Amavizca et al., 2017; Cho et al., 2019; Sharma et al., 2020). In turn, microalgae provide photosynthates, including fixed carbon, as nutrient sources for soil-borne microbes (Gobler et al., 2007; Kazamia et al., 2012). Taken together, these studies imply that exogenous microalgae can interact with other soil–borne microbes in plant microbiome, as do traditional plant-associated bacteria and fungi.

RHIZOSPHERE MICROBIOME ENGINEERING WITH ALGAE

Modification of the rhizosphere using microalgae, including cyanobacteria and eukaryotic microalgae, will potentially allow us to engineer and change the structure and effectiveness of the rhizosphere microbiome, thereby improving plant health. Previously, the effect of soil algae diversity on plants was investigated by application of a commercial proprietary suspension of microalgae called GOgreen® (Hastings et al., 2014). Four algal groups, including green algae (Chlorophyta), blue–green algae (Cyanophyta), yellow–green algae (Xanthophyta), and diatoms (Bacillariophyta), are mainly found in soil (Paul and Clark, 1989). The application of GOgreen® to maize roots under field conditions increased the number and diversity of diatoms and reduced the soil pH with a pH higher than 7. Since the connection between species diversity and their influence on ecological function is unclear, the authors measured two indicators of soil quality: organic matter content (OM) and cation exchange capacity (CEC). The values of OM and CEC were improved significantly by algae treatment (Hastings et al., 2014). In addition, inoculation of rice plants with the cyanobacterium *Calothrix elenkenii* increased the bacterial population diversity in the microbiome by 10-fold (Priya et al., 2015). Fatty acid methyl ester analysis and 16S rRNA sequencing data indicated that Bacillaceae was the most abundant bacterial group induced by cyanobacteria inoculation. Moreover, *C. elenkenii* inoculation increased the shoot length, root length, fresh weight, and dry weight of plants as well as enhanced the level of plant hormones

(IAA and ABA), chlorophyll, and antioxidant enzymes (POD, polyphenol oxidase [PPO], and PAL). However, direct evidence based on experiments using the gnotobiotic system was not provided. In the line of this study, more direct approaches were also attempted. Next-generation sequencing of 16S rRNA amplicons was conducted to determine the effect of *C. vulgaris* application on bean root microbiota (Kublanovskaya et al., 2019). Interestingly, no significant changes were detected in bacterial diversity in the bean rhizosphere upon the application of *C. vulgaris*. Algae-mediated microbiome engineering for promoting plant health is in its infancy. Fine-tuning microbiome engineering for keystone taxa that affect plant growth and health is necessary, and algae and their products can be utilized for this purpose.

A synthetic microbiome comprising algae and bacteria represents a promising tool for the sustainable development of soil fertility, water preservation, and plant growth, especially under stress conditions (Nain et al., 2010; Rana et al., 2015; Perea et al., 2018). A consortium of eukaryotic microalgae, cyanobacteria, and bacteria will provide organic carbon for plant growth (Belnap, 2003; Bashan and de-Bashan, 2010), fix atmospheric nitrogen (Issa et al., 2001; Pointing and Belnap, 2012), and promote seedling survival (Godínez-Alvarez et al., 2012). Detailed investigation of the algae–bacteria network and their effect on the plant microbiome is required to maximize plant growth and protect plants against pathogens (Krug et al., 2020).

POTENTIAL APPLICATIONS OF ALGAE

The beneficial effects of algae on plants and agriculture have been described above. Large-scale production of algae has been optimized for improving human health; however, the application of algae for large-scale crop cultivation has not been elucidated. We summarized the determinants of algae that augment plant growth and immunity, and classified these determinants as secreted products and the cell itself (**Figure 2**). The inoculation of plants with cell wall components such as glucans, increased plant growth and activated plant defense responses (Mercier et al., 2001; Sangha et al., 2010, 2015; Vera et al., 2012; Ghannam et al., 2013; Shukla et al., 2016; Pettongkhao et al., 2019; Zou et al., 2019, **Figure 3**). The products secreted by algae can be harvested in large amounts when algae are grown in liquid media. D-lactic acid was recently identified as an algal determinant that elicits plant immune response against bacterial pathogens (Lee et al., 2020a, **Figure 3**). Additionally, plant defense hormone-mimicking compounds, such as JA, benzoic acid and ET, were also detected in algae culture (Rodgers et al., 1979; Kreslavsky et al., 1997; Tsavkelova et al., 2006; Natarajan et al., 2012, **Figure 3**). These defense hormones strongly activate plant defense when supplied exogenously. Cell and cell envelope components of algae can be used for limited applications in the greenhouse and field to reduce the high production cost, although these products demonstrate high efficacy (Choleva et al., 2005, 2007; Dubey and Dubey, 2010; Bileva, 2013; Coppens et al., 2016). Products secreted in the liquid culture of algae also show a great potential for application in the field (Shaaban, 2001a,b; Barone et al., 2018; Mutale-joan et al., 2020). Generally, large-scale production of algae, mostly by heterotrophic cultivation, is performed to harvest algal cells (Lee et al., 2020a). The cell-free components are considered waste products that need to be detoxified. If the cell-free extracts can be reused for plants, their potential applications will increase greatly.

However, several issues must be addressed prior to application of algae on crop plants. First, the potential harmful effects of cell-free extracts of algae should be evaluated and eliminated. In many cases, algae produce toxic compounds during cultivation. For instance, at high concentrations, 2,4-D (auxin) acts as a herbicide (Marth and Mitchell, 1944). Thus, quality control of the liquid culture of algae is critical. Second, production of algal determinants should be optimized for large-scale production. Third, formulation of cell-free extracts should be carefully considered. The cell-free extract could simply be applied by drench application or by using the drip-irrigation system. However, the delivery of a large volume of extract is problematic. Therefore, the extract should be vaporized and purified using chemical and physical procedures, and the final product showing high effectiveness should be used for agricultural applications. Furthermore, granulation of determinants is similar to that of other agricultural products such as fertilizers and agrochemicals. Finally, the specific procedure how to isolate effective algae in plant health improvement also must be developed in near future.

Previously, algae were not considered as a member of the beneficial plant microbiome. However, with recent progress in metagenome analysis, algae are now recognized as important members of the plant microbiome. While microbes such as bacteria and fungi have been used to improve plant fitness, new data indicate that algae also promote plant growth and act as biological control agents against pathogens by directly inhibiting pathogen growth and activating plant immune responses. Thus, algae represent a new bioactive material that can be utilized as biofertilizers and plant protectants, which implies that algae should be classified as a member of the beneficial plant microbiome.

AUTHOR CONTRIBUTIONS

C-MR designed the review. S-ML created the figures and tables. C-MR and S-ML wrote the manuscript. Both authors contributed to the article and approved the submitted version.

FUNDING

This research was supported by grants from the Rural Development Administration, Strategic Initiative for Microbiomes in Agriculture and Food, Ministry of Agriculture, Food and Rural Affairs, Republic of Korea (as part of the multi-ministerial Genome Technology to Business Translation Program) (918017-4), Center for Agricultural Microorganism and Enzyme (Project No. PJ015049) of Rural Development Administration, and the KRIBB Initiative Program, South Korea.

REFERENCES

Aaronson, S., Dhawale, S., Patni, N., DeAngelis, B., Frank, O., and Baker, H. (1977). The cell content and secretion of water-soluble vitamins by several freshwater algae. *Arch. Microbiol.* 112, 57–59. doi: 10.1007/bf00446654

Abd El-Baky, H. H., El-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 Agricult.* 90, 299–303. doi: 10.1002/jsfa.3815

Adesalu, T., and Olugbemi, O. (2015). Soil algae: a case study of two vegetable farmlands in Lagos and Ogun states, southwest Nigeria. *IFE J. Sci.* 17, 765–772.

Ahmed, M., Stal, L. J., and Hasnain, S. (2010). Association of non-heterocystous cyanobacteria with crop plants. *Plant Soil* 336, 363–375. doi: 10.1007/s11104-010-0488-x

Ahn, I.-P., Kim, S., and Lee, Y.-H. (2005). Vitamin B1 functions as an activator of plant disease resistance. *Plant Physiol.* 138, 1505–1515. doi: 10.1104/pp.104.058693

Ali, S., Charles, T., and Glick, B. (2012). Delay of flower senescence by bacterial endophytes expressing 1-aminocyclopropane-1-carboxylate deaminase. *J. Appl. Microbiol.* 113, 1139–1144. doi: 10.1111/j.1365-2672.2012.05409.x

Amavizca, E., Bashan, Y., Ryu, C. M., Farag, M. A., Bebout, B. M., and de-Bashan, L. E. (2017). Enhanced performance of the microalga *Chlorella sorokiniana* remotely induced by the plant growth-promoting bacteria Azospirillum brasilense and Bacillus pumilus. *Sci. Rep.* 7, 1–11.

An, B., Lan, J., Deng, X., Chen, S., Ouyang, C., Shi, H., et al. (2017). Silencing of D-lactate dehydrogenase impedes glyoxalase system and leads to methylglyoxal accumulation and growth inhibition in rice. *Front. Plant Sci.* 8:2071. doi: 10.3389/fpls.2017.02071

Arroussi, H. E., Benhima, R., Elbaouchi, A., Sijilmassi, B., Mernissi, N. E., 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

Atlante, A., de Bari, L., Valenti, D., Pizzuto, R., Paventi, G., and Passarella, S. (2005). Transport and metabolism of D-lactate in *Jerusalem artichoke* mitochondria. *Biochim. Biophys. Acta Bioenerg.* 1708, 13–22. doi: 10.1016/j.bbabio.2005.03.003

Babu, S., Prasanna, R., Bidyarani, N., and Singh, R. (2015). Analysing the colonisation of inoculated cyanobacteria in wheat plants using biochemical and molecular tools. *J. Appl. Phycol.* 27, 327–338. doi: 10.1007/s10811-014-0322-6

Barone, V., Baglieri, A., Stevanato, P., Broccanello, C., Bertoldo, G., Bertaggia, M., et al. (2018). Root morphological and molecular responses induced by microalgae extracts in sugar beet (Beta vulgaris L.). *J. Appl. Phycol.* 30, 1061–1071. doi: 10.1007/s10811-017-1283-3

Barone, V., Puglisi, I., Fragalà, F., Piero, A. R. L., Giuffrida, F., and Baglieri, A. (2019). Novel bioprocess for the cultivation of microalgae in hydroponic growing system of tomato plants. *J. Appl. Phycol.* 31, 465–470. doi: 10.1007/s10811-018-1518-y

Bashan, Y., and de-Bashan, L. E. (2010). "Microbial populations of arid lands and their potential for restoration of deserts," in *Soil biology and agriculture in the tropics*, ed. P. Dion (Berlin: Springer), 109–137. doi: 10.1007/978-3-642-05076-3_6

Belnap, J. (2003). The world at your feet: desert biological soil crusts. *Front. Ecol. Environ.* 1, 181–189. doi: 10.2307/3868062

Benderliev, K. (1999). Algae and cyanobacteria release organic chelators in the presence of inorganic Fe (III) thus keeping iron dissolved. *Bulg. J. Plant Physiol.* 25, 65–75.

Benderliev, K., Ivanova, N., and Pilarski, P. (2003). Singlet oxygen and other reactive oxygen species are involved in regulation of release of iron-binding chelators from Scenedesmus cells. *Biologia Plant.* 47, 523–526. doi: 10.1023/b:biop.0000041056.07819.df

Benjamins, R., and Scheres, B. (2008). Auxin: the looping star in plant development. *Annu. Rev. Plant Biol.* 59, 443–465. doi: 10.1146/annurev.arplant.58.032806.103805

Bérard, A., Dorigo, U., Humbert, J. F., and Martin-Laurent, F. (2005). Microalgae community structure analysis based on 18S rDNA amplification from DNA extracted directly from soil as a potential soil bioindicator. *Agronomy Sustainable Dev.* 25, 285–291. doi: 10.1051/agro:2005004

Berg, G., Rybakova, D., Fischer, D., Cernava, T., Vergès, M.-C. C., Charles, T., et al. (2020). Microbiome definition re-visited: old concepts and new challenges. *Microbiome* 8, 1–22.

Bergman, B., Johansson, C., and Soderback, E. (1992). Tansley Review No. 42. The Nostoc-Gunnera symbiosis. *N. Phytol.* 122, 379–400. doi: 10.1111/j.1469-8137.1992.tb00067.x

Bhowal, B., Singla-Pareek, S. L., Sopory, S. K., and Kaur, C. (2020). From methylglyoxal to pyruvate: a genome-wide study for the identification of glyoxalases and D-lactate dehydrogenases in Sorghum bicolor. *BMC Genomics* 21:145. doi: 10.1186/s12864-020-6547-7

Bidyarani, N., Prasanna, R., Chawla, G., Babu, S., and Singh, R. (2015). Deciphering the factors associated with the colonization of rice plants by cyanobacteria. *J. Basic Microbiol.* 55, 407–419. doi: 10.1002/jobm.201400591

Bileva, T. (2013). Influence of green algae *Chlorella vulgaris* on infested with xiphinema index grape seedlings. *J. Earth Sci. Clim. Change* 4, 136–138.

Biondi, N., Piccardi, R., Margheri, M. C., Rodolfi, L., Smith, G. D., and Tredici, M. R. (2004). Evaluation of Nostoc strain ATCC 53789 as a potential source of natural pesticides. *Appl. Environ. Microbiol.* 70, 3313–3320. doi: 10.1128/aem.70.6.3313-3320.2004

Blaby-Haas, C. E., and Merchant, S. S. (2019). Comparative and functional algal genomics. *Annu. Rev. Plant Biol.* 70, 605–638.

Bradley, I. M., Pinto, A. J., and Guest, J. S. (2016). Design and evaluation of Illumina MiSeq-compatible, 18S rRNA gene-specific primers for improved characterization of mixed phototrophic communities. *Appl. Environ. Microbiol.* 82, 5878–5891. doi: 10.1128/aem.01630-16

Braun-Howland, E. B., and Nierzwicki-Bauer, S. A. (1990). *Azolla-Anabaena symbiosis: biochemistry, physiology, ultrastructure, and molecular biology*. Boca Raton: CRC Press.

Bumandalai, O., and Tserennadmid, R. (2019). Effect of *Chlorella vulgaris* as a biofertilizer on germination of tomato and cucumber seeds. *Int. J. Aquat. Biol.* 7, 95–99.

Carlson, A. S., Dole, J. M., Matthysse, A. G., Hoffmann, W. A., and Kornegay, J. L. (2015). Bacteria species and solution pH effect postharvest quality of cut Zinnia elegans. *Sci. Horticult.* 194, 71–78. doi: 10.1016/j.scienta.2015.07.044

Carmichael, W. W. (1994). The toxin of cyanobacteria. *Sci. Am.* 270, 64–70.

Chaudhary, V., Prasanna, R., Nain, L., Dubey, S., Gupta, V., Singh, R., et al. (2012). Bioefficacy of novel cyanobacteria-amended formulations in suppressing damping off disease in tomato seedlings. *World J. Microbiol. Biotechnol.* 28, 3301–3310. doi: 10.1007/s11274-012-1141-z

Cheng, C. Y., Mathews, D. E., Eric Schaller, G., and Kieber, J. J. (2013). Cytokinin-dependent specification of the functional megaspore in the Arabidopsis female gametophyte. *Plant J.* 73, 929–940. doi: 10.1111/tpj.12084

Cho, K., Heo, J., Cho, D. H., Tran, Q. G., Yun, J. H., Lee, S. M., et al. (2019). Enhancing algal biomass and lipid production by phycospheric bacterial volatiles and possible growth enhancing factor. *Algal Res.* 37, 186–194. doi: 10.1016/j.algal.2018.11.011

Choix, F. J., de-Bashan, L. E., and Bashan, Y. (2012). Enhanced accumulation of starch and total carbohydrates in alginate-immobilized *Chlorella* spp. induced by *Azospirillum brasilense*: II. Heterotrophic conditions. *Enzyme Microb. Technol.* 51, 300–309. doi: 10.1016/j.enzmictec.2012.07.012

Choleva, B., Bileva, T., and Tsvetkov, J. (2007). Organo-biological means and methods for control of plant parasitic nematodes as alternative of agrochemicals. *Ecol. Fut.* 6, 43–49.

Choleva, B., Bileva, T., Tzvetkov, Y., and Barakov, P. (2005). Preliminary study of the green algae chlorella (*Chlorella vulgaris*) for control on the root-knot nematode (*Meloidogyne arenaria*) in tomato plants and ectoparasite Xiphinema indexin grape seedlings. *Commun.Cations Agricult. Appl. Biol. Sci.* 70, 915–926.

Coppens, J., Grunert, O., Van Den Hende, S., Vanhoutte, I., Boon, N., Haesaert, G., et al. (2016). 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

Cuddy, W. S., Neilan, B. A., and Gehringer, M. M. (2012). Comparative analysis of cyanobacteria in the rhizosphere and as endosymbionts of cycads in drought-affected soils. *FEMS Microbiol. Ecol.* 80, 204–215. doi: 10.1111/j.1574-6941.2011.01288.x

Davey, M. C. (1989). The effects of freezing and desiccation on photosynthesis and survival of terrestrial Antarctic algae and cyanobacteria. *Polar Biol.* 10, 29–36.

Davey, M. C. (1991). The seasonal periodicity of algae on Antarctic fellfield soils. *Ecography* 14, 112–120. doi: 10.1111/j.1600-0587.1991.tb00641.x

Davey, M., and Clarke, K. (1991). The spatial distribution of microalgae on Antarctic fellfield soils. *Antarctic Sci.* 3, 257–263. doi: 10.1017/s0954102091000317

De Meyer, G., Capieau, K., Audenaert, K., Buchala, A., Métraux, J.-P., and Höfte, M. (1999). Nanogram amounts of salicylic acid produced by the rhizobacterium *Pseudomonas aeruginosa* 7NSK2 activate the systemic acquired resistance pathway in bean. *Mol. Plant Microbe Interact.* 12, 450–458. doi: 10.1094/mpmi.1999.12.5.450

Dessaux, Y., Grandclément, C., and Faure, D. (2016). Engineering the rhizosphere. *Trends Plant Sci.* 21, 266–278.

Dimitrov, P., and Zucker, S. W. (2006). A constant production hypothesis guides leaf venation patterning. *Proc. Natl. Acad. Sci.* 103, 9363–9368. doi: 10.1073/pnas.0603559103

Dow, C. S., and Swoboda, U. K. (2000). *Cyanotoxins. In The ecology of Cyanobacteria*. Berlin: Springer, 613–632.

Dubey, A., and Dubey, D. (2010). *Evaluation of cost effective organic fertilizers*. India: Kilpest India Ltd.

Duijff, B. J., Meijer, J. W., Bakker, P. A., and Schippers, B. (1993). Siderophore-mediated competition for iron and induced resistance in the suppression of Fusarium wilt of carnation by fluorescent *Pseudomonas* spp. *Netherl. J. Plant Pathol.* 99, 277–289. doi: 10.1007/bf01974309

Dukare, A. S., Prasanna, R., Dubey, S. C., Nain, L., Chaudhary, V., Singh, R., et al. (2011). Evaluating novel microbe amended composts as biocontrol agents in tomato. *Crop Protect.* 30, 436–442. doi: 10.1016/j.cropro.2010.12.017

Elarroussia, H., Elmernissia, N., Benhimaa, R., El Kadmiria, I. M., Bendaou, N., Smouni, A., et al. (2016). Microalgae polysaccharides a promising plant growth biostimulant. *J. Algal Biomass Utilizat.* 7, 55–63.

Elster, J. (2002). "Ecological classification of terrestrial algal communities in polar environments," in *Geoecology of Antarctic ice-free coastal landscapes*, eds L. Beyer and M. Bölter (Berlin: Springer), 303–326. doi: 10.1007/978-3-642-56318-8_17

Erbs, G., and Newman, M. A. (2003). The role of lipopolysaccharides in induction of plant defence responses. *Mol. Plant Pathol.* 4, 421–425. doi: 10.1046/j.1364-3703.2003.00179.x

Faheed, F. A., and Fattah, Z. A. (2008). Effect of Chlorella vulgaris as bio-fertilizer on growth parameters and metabolic aspects of lettuce plant. *J. Agricult. Soc. Sci.* 4, 165–169.

Farid, R., Mutale-joan, C., Redouane, B., Najib, E. M., Abderahime, A., Laila, S., et al. (2019). Effect of microalgae polysaccharides on biochemical and metabolomics pathways related to plant defense in Solanum lycopersicum. *Appl. Biochem. Biotechnol.* 188, 225–240. doi: 10.1007/s12010-018-2916-y

Gantar, M. (2000). Mechanical damage of roots provides enhanced colonization of the wheat endorhizosphere by the dinitrogen-fixing cyanobacterium Nostoc sp. strain 2S9B. *Biol. Fertility Soils* 32, 250–255. doi: 10.1007/s003740000243

Gantar, M., and Elhai, J. (1999). Colonization of wheat para-nodules by the N 2-fixing cyanobacterium Nostoc sp. strain 2S9B. *N. Phytol.* 141, 373–379. doi: 10.1046/j.1469-8137.1999.00352.x

Gantar, M., Kerby, N., and Rowell, P. (1991). Colonization of wheat (Triticum vulgare L.) by N2-fixing cyanobacteria: II. An ultrastructural study. *N. Phytol.* 118, 485–492. doi: 10.1111/j.1469-8137.1991.tb00031.x

Gantar, M., Kerby, N., and Rowell, P. (1993). Colonization of wheat (Triticum vulgare L.) by N2-fixing cyanobacteria: III. The role of a hormogonia-promoting factor. *N. Phytol.* 124, 505–513. doi: 10.1111/j.1469-8137.1993.tb03842.x

Gantar, M., Rowell, P., Kerby, N. W., and Sutherland, I. W. (1995). Role of extracellular polysaccharide in the colonization of wheat (Triticum vulgare L.) roots by N 2-fixing cyanobacteria. *Biol. Fertility Soils* 19, 41–48. doi: 10.1007/bf00336345

Ghannam, A., Abbas, A., Alek, H., Al-Waari, Z., and Al-Ktaifani, M. (2013). Enhancement of local plant immunity against tobacco mosaic virus infection after treatment with sulphated-carrageenan from red alga (Hypnea musciformis). *Physiol. Mol. Plant Pathol.* 84, 19–27. doi: 10.1016/j.pmpp.2013.07.001

Gobler, C. J., Norman, C., Panzeca, C., Taylor, G. T., and Sañudo-Wilhelmy, S. A. (2007). Effect of B-vitamins (B1, B12) and inorganic nutrients on algal bloom dynamics in a coastal ecosystem. *Aquat. Microb. Ecol.* 49, 181–194. doi: 10.3354/ame01132

Godínez-Alvarez, H., Morín, C., and Rivera-Aguilar, V. (2012). Germination, survival and growth of three vascular plants on biological soil crusts from a Mexican tropical desert. *Plant Biol.* 14, 157–162.

Goldman, S., Lammers, P., Berman, M., and Sanders-Loehr, J. (1983). Siderophore-mediated iron uptake in different strains of Anabaena sp. *J. Bacteriol.* 156, 1144–1150. doi: 10.1128/jb.156.3.1144-1150.1983

Gonzalez, L. E., and Bashan, Y. (2000). Increased growth of the microalga chlorella vulgariswhen coimmobilized and cocultured in alginate beads with the plant-growth-promoting bacterium Azospirillum brasilense. *Appl. Environ. Microbiol.* 66, 1527–1531. doi: 10.1128/aem.66.4.1527-1531.2000

Goyer, A. (2010). Thiamine in plants: aspects of its metabolism and functions. *Phytochemistry* 71, 1615–1624. doi: 10.1016/j.phytochem.2010.06.022

Gruber, P. J., Frederick, S. E., and Tolbert, N. (1974). Enzymes related to lactate metabolism in green algae and lower land plants. *Plant Physiol.* 53, 167–170. doi: 10.1104/pp.53.2.167

Gupta, V., Ratha, S. K., Sood, A., Chaudhary, V., and Prasanna, R. (2013). New insights into the biodiversity and applications of cyanobacteria (blue-green algae)—prospects and challenges. *Algal Res.* 2, 79–97. doi: 10.1016/j.algal.2013.01.006

Hamann, T., Benkova, E., Bäurle, I., Kientz, M., and Jürgens, G. (2002). The Arabidopsis BODENLOS gene encodes an auxin response protein inhibiting MONOPTEROS-mediated embryo patterning. *Genes Dev.* 16, 1610–1615. doi: 10.1101/gad.229402

Hamouda, R. A., and El-Ansary, M. (2013). Biocontrol of Root knot Nematode, Meloidogyne incognita infected banana plants by Cyanobacteria. *Egypt. J. Agronematol.* 12, 113–129.

Hamouda, R. A., and El-Ansary, M. (2017). Potential of Plant-Parasitic Nematode Control in Banana Plants by Microalgae as a New Approach Towards Resistance. *Egypt. J. Biol. Pest Control* 27, 165–172.

Hashtroudi, M. S., Ghassempour, A., Riahi, H., Shariatmadari, Z., and Khanjir, M. (2013). Endogenous auxins in plant growth-promoting Cyanobacteria—Anabaena vaginicola and Nostoc calcicola. *J. Appl. Phycol.* 25, 379–386. doi: 10.1007/s10811-012-9872-7

Hastings, K. L., Smith, L. E., Lindsey, M. L., Blotsky, L. C., Downing, G. R., et al. (2014). Effect of microalgae application on soil algal species diversity, cation exchange capacity and organic matter after herbicide treatments. *F1000Research* 3:281. doi: 10.12688/f1000research.4016.1

Havaux, M., Ksas, B., Szewczyk, A., Rumeau, D., Franck, F., et al. (2009). Vitamin B6 deficient plants display increased sensitivity to high light and photo-oxidative stress. *BMC Plant Biol.* 9:130. doi: 10.1186/1471-2229-9-130

Holajjer, P., Kamra, A., Gaur, H., and Manjunath, M. (2013). Potential of cyanobacteria for biorational management of plant parasitic nematodes: a review. *Crop Protect.* 53, 147–151. doi: 10.1016/j.cropro.2013.07.005

Husic, D. W., and Tolbert, N. (1987). Inhibition of glycolate and D-lactate metabolism in a Chlamydomonas reinhardtii mutant deficient in mitochondrial respiration. *Proc. Natl. Acad. Sci.* 84, 1555–1559. doi: 10.1073/pnas.84.6.1555

Hussain, A., and Hasnain, S. (2011). Phytostimulation and biofertilization in wheat by cyanobacteria. *J. Industr. Microbiol. Biotechnol.* 38, 85–92. doi: 10.1007/s10295-010-0833-3

Hussain, A., Hamayun, M., and Shah, S. T. (2013). Root colonization and phytostimulation by phytohormones producing entophytic Nostoc sp. AH-12. *Curr. Microbiol.* 67, 624–630. doi: 10.1007/s00284-013-0408-4

Hussain, A., Krischke, M., Roitsch, T., and Hasnain, S. (2010). Rapid determination of cytokinins and auxin in cyanobacteria. *Curr. Microbiol.* 61, 361–369. doi: 10.1007/s00284-010-9620-7

Hussain, A., Shah, S. T., Rahman, H., Irshad, M., and Iqbal, A. (2015). Effect of IAA on in vitro growth and colonization of Nostoc in plant roots. *Front. Plant Sci.* 6:46. doi: 10.3389/fpls.2015.00046

Innok, S., Chunleuchanon, S., Boonkerd, N., and Teaumroong, N. (2009). Cyanobacterial akinete induction and its application as biofertilizer for rice cultivation. *J. Appl. Phycol.* 21:737. doi: 10.1007/s10811-009-9409-x

Issa, O. M., Stal, L. J., Défarge, C., Couté, A., and Trichet, J. (2001). Nitrogen fixation by microbial crusts from desiccated Sahelian soils (Niger). *Soil Biol. Biochem.* 33, 1425–1428. doi: 10.1016/s0038-0717(01)00046-3

Jain, M., Aggarwal, S., Nagar, P., Tiwari, R., and Mustafiz, A. (2020). A D-lactate dehydrogenase from rice is involved in conferring tolerance to

multiple abiotic stresses by maintaining cellular homeostasis. *Sci. Rep.* 10, 1–17.

Jain, M., Nagar, P., Sharma, A., Batth, R., Aggarwal, S., Kumari, S., et al. (2018). GLYI and D-LDH play key role in methylglyoxal detoxification and abiotic stress tolerance. *Sci. Rep.* 8, 1–9.

Jaki, B., Orjala, J., Heilmann, J., Linden, A., Vogler, B., and Sticher, O. (2000). Novel Extracellular Diterpenoids with Biological Activity from the Cyanobacterium Nostoc c ommune. *J. Nat. Products* 63, 339–343. doi: 10.1021/np9903090

Karthikeyan, N., Prasanna, R., Nain, L., and Kaushik, B. D. (2007). Evaluating the potential of plant growth promoting cyanobacteria as inoculants for wheat. *Eur. J. Soil Biol.* 43, 23–30. doi: 10.1016/j.ejsobi.2006.11.001

Karthikeyan, N., Prasanna, R., Sood, A., Jaiswal, P., Nayak, S., and Kaushik, B. (2009). Physiological characterization and electron microscopic investigation of cyanobacteria associated with wheat rhizosphere. *Folia Microbiol.* 54, 43–51. doi: 10.1007/s12223-009-0007-8

Kazamia, E., Czesnick, H., Nguyen, T. T. V., Croft, M. T., Sherwood, E., et al. (2012). Mutualistic interactions between vitamin B12-dependent algae and heterotrophic bacteria exhibit regulation. *Environ. Microbiol.* 14, 1466–1476. doi: 10.1111/j.1462-2920.2012.02733.x

Khan, Z., Kim, Y., Kim, S., and Kim, H. (2007). Observations on the suppression of root-knot nematode (Meloidogyne arenaria) on tomato by incorporation of cyanobacterial powder (Oscillatoria chlorina) into potting field soil. *Bioresour. Technol.* 98, 69–73. doi: 10.1016/j.biortech.2005.11.029

Khan, Z., Park, S., Shin, S., Bae, S., Yeon, I., and Seo, Y. (2005). Management of Meloidogyne incognita on tomato by root-dip treatment in culture filtrate of the blue-green alga, Microcoleus vaginatus. *Bioresour. Technol.* 96, 1338–1341.

Khaw, Y. S., Khong, N. M., Shaharuddin, N. A., and Yusoff, F. M. (2020). A simple 18S rDNA approach for the identification of cultured eukaryotic microalgae with an emphasis on primers. *J. Microbiol. Methods* 172:105890.

Kim, J., and Kim, J.-D. (2008). Inhibitory effect of algal extracts on mycelial growth of the tomato-wilt pathogen, Fusarium oxysporum f. sp. lycopersici. *Mycobiology* 36, 242–248.

Kim, M., Shim, C., Kim, Y., Park, J., Hong, S., Ji, H., et al. (2014). Effect of Chlorella vulgaris CHK0008 fertilization on enhancement of storage and freshness in organic strawberry and leaf vegetables. *Kor. J. Horticult. Sci. Technol.* 32, 872–878.

Kim, M.-J., Shim, C.-K., Kim, Y.-K., Jee, H.-J., Park, J.-H., Hong, S.-J., et al. (2012). Effect of Green Algae, Chlorella fusca on Improving Seed Germination and Seedling Growth in Vegetable Crop and Minor Cereal. *Kor. J. Org. Agric.* 2012, 491–492.

Kim, M.-J., Shim, C.-K., Kim, Y.-K., Ko, B.-G., Park, J.-H., Hwang, S.-G., et al. (2018a). Effect of biostimulator Chlorella fusca on improving growth and qualities of chinese chives and spinach in organic farm. *Plant Pathol. J.* 34:567.

Kim, S. J., Ko, E. J., Hong, J. K., and Jeun, Y. C. (2018b). Ultrastructures of Colletotrichum orbiculare in cucumber leaves expressing systemic acquired resistance mediated by Chlorella fusca. *Plant Pathol. J.* 34:113.

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.

Kloepper, J. W., Ryu, C.-M., and Zhang, S. (2004). Induced systemic resistance and promotion of plant growth by Bacillus spp. *Phytopathology* 94, 1259–1266.

Konst, H., McKercher, P., Gorham, P., Robertson, A., and Howell, J. (1965). Symptoms and pathology produced by toxic Microcystis aeruginosa NRC-1 in laboratory and domestic animals. *Can. J. Comparat. Med. Vet. Sci.* 29:221.

Koptera, Z. (1970). Biosynthesis of biotin, pyredoxin, nicotinic acid and pantothenic acids by some blue-green algae. *Microbiol. Z* 32, 555–560.

Kreslavsky, V., Kobzar, E., and Muzafarov, E. (1997). Effect of red radiation, kinetin and linuron on growth and ethylene production in Chlorella. *Biol. Plant.* 39, 427–430.

Krings, M., Hass, H., Kerp, H., Taylor, T. N., Agerer, R., and Dotzler, N. (2009). Endophytic cyanobacteria in a 400-million-yr-old land plant: A scenario for the origin of a symbiosis? *Rev. Palaeobot. Palynol.* 153, 62–69.

Krug, L., Erlacher, A., Markut, K., Berg, G., and Cernava, T. (2020). The microbiome of alpine snow algae shows a specific inter-kingdom connectivity and algae-bacteria interactions with supportive capacities. *ISME J.* 14, 2197–2210.

Kuan, K. B., Othman, R., Abdul Rahim, K., and Shamsuddin, Z. H. (2016). Plant growth-promoting rhizobacteria inoculation to enhance vegetative growth, nitrogen fixation and nitrogen remobilisation of maize under greenhouse conditions. *PLoS One* 11:e0152478. doi: 10.1371/journal.pone.0152478

Kublanovskaya, A., Khapchaeva, S., Zotov, V., Zaytsev, P., Lobakova, E., and Solovchenko, A. (2019). The Effect of the Microalga Chlorella vulgaris Ippas C-1 Biomass Application on Yield, Biological Activity, and the Microbiome of the Soil during Bean Growing. *Moscow Univ. Biol. Sci. Bull.* 74, 227–234.

Kumar, M., Prasanna, R., Bidyarani, N., Babu, S., Mishra, B. K., Kumar, A., et al. (2013). Evaluating the plant growth promoting ability of thermotolerant bacteria and cyanobacteria and their interactions with seed spice crops. *Sci. Horticult.* 164, 94–101.

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

Lee, S. M., Kim, S. K., Lee, N., Ahn, C. Y., and Ryu, C. M. (2020a). D-Lactic acid secreted by Chlorella fusca primes pattern-triggered immunity against *Pseudomonas* syringae in Arabidopsis. *Plant J.* 102, 761–778. doi: 10.1111/tpj.14661

Lee, S. M., Lee, B., Shim, C. K., Chang, Y. K., and Ryu, C. M. (2020b). Plant anti-aging: Delayed flower and leaf senescence in Erinus alpinus treated with cell-free Chlorella cultivation medium. *Plant Signal. Behav.* 15:1763005. doi: 10.1080/15592324.2020.1763005

Lee, Y. J., Kim, S. J., and Jeun, Y. C. (2017). Ultra-structural Observations of Colletotrichum orbiculare on Cucumber Leaves Pre-treated with Chlorella fusca. *Res. Plant Dis.* 23, 42–48. doi: 10.5423/RPD.2017.23.1.42

Lee, Y. J., Ko, Y. J., and Jeun, Y. C. (2016). Illustration of disease suppression of anthracnose on cucumber leaves by treatment with Chlorella fusca. *Res. Plant Dis.* 22, 257–263. doi: 10.5423/RPD.2016.22.4.257

Lemanceau, P., and Alabouvette, C. (1993). Suppression of Fusarium wilts by fluorescent pseudomonads: mechanisms and applications. *Biocontr. Sci. Technol.* 3, 219–234. doi: 10.1080/09583159309355278

Li, Y., Xu, S.-S., Gao, J., Pan, S., and Wang, G.-X. (2014). Chlorella induces stomatal closure via NADPH oxidase-dependent ROS production and its effects on instantaneous water use efficiency in Vicia faba. *PLoS One* 9:e93290. doi: 10.1371/journal.pone.0093290

Lim, P. O., Kim, H. J., and Gil Nam, H. (2007). Leaf senescence. *Annu. Rev. Plant Biol.* 58, 115–136. doi: 10.1146/annurev.arplant.57.032905.105316

Lindblad, P., Bergman, B., and Rai, A. N. (1990). "The cycad-cyanobacterial symbiosis," in *CRC handbook of symbiotic cyanobacteria*, ed. A. N. Rai (Florida: CRC Press), 137–159. doi: 10.1201/9781351071185-6

Liu, F., Wei, F., Wang, L., Liu, H., Zhu, X., and Liang, Y. (2010). Riboflavin activates defense responses in tobacco and induces resistance against Phytophthora parasitica and Ralstonia solanacearum. *Physiol. Mol. Plant Pathol.* 74, 330–336. doi: 10.1016/j.pmpp.2010.05.002

Liu, J., and Chen, F. (2016). Biology and industrial applications of Chlorella: advances and prospects. *Adv. Biochem. Eng. Biotechnol.* 153, 1–35. doi: 10.1007/10_2014_286

Longford, S. R., Campbell, A. H., Nielsen, S., Case, R. J., Kjelleberg, S., and Steinberg, P. D. (2019). Interactions within the microbiome alter microbial interactions with host chemical defences and affect disease in a marine holobiont. *Sci. Rep.* 9, 1–13. doi: 10.1038/s41598-018-37062-z

López, A., Ming, D. S., and Towers, G. N. (2002). Antifungal Activity of Benzoic Acid Derivatives from Piper l anceaefolium. *J. Nat. Prod.* 65, 62–64. doi: 10.1021/np010410g

Lugtenberg, B., and Kamilova, F. (2009). Plant-growth-promoting rhizobacteria. *Annu. Rev. Microbiol.* 63, 541–556. doi: 10.1146/annurev.micro.62.081307.162918

Manjunath, M., Prasanna, R., Nain, L., Dureja, P., Singh, R., Kumar, A., et al. (2010). Biocontrol potential of cyanobacterial metabolites against damping off disease caused by Pythium aphanidermatum in solanaceous vegetables. *Archiv. Phytopathol. Plant Protect.* 43, 666–677. doi: 10.1080/03235400802075815

Mankiewicz, J., Tarczynska, M., Walter, Z., and Zalewski, M. (2003). Natural toxins from cyanobacteria. *Acta Biol. Cracov. Bot.* 45, 9–20.

Marsch-Martínez, N., Ramos-Cruz, D., Reyes-Olalde, J. I., Lozano-Sotomayor, P., Zúñiga-Mayo, V. M., and de Folter, S. (2012). The role of cytokinin during Arabidopsis gynoecia and fruit morphogenesis and patterning. *Plant J.* 72, 222–234. doi: 10.1111/j.1365-313X.2012.05062.x

Marth, P. C., and Mitchell, J. W. (1944). 2, 4-Dichlorophenoxyacetic acid as a differential herbicide. *Bot. Gazette* 106, 224–232. doi: 10.1086/335289

Maurino, V. G., and Engqvist, M. K. (2015). 2-Hydroxy acids in plant metabolism. *Am. Soc. Plant Biol.* 13:e0182. doi: 10.1199/tab.0182

Mazur, H., Konop, A., and Synak, R. (2001). Indole-3-acetic acid in the culture medium of two axenic green microalgae. *J. Appl. Phycol.* 13, 35–42. doi: 10.1023/A:1008199409953

McKnight, D. M., and Morel, F. M. (1980). Copper complexation by siderophores from filamentous blue-green algae 1. *Limnol. Oceanogr.* 25, 62–71. doi: 10.4319/lo.1980.25.1.0062

Meeks, J. C., and Elhai, J. (2002). Regulation of cellular differentiation in filamentous cyanobacteria in free-living and plant-associated symbiotic growth states. *Microbiol. Mol. Biol. Rev.* 66, 94–121. doi: 10.1128/MMBR.66.1.94-121.2002

Meeks, J. C., and Rai, A. (1990). "Cyanobacterial-bryophyte associations," in *CRC handbook of symbiotic cyanobacteria*, ed. A. N. Rai (Florida: CRC Press), 43–63. doi: 10.1201/9781351071185-3

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

Mercier, L., Lafitte, C., Borderies, G., and Briand, X. (2001). Esquerré-Tugayé MT, Fournier J: The algal polysaccharide carrageenans can act as an elicitor of plant defence. *N. Phytol.* 149, 43–51. doi: 10.1046/j.1469-8137.2001.00011.x

Misra, S., and Kaushik, B. (1989). Growth promoting substances of cyanobacteria. I: vitamins and their influence on rice plant. *Proc. Ind. Natl. Sci. Acad. Part B Biol. Sci.* 55, 295–300.

Mutale-joan, C., Redouane, B., Najib, E., Yassine, K., Lyamlouli, K., Laila, S., et al. (2020). Screening of microalgae liquid extracts for their bio stimulant properties on plant growth, nutrient uptake and metabolite profile of Solanum lycopersicum L. *Sci. Rep.* 10, 1–12. doi: 10.1038/s41598-020-59840-4

Nain, L., Rana, A., Joshi, M., Jadhav, S. D., Kumar, D., Shivay, Y., et al. (2010). Evaluation of synergistic effects of bacterial and cyanobacterial strains as biofertilizers for wheat. *Plant Soil* 331, 217–230. doi: 10.1007/s11104-009-0247-z

Naing, A. H., Win, N. M., Han, J.-S., Lim, K. B., and Kim, C. K. (2017). Role of nano-silver and the bacterial strain *Enterobacter cloacae* in increasing vase life of cut carnation 'Omea'. *Front. Plant Sci.* 8:1590. doi: 10.3389/fpls.2017.01590

Natarajan, C., Prasanna, R., Gupta, V., Dureja, P., and Nain, L. (2012). Characterization of the fungicidal activity of Calothrix elenkinii using chemical methods and microscopy. *Appl. Biochem. Microbiol.* 48, 51–57. doi: 10.1134/S0003683812010115

Nilsson, M., Bhattacharya, J., Rai, A., and Bergman, B. (2002). Colonization of roots of rice (Oryza sativa) by symbiotic Nostoc strains. *N. Phytol.* 156, 517–525. doi: 10.1046/j.1469-8137.2002.00534.x

Ohta, T., Sueoka, E., Iida, N., Komori, A., Suganuma, M., Nishiwaki, R., et al. (1994). Nodularin, a potent inhibitor of protein phosphatases 1 and 2A, is a new environmental carcinogen in male F344 rat liver. *Cancer Res.* 54, 6402–6406.

Pagnussat, G. C., Alandete-Saez, M., Bowman, J. L., and Sundaresan, V. (2009). Auxin-dependent patterning and gamete specification in the Arabidopsis female gametophyte. *Science* 324, 1684–1689. doi: 10.1126/science.1167324

Park, K., Kloepper, J. W., and Ryu, C.-M. (2008). Rhizobacterial exopolysaccharides elicit induced resistance on cucumber. *J. Microbiol. Biotechnol.* 18, 1095–1100.

Parker, M. S., Mock, T., and Armbrust, E. V. (2008). Genomic insights into marine microalgae. *Annu. Rev. Genet.* 42, 619–645. doi: 10.1146/annurev.genet.42.110807.091417

Paudel, Y., Pradhan, S., Pant, B., and Prasad, B. (2012). Role of blue green algae in rice productivity. *Agricult. Biol. J. North Am.* 3, 332–335. doi: 10.5251/abjna.2012.3.8.332.335

Paul, E. A., and Clark, F. E. (1989). *Soil Microbiology and Biochemistry*. San Diego: Academic Press, Inc, 273. doi: 10.1016/B978-0-12-546805-3.50004-7

Perea, J. R., Llorens-Martín, M., Ávila, J., and Bolós, M. (2018). The role of microglia in the spread of tau: relevance for tauopathies. *Front. Cell. Neurosci.* 12:172. doi: 10.3389/fncel.2018.00172

Pettongkhao, S., Bilanglod, A., Khompatara, K., and Churngchow, N. (2019). Sulphated Polysaccharide from Acanthophora spicifera Induced Hevea brasiliensis Defense Responses Against Phytophthora palmivora Infection. *Plants* 8:73. doi: 10.3390/plants8030073

Pieterse, C. M., Van Wees, S. C., Van Pelt, J. A., Knoester, M., Laan, R., Gerrits, H., et al. (1998). A novel signaling pathway controlling induced systemic resistance in Arabidopsis. *Plant Cell* 10, 1571–1580. doi: 10.1105/tpc.10.9.1571

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

Pointing, S. B., and Belnap, J. (2012). Microbial colonization and controls in dryland systems. *Nat. Rev. Microbiol.* 10, 551–562. doi: 10.1038/nrmicro2831

Prasanna, R., Chaudhary, V., Gupta, V., Babu, S., Kumar, A., Singh, R., et al. (2013). Cyanobacteria mediated plant growth promotion and bioprotection against Fusarium wilt in tomato. *Eur. J. Plant Pathol.* 136, 337–353. doi: 10.1007/s10658-013-0167-x

Prasanna, R., Kanchan, A., Ramakrishnan, B., Ranjan, K., Venkatachalam, S., Hossain, F., et al. (2016). Cyanobacteria-based bioinoculants influence growth and yields by modulating the microbial communities favourably in the rhizospheres of maize hybrids. *Eur. J. Soil Biol.* 75, 15–23. doi: 10.1016/j.ejsobi.2016.04.001

Prasanna, R., Nain, L., Ancha, R., Srikrishna, J., Joshi, M., and Kaushik, B. D. (2009). Rhizosphere dynamics of inoculated cyanobacteria and their growth-promoting role in rice crop. *Egypt. J. Biol.* 11, 26–36.

Prasanna, R., Nain, L., Tripathi, R., Gupta, V., Chaudhary, V., Middha, S., et al. (2008). Evaluation of fungicidal activity of extracellular filtrates of cyanobacteria–possible role of hydrolytic enzymes. *J. Basic Microbiol.* 48, 186–194. doi: 10.1002/jobm.200700199

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

Pushkareva, E., Johansen, J. R., and Elster, J. (2016). A review of the ecology, ecophysiology and biodiversity of microalgae in Arctic soil crusts. *Polar Biol.* 39, 2227–2240. doi: 10.1007/s00300-016-1902-5

Rachidi, F., Benhima, R., Sbabou, L., and El Arroussi, H. (2020). Microalgae polysaccharides bio-stimulating effect on tomato plants: Growth and metabolic distribution. *Biotechnol. Rep.* 25:e00426. doi: 10.1016/j.btre.2020.e00426

Rana, A., Kabi, S. R., Verma, S., Adak, A., Pal, M., Shivay, Y. S., et al. (2015). Prospecting plant growth promoting bacteria and cyanobacteria as options for enrichment of macro-and micronutrients in grains in rice–wheat cropping sequence. *Cogent Food Agricult.* 1:1037379. doi: 10.1080/23311932.2015.1037379

Rasmussen, U., Johansson, C., Renglin, A., Petersson, C., and Bergman, B. (1996). A molecular characterization of the Gunnera–Nostoc symbiosis: comparison with Rhizobium–and Agrobacterium–plant interactions. *N. Phytol.* 133, 391–398. doi: 10.1111/j.1469-8137.1996.tb01906.x

Renuka, N., Prasanna, R., Sood, A., Ahluwalia, A. S., Bansal, R., Babu, S., et al. (2016). Exploring the efficacy of wastewater-grown microalgal biomass as a biofertilizer for wheat. *Environ. Sci. Poll. Res.* 23, 6608–6620. doi: 10.1007/s11356-015-5884-6

Robbins, W. J., Hervey, A., and Stebbins, M. E. (1951). Further observations on Euglena and B12. *Bull. Torrey Bot. Club* 78, 363–375. doi: 10.2307/2482017

Rodgers, G., Bergman, B., Henriksson, E., and Udris, M. (1979). Utilisation of blue-green algae as biofertilisers. *Plant Soil* 52, 99–107. doi: 10.1007/BF02197736

Rodríguez, A., Stella, A., Storni, M., Zulpa, G., and Zaccaro, M. (2006). Effects of cyanobacterial extracellular products and gibberellic acid on salinity tolerance in Oryza sativaL. *Saline Syst.* 2:7. doi: 10.1186/1746-1448-2-7

Sangha, J. S., Kandasamy, S., Khan, W., Bahia, N. S., Singh, R. P., Critchley, A. T., et al. (2015). λ-carrageenan suppresses tomato chlorotic dwarf viroid (TCDVd) replication and symptom expression in tomatoes. *Mar. Drugs* 13, 2875–2889. doi: 10.3390/md13052875

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

Santi, S., De Marco, F., Polizzotto, R., Grisan, S., and Musetti, R. (2013). Recovery from stolbur disease in grapevine involves changes in sugar transport and metabolism. *Front. Plant Sci.* 4:171. doi: 10.3389/fpls.2013.00171

Santos, C. A., and Reis, A. (2014). Microalgal symbiosis in biotechnology. *Appl. Microbiol. Biotechnol.* 98, 5839–5846. doi: 10.1007/s00253-014-5764-x

Sathiyamoorthy, P., and Shanmugasundaram, S. (1996). Preparation of cyanobacterial peptide toxin as a biopesticide against cotton pests. *Appl. Microbiol. Biotechnol.* 46, 511–513. doi: 10.1007/s002530050852

Schaller, G. E., Street, I. H., and Kieber, J. J. (2014). Cytokinin and the cell cycle. *Curr. Opin. Plant Biol.* 21, 7–15. doi: 10.1016/j.pbi.2014.05.015

Shaaban, M. M. (2001a). Green microalgae water extract as foliar feeding to wheat plants. *Pak. J. Biol. Sci.* 4, 628–632. doi: 10.3923/pjbs.2001.628.632

Shaaban, M. M. (2001b). Nutritional status and growth of maize plants as affected by green microalgae as soil additives. *J. Biol. Sci.* 1, 475–479. doi: 10.3923/jbs.2001.475.479

Shah, A., and Vaidya, B. (1977). Detection of vitamin B 12 and pantothenic acid in cell exudates of blue-green algae. *Biol. Plant.* 19, 426–429. doi: 10.1007/BF02922978

Shahri, W., and Tahir, I. (2014). Flower senescence: some molecular aspects. *Planta* 239, 277–297. doi: 10.1007/s00425-013-1984-z

Sharma, V., Prasanna, R., Hossain, F., Muthusamy, V., Nain, L., et al. (2020). Priming maize seeds with cyanobacteria enhances seed vigour and plant growth in elite maize inbreds. *3 Biotech.* 10, 1–15. doi: 10.1007/s13205-020-2141-6

Shukla, P. S., Borza, T., Critchley, A. T., and Prithiviraj, B. (2016). Carrageenans from red seaweeds as promoters of growth and elicitors of defense response in plants. *Front. Mar. Sci.* 3:81. doi: 10.3389/fmars.2016.00081

Silverster, W., and Smith, D. (1969). Nitrogen fixation by Gunnera–Nostoc symbiosis. *Nature* 224, 1231–1231. doi: 10.1038/2241231a0

Silvester, W. B. (1976). "Endophyte adaptation in Gunnera-Nostocsymbiosis," in *Symbiotic Nitrogen Fixation in Plants*, Vol. 7, ed. S. Nutman (Cambridge: Cambridge University Press), 521–538.

Singh, P., and Bisoyi, R. (1989). Blue green algae in rice fields. *Phykos* 28, 181–195.

Singh, S., and Datta, P. (2007). Outdoor evaluation of herbicide resistant strains of Anabaena variabilis as biofertilizer for rice plants. *Plant Soil* 296, 95–102. doi: 10.1007/s11104-007-9293-6

Song, G. C., Choi, H. K., and Ryu, C.-M. (2013). The folate precursor para-aminobenzoic acid elicits induced resistance against Cucumber mosaic virus and Xanthomonas axonopodis. *Ann. Bot.* 111, 925–934. doi: 10.1093/aob/mct049

Spiller, H., Stallings, W., Woods, T., and Gunasekaran, M. (1993). Requirement for direct association of ammonia-excreting Anabaena variabilis mutant (SA-1) with roots for maximal growth and yield of wheat. *Appl. Microbiol. Biotechnol.* 40, 557–566. doi: 10.1007/BF00175748

Stirk, W. A., Ördög, V., Novák, O., Rolčík, J., Strnad, M., Bálint, P., et al. (2013). Auxin and cytokinin relationships in 24 microalgal strains1. *J. Phycol.* 49, 459–467. doi: 10.1111/jpy.12061

Stirk, W., Ördög, V., Van Staden, J., and Jäger, K. (2002). Cytokinin-and auxin-like activity in Cyanophyta and microalgae. *J. Appl. Phycol.* 14, 215–221. doi: 10.1023/A:1019928425569

Strzelczyk, E., Dahm, H., and Pachlewski, R. (1991). B-group vitamins production by mycorrhizal fungi in response to pH (in vitro studies). *Plant Soil* 137, 237–241. doi: 10.1007/BF00011202

Taheri, P., and Hofte, M. (2007). Induction of systemic defense responses in rice against the sheath blight pathogen Rhizoctonia solani, by means of riboflavin. *Commun. Agricult. Appl. Biol. Sci.* 72:983.

Taheri, P., and Tarighi, S. (2010). Riboflavin induces resistance in rice against Rhizoctonia solani via jasmonate-mediated priming of phenylpropanoid pathway. *J. Plant Physiol.* 167, 201–208. doi: 10.1016/j.jplph.2009.08.003

Tchan, Y. T., Kennedy, I. R. (1989). IR Possible N_2-fixing root nodules induced in non-legumes. *Agric. Sci.* 2, 57–59.

Treves, H., Raanan, H., Kedem, I., Murik, O., Keren, N., Zer, H., et al. (2016). The mechanisms whereby the green alga Chlorella ohadii, isolated from desert soil crust, exhibits unparalleled photodamage resistance. *N. Phytol.* 210, 1229–1243. doi: 10.1111/nph.13870

Tripathi, R., Dwivedi, S., Shukla, M., Mishra, S., Srivastava, S., Singh, R., et al. (2008). Role of blue green algae biofertilizer in ameliorating the nitrogen demand and fly-ash stress to the growth and yield of rice (Oryza sativa L.) plants. *Chemosphere* 70, 1919–1929. doi: 10.1016/j.chemosphere.2007.07.038

Tsavkelova, E., Klimova, S. Y., Cherdyntseva, T., and Netrusov, A. (2006). Microbial producers of plant growth stimulators and their practical use: a review. *Appl. Biochem. Microbiol.* 42, 117–126. doi: 10.1134/S0003683806020013

van Loon, L. C., Geraats, B. P., and Linthorst, H. J. (2006). Ethylene as a modulator of disease resistance in plants. *Trends Plant Sci.* 11, 184–191.

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.

Volk, R.-B., and Furkert, F. H. (2006). Antialgal, antibacterial and antifungal activity of two metabolites produced and excreted by cyanobacteria during growth. *Microbiol. Res.* 161, 180–186.

Watanabe, F., and Bito, T. (2018). Vitamin B12 sources and microbial interaction. *Exp. Biol. Med.* 243, 148–158.

Welchen, E., Schmitz, J., Fuchs, P., García, L., Wagner, S., Wienstroer, J., et al. (2016). D-Lactate dehydrogenase links methylglyoxal degradation and electron transport through cytochrome C. *Plant Physiol.* 172, 901–912.

Werner, T., Motyka, V., Strnad, M., and Schmülling, T. (2001). Regulation of plant growth by cytokinin. *Proc. Natl. Acad. Sci.* 98, 10487–10492.

Whipps, J., Lewis, K., and Cooke, R. (1988). "Mycoparasitism and plant disease control 161–187," in *Fungi in Biological Control Systems*, ed. N. M. Burge (Manchester: Manchester University Press), 176.

Wilpiszeski, R. L., Aufrecht, J. A., Retterer, S. T., Sullivan, M. B., Graham, D. E., Pierce, E. M., et al. (2019). Soil aggregate microbial communities: towards understanding microbiome interactions at biologically relevant scales. *Appl. Environ. Microbiol.* 85, 324–319e.

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.

Xu, J., Zhang, Y., Zhang, P., Trivedi, P., Riera, N., Wang, Y., et al. (2018). The structure and function of the global citrus rhizosphere microbiome. *Nat. Commun.* 9, 1–10.

Zhang, B., Zhang, Y., Downing, A., and Niu, Y. (2011). Distribution and composition of cyanobacteria and microalgae associated with biological soil crusts in the Gurbantunggut Desert, China. *Arid Land Res. Manag.* 25, 275–293.

Zhang, J., Wang, X., and Zhou, Q. (2017). Co-cultivation of Chlorella spp and tomato in a hydroponic system. *Biomass Bioener.* 97, 132–138.

Zhu, H., Li, S., Hu, Z., and Liu, G. (2018). Molecular characterization of eukaryotic algal communities in the tropical phyllosphere based on real-time sequencing of the 18S rDNA gene. *BMC Plant Biol.* 18, 1–14. doi: 10.1186/s12870-018-1588-7

Zou, P., Lu, X., Zhao, H., Yuan, Y., Meng, L., Zhang, C., et al. (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

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

ORIGINAL RESEARCH
published: 03 March 2021
doi: 10.3389/fpls.2021.632689

Verticillium dahliae Inoculation and *in vitro* Propagation Modify the Xylem Microbiome and Disease Reaction to Verticillium Wilt in a Wild Olive Genotype

*Manuel Anguita-Maeso[1], José Luis Trapero-Casas[1], Concepción Olivares-García[1], David Ruano-Rosa[1], Elena Palomo-Ríos[2], Rafael M. Jiménez-Díaz[1,3], Juan A. Navas-Cortés[1] and Blanca B. Landa[1]**

[1] Institute for Sustainable Agriculture, Spanish National Research Council (Consejo Superior de Investigaciones Científicas), Córdoba, Spain, [2] Institute for Mediterranean and Subtropical Horticulture "La Mayora" (IHSM-UMA-Consejo Superior de Investigaciones Científicas), Department of Botany and Plant Physiology, University of Malaga, Málaga, Spain, [3] Agronomy Department, University of Córdoba, Córdoba, Spain

Host resistance is the most practical, long-term, and economically efficient disease control measure for Verticillium wilt in olive caused by the xylem-invading fungus *Verticillium dahliae* (*Vd*), and it is at the core of the integrated disease management. Plant's microbiome at the site of infection may have an influence on the host reaction to pathogens; however, the role of xylem microbial communities in the olive resistance to *Vd* has been overlooked and remains unexplored to date. This research was focused on elucidating whether *in vitro* olive propagation may alter the diversity and composition of the xylem-inhabiting microbiome and if those changes may modify the resistance response that a wild olive clone shows to the highly virulent defoliating (D) pathotype of *Vd*. Results indicated that although there were differences in microbial communities among the different propagation methodologies, most substantial changes occurred when plants were inoculated with *Vd*, regardless of whether the infection process took place, with a significant increase in the diversity of bacterial communities when the pathogen was present in the soil. Furthermore, it was noticeable that olive plants multiplied under *in vitro* conditions developed a susceptible reaction to D *Vd*, characterized by severe wilting symptoms and 100% vascular infection. Moreover, those *in vitro* propagated plants showed an altered xylem microbiome with a decrease in total OTU numbers as compared to that of plants multiplied under non-aseptic conditions. Overall, 10 keystone bacterial genera were detected in olive xylem regardless of infection by *Vd* and the propagation procedure of plants (*in vitro* vs nursery), with *Cutibacterium* (36.85%), *Pseudomonas* (20.93%), *Anoxybacillus* (6.28%), *Staphylococcus* (4.95%), *Methylobacterium-Methylorubrum* (3.91%), and *Bradyrhizobium* (3.54%) being the most abundant. *Pseudomonas* spp. appeared as the most predominant bacterial group in

OPEN ACCESS

Edited by:
Roeland Lucas Berendsen, Utrecht University, Netherlands

Reviewed by:
Sotiris Tjamos, Agricultural University of Athens, Greece
Antonio José Fernández-González, Consejo Superior de Investigaciones Científicas (CSIC), Spain
Wu Xiong, Utrecht University, Netherlands
Hanna Rövenich, University of Cologne, Germany

***Correspondence:**
Blanca B. Landa
blanca.landa@csic.es

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

Received: *23 November 2020*
Accepted: *01 February 2021*
Published: *03 March 2021*

Citation:
Anguita-Maeso M, Trapero-Casas JL, Olivares-García C, Ruano-Rosa D, Palomo-Ríos E, Jiménez-Díaz RM, Navas-Cortés JA and Landa BB (2021) Verticillium dahliae Inoculation and in vitro Propagation Modify the Xylem Microbiome and Disease Reaction to Verticillium Wilt in a Wild Olive Genotype. Front. Plant Sci. 12:632689. doi: 10.3389/fpls.2021.632689

micropropagated plants and *Anoxybacillus* appeared as a keystone bacterium in *Vd*-inoculated plants irrespective of their propagation process. Our results are the first to show a breakdown of resistance to *Vd* in a wild olive that potentially may be related to a modification of its xylem microbiome and will help to expand our knowledge of the role of indigenous xylem microbiome on host resistance, which can be of use to fight against main vascular diseases of olive.

Keywords: microbiome, xylem, olive, *Verticillium dahliae*, micropropagation, host resistance

INTRODUCTION

Verticillium wilt, caused by the vascular-colonizing, soil-borne fungus, *Verticillium dahliae* (*Vd*), is one of the main diseases threatening the health and growth of olive (*Olea europaea* subsp. *europaea* var. *europaea*) production worldwide. This disease, first reported in Italy in 1946, has steadily increased in prevalence and incidence to become an actual threat to olive cultivation in the Mediterranean Basin due to the high rates of tree mortality and important reductions in yields (Jiménez-Díaz et al., 2011; Landa et al., 2019). Infection of olive plants by *Vd* takes place through the root system; then, the pathogen colonizes the xylem vessels and impairs the sap flow by means of mycelial proliferation, the formation of occlusions and tyloses that ultimately cause the tree death (Báidez et al., 2007; Jiménez-Díaz et al., 2011). Two pathotypes have been identified among *Vd* isolates in olive, namely, defoliating (D) and non-defoliating (ND), which differ much in virulence and determine the severity of Verticillium wilt. The ND pathotype induces moderately severe branch die back and leaf necrosis, whereas the highly virulent D pathotype induces severe falling of green leaves, necrosis of entire plant canopy sectors, and eventually tree death (Navas-Cortés et al., 2008; Jiménez-Díaz et al., 2011).

The most practical and economically efficient method for the management of Verticillium wilt is the use of resistant cultivars. However, most of the olive cultivars widely grown in Spain are moderately to highly susceptible to D *Vd* (López-Escudero et al., 2010; Jiménez-Díaz et al., 2011; Ostos et al., 2020). Recently, a few wild olive genotypes were identified as highly resistant to D *Vd* that may have a valuable potential as rootstocks for the management of Verticillium wilt (Jiménez-Fernández et al., 2016). Nevertheless, use of a single control measure may not be fully effective for the management of Verticillium wilt in olives, as shown for other wilt diseases (Jiménez-Díaz et al., 2011). Thus, an integrated management strategy is advisable, combining the use of resistant olive cultivars, or of tolerant ones grafted onto resistant rootstocks, with adequate irrigation management and agricultural practices that prevent the spread of inoculum of the pathogen (Jiménez-Díaz et al., 2011; Bubici and Cirulli, 2012; Jiménez-Fernández et al., 2016).

Although plants have evolved their own adaptations to alleviate most biotic and abiotic stresses in nature, they also rely on their microbial partners to survive and defend themselves against microbial invaders and pathogens (Hassani et al., 2018). In nature, healthy plants live in association and interact with a myriad of microorganisms, collectively called the plant microbiome, which is now known to bear important roles in plant health. Endophytes are bacteria and fungi that live within plants where they establish non-pathogenic relationships with their hosts (Azevedo et al., 2000) and promote plant growth directly by phytostimulation and biofertilization, and/or indirectly by inducing stress tolerance and disease suppression (Compant et al., 2016; Hassani et al., 2018). Therefore, a thorough knowledge of the microbial communities residing within the xylem vessels of olive trees may be crucial for understanding their potential influence on the healthy growth of this plant as well as on the resistance shown by specific olive genotypes against D *Vd* or other vascular plant pathogens (Hong and Park, 2016). Different methodological approaches, including culture-dependent procedures complemented with next-generation sequencing (NGS) technologies, have recently made it possible to characterize microbial communities associated with olive xylem tissue (Hardoim et al., 2015; Fausto et al., 2018; Anguita-Maeso et al., 2020; Zicca et al., 2020). However, although it can be considered that most of the olive xylem microbiota should be assembled by microorganisms with neutral or positive effects, their mechanistic role in defense against vascular pathogens has not yet been addressed.

The modification or attenuation of the diversity and composition of xylem microbial communities might result in different responses from the plant host to cope with vascular pathogens. The transmission of microbiota to the progeny in plants vegetatively propagated represents a way to ensure the presence of beneficial symbionts within the plant (Vannier et al., 2018; Liu et al., 2019). However, it is unknown how xylem endophytic microbiota in olive may be transmitted from shoot tips to explants, as well as to mature plants, and how stable would that be during this process. Micropropagation has become an important tool to reproduce selected olive genotypes and guarantees true type and pathogen-free plants (Fabbri et al., 2009). Olive micropropagation, through tissue culture, which is conducted *in vitro* under aseptic conditions, for at least a certain period of time, can potentially induce changes and alter the composition of the xylem microbiome. Thus, by producing olive plants by tissue culture, some beneficial and non-pathogenic endophytes may be excluded, since tissue cultures are initiated from shoots after extensive surface sterilization and then plants are maintained under aseptic conditions. Consequently, this propagation procedure represents an ideal experimental approach to test whether the xylem microbiome has a role on the resistance shown by specific olive genotypes to vascular pathogens.

This present research was conceived to elucidate how plants protect themselves by shaping their xylem microbiome in a resistant wild olive genotype as a first step to assess the complex plant–microbe interactions in the xylem that can contribute to maintain olive health and its resistance response against vascular pathogens. The specific objectives of this work were to determine whether (i) *in vitro* propagation methodology can modify the diversity and composition of the xylem microbiome in olive; (ii) those changes may alter host resistance response to the vascular pathogen *Vd*; and (iii) inoculation and vascular infection of olive by *Vd* may induce changes in the olive xylem microbiome. Determining those effects may be essential to identify microbial communities that are triggered after pathogen infection and could act as antagonists against *Vd*. Furthermore, understanding the tight relationships between xylem-inhabiting microorganisms and vascular pathogens will help to reveal determinant microbial players that may contribute to produce olive plants more resilient to infection by vascular pathogens.

MATERIALS AND METHODS

Olive Plant Material

A wild olive (*O. europaea* var. *sylvestris*) clone "Ac-18" highly resistant to the D pathotype of *Vd* (Jiménez-Fernández et al., 2016) was used in this study. The high resistance of "Ac-18" plants to D *Vd* is characterized by symptomless infection, together with plugging of xylem vessels, no re-isolation of the fungus from stem vascular tissue, and the plant's ability to quantitatively reduce the extent of stem colonization by the pathogen (Jiménez-Fernández et al., 2016). Also, olive cv. "Picual" highly susceptible to D and susceptible to ND *Vd* pathotypes (Calderon et al., 2014) and a wild olive clone "Ac-15" highly susceptible to D *Vd* (Narváez et al., 2019) were used in the pathogenicity experiments as controls of disease reaction.

Three types of "Ac-18" plant materials were used in the study, which were derived from shoots of the same mother adult plant, namely: (i) *in vitro*-standard plants: plants micropropagated using the standard olive methodology of axillary shoot elongation; (ii) *in vitro*-adapted plants: plants micropropagated and subsequently adapted to greenhouse conditions; and (iii) nursery propagated: plants propagated following standard semi-woody stacking procedure at a commercial olive nursery.

For *in vitro*-standard propagation of "Ac-18" and "Ac-15" plants, 1.5- to 2.0-cm-long shoots bearing two nodes were multiplied as in Narváez et al. (2020) using RP medium [DKW macro- and micronutrients and vitamins as modified by Roussos and Pontikis (2002)] supplemented with 2 mg/L zeatin riboside (Vidoy-Mercado et al., 2012). For rooting, 2-cm-long shoots were cultured for 3 days in basal RP liquid medium supplemented with 10 mg/L IBA and subsequently transferred to basal solid RP medium supplemented with 1 g/L activated charcoal. Plants were incubated under a 16-h photoperiod at 40 μmol m^{-2} s^{-1} at 25 $\pm$ 2°C for 8 weeks until ensuring at least three to four roots. Adaptation to *ex vitro* conditions was carried out initially using 35 $\times$ 24 $\times$ 18 cm germination boxes under high levels of sterility. Propagated "Ac-18" rootlets were transplanted to 300-mL pots filled with a sterilized (121°C for 20 min) perlite and vermiculite (1:1) mixture. Plants were sprayed with sterile water, and the cover was sealed with transparent film, and incubated at 25 $\pm$ 2°C in darkness in a growth chamber. After 2 days, a 2-h cycle of indirect fluorescent light of 360 μmol m^{-2} s^{-1} was provided, the duration of which was increased daily until reaching a 12-h light cycle within 1 week. The starting high relative humidity provided by the closed environment made it unnecessary to water the plants for 3 weeks. Afterward, plants were watered (3 mL per pot) weekly with sterile water using a 10-mL syringe and fertilized once per month with Hoagland's nutrient solution (Hoagland and Arnon, 1950). After 8 weeks, plants were transplanted to 500-mL pots filled with a sterile peat:perlite (3:1) mixture. Plants grew for additional 6 months in a growth chamber adjusted to 22 $\pm$ 2°C, 60–80% relative humidity, and a 14-h photoperiod of fluorescent light of 360 μmol m^{-2} s^{-1}. Plants were watered as needed and fertilized weekly with 100 ml of Hoagland's nutrient solution.

In vitro-adapted "Ac-18" plants were produced using the same methodology than for *in vitro*-standard plants, with the exception that plants were grown for additional 12 months in a glass greenhouse at a fluctuating minimum/maximum temperature of 15 $\pm$ 5 and 25 $\pm$ 5°C across the entire growing period and daylight conditions. Plants were watered using tap water as needed and fertilized weekly as described before.

Finally, nursery propagated "Ac-18" and "Picual" plants followed standard semi-woody stacking procedure at a commercial olive nursery (Plantas Continental S.A, Córdoba, Spain). Briefly, semi-hard stem cuttings with two active leaves on the top were dipped in an indole butyric acid solution to stimulate rooting and planted on peat:coconut fiber (1:5) pellets under mist conditions in plastic tunnels (Caballero and Del Río, 2010). Once callus was formed and roots appeared (three to four roots), plants were transplanted to 500-mL pots containing a perlite:coconut fiber:peat (1.5:5:3.5) mixture amended with 4 g L^{-1} of slow release fertilizer (Osmocote® Exact standard 15-9-12 + 2MgO; ICL Specialty Fertilizers, Netherlands). Plants were incubated under natural environmental conditions for 6 months in a plastic greenhouse. During this time, plants received water as needed but no additional fertilizers.

At the time of inoculation with the pathogen *in vitro*-standard, nursery propagated and *in vitro*-adapted "Ac-18" plants were 10, 10, and 18 months old, respectively. Apparently, plants from all types of propagation procedures had a similar degree of bark lignification of the main stem and root development (*data not shown*).

Pathogenicity Experiment

A monoconidial *Vd* isolate (V138I) from defoliated "Coker 310" cotton plants at Córdoba (Spain) and a representative of the D pathotype was used in the experiment. This isolate proved highly virulent on olive in a previous work (Jiménez-Fernández et al., 2016). Inoculum consisted of an infested cornmeal–sand mixture (CMS; sand:cornmeal:deionized water, 9:1:2, w/w) produced as described by Jiménez-Fernández et al. (2016). The infested CMS was homogenized, allowed to desiccate in an incubator adjusted to 33°C for 3 days, and thoroughly

mixed with a pasteurized soil mixture (clay loam:peat, 2:1, v/v) at a rate of approximately 1:20 (w/w) to reach an inoculum density of 5×10^7 CFU g^{-1} soil of *Vd* as determined by dilution-plating on chlortetracycline-amended water agar (CWA; 1 L distilled water, 20 g agar, 30 mg chlortetracycline) (Jiménez-Fernández et al., 2016).

Plants were then transplanted to pots filled with the pasteurized soil mixture. For transplanting inoculation, plants uprooted from the potting substrate were shaken to retain the rhizosphere soil and placed in pots filled with the infested soil mixture. Control plants were treated similarly with sterile distilled water (root-dip inoculation), or grown in non-infested CMS mixed with the pasteurized soil mixture at the same rate as infested CMS (transplanting inoculation). Inoculated and control plants were incubated in the growth chamber adjusted to the above conditions for 3 months.

Plants of "Ac-18" clone, representative of each propagation procedure, and susceptible "Picual" and "Ac-15" plants, were then transplanted to 1500-mL pots filled with the D *Vd*-infested soil mixture. Before transplanting, plants were uprooted from the potting substrate, gently shaken to retain only the rhizosphere soil, and placed in pots filled with the infested soil mixture. Non-inoculated plants were treated similarly and transplanted to the pasteurized soil mixture mixed with non-infested CMS at the same rate as infested CMS. Inoculated and control plants were incubated in a growth chamber adjusted to 22 ± 2°C, 60–80% relative humidity, and a 14-h photoperiod of fluorescent light of 360 μmol m^{-2} s^{-1} for 3 months. During this time, plants were watered as needed with tap water and fertilized weekly as previously described. There were six replicated pots (one plant per pot) for inoculated and non-inoculated plants of each plant genotype, respectively, in a completely randomized design.

Disease reaction was assessed by the incidence (percentage of plants showing disease symptoms) and severity of foliar symptoms. Symptoms were assessed on individual plants on a 0–4 rating scale according to the percentage of affected leaves and twigs at 2- to 3-day intervals throughout the duration of the experiment (Jiménez-Fernández et al., 2016). Upon termination of the experiment, the extent of colonization by *Vd* was determined by isolations of the fungus in CWA (Jiménez-Fernández et al., 2016) from 6-cm-long stem pieces sampled from the main stem at the same time as similar samples were processed for extraction of xylem microbiome (see below). Data of pathogen isolation from the stem were used to calculate the intensity of stem vascular colonization for each individual plant, according to a stem colonization index (SCI) as described before (Jiménez-Fernández et al., 2016).

Additionally, the amount of *Vd* present in "Ac-18" stem samples was determined by using the TaqMan qPCR assay developed by Bilodeau et al. (2012) as described in Gramaje et al. (2013). The same DNA samples used for the xylem microbiome characterization were used for pathogen quantification, with each sample being analyzed in duplicate. All qPCR assays were performed in a LightCycler480 (Roche Diagnostics) apparatus. The cycle threshold (Ct) values for each qPCR reaction were calculated using the default estimation criteria in the manufacturer's software. The quantification limit of the TaqMan qPCR assay was fixed at a Ct of 36 (0.1 pg of *Vd* DNA μL^{-1}) (Gramaje et al., 2013).

DNA Xylem Microbiome Extraction and Sequencing

The xylem microbiome was extracted following the procedure described by Anguita-Maeso et al. (2020). Briefly, a number of three 6-cm-long pieces from the bottom, middle, and upper stem of each "Ac-18" plant were debarked and xylem chips were obtained by scraping the most external layer of the debarked woody pieces with a sterile scalpel. Xylem chips from an "Ac-18" plant were mixed together, and a 0.5-g sample was placed in a Bioreba bag containing 5 mL of sterile phosphate-buffered saline (PBS); the bags were closed with a thermal sealer and the content was macerated with a hand homogenizer (BIOREBA, Reinach, Switzerland). Extracts were stored at −80°C until DNA extraction. All the processes described above took place under sterile conditions within a flow hood chamber (Anguita-Maeso et al., 2020). Aliquots (0.5 mL) of macerated xylem chips in PowerBead tubes (DNeasy PowerSoil Kit, QIAGEN) were homogenized 7 min at 50 pulses s^{-1} with the Tissuelyser LT (QIAGEN) prior to incubation in lysis buffer for 1 h at 60°C for increasing cell lysis, and then processed following the DNeasy PowerSoil Kit manufacturer's protocol.

Extracted DNA was used directly to amplify the V5–V6 rRNA region with the primers 799F (5'-AAC MGGATTAGATACCCKG-3') and 1115R (5'-AGGGTTGCG CTCGTTG-3'). PCR products were purified using Agencourt AMPure XP (Beckman Coulter) following manufacturer instructions. Purified PCR products were quantified using Quant-iT^{TM} PicoGreen^{TM} dsDNA Assay Kit (Thermo Fisher Scientific) and a Tecan Safire microplate reader (Tecan Group, Männedorf, Switzerland). Equimolecular amounts from each individual sample in 10 mM of Tris were combined, and the pooled library was sequenced by the Genomics Unit at "Fundación Parque Científico de Madrid," Madrid, Spain using the Illumina MiSeq platform (Nano-V2; PE 2 × 250 bp). The ZymoBIOMICS microbial standard (Zymo Research Corp., Irvine, CA, United States) and water (no template DNA) were used as internal positive and negative controls, respectively, for library construction and sequencing. Raw sequence data have been deposited in the Sequence Read Archive (SRA) database at the NCBI under BioProject accession number PRJNA679263.

Statistical and Bioinformatics Analyses

Quality control and adapter trimming of demultiplexed raw fastq 16S rRNA sequences obtained from MiSeq output was performed with FastQC and TrimGalore tools. Truncation length in 225 bp of the forward and reverse reads was needed to increase the Phred quality ($Q > 30$) score visualized in MultiQC tool. No trimming length was needed. Quality reads were then analyzed using the Quantitative Insights into Microbial Ecology bioinformatics pipeline, QIIME2 (version 2020.2[1]) (Caporaso et al., 2010; Bolyen et al., 2019) with default

[1]https://qiime2.org/

parameters unless otherwise noted. DADA2 pipeline was used for denoising fastq paired-end sequences along with filtering chimeras. Operational taxonomic units (OTUs) were obtained at 99% similarity and were taxonomically classified using VSEARCH consensus taxonomy classifier (Rognes et al., 2016) against Silva SSU v.138 database. Singletons were discarded for taxonomy and statistical analysis.

Differences in bacterial communities were calculated in QIIME2 using rarefaction curves of alpha-diversity indexes (Richness, Shannon, Evenness, and Simpson) at OTU level. Alpha and beta diversity as well as alpha rarefaction curves were conducted rarefying all samples to the minimum number of reads found. The non-parametric Scheirer–Ray–Hare test ($P < 0.05$) was used to assess the effects of the inoculation treatment, propagation method, and their interaction in alpha diversity indexes, using the rcompanion package (Mangiafico, 2020) in R. Dunn's Kruskal–Wallis multiple comparisons were performed for *post hoc* analysis. The *P*-value was adjusted with the Benjamini–Hochberg method (Benjamini and Hochberg, 1995). Venn diagrams were generated from non-rarefied data using Venn package (Dusa, 2018) in R, and were used to identify shared (core microbiome) or unique taxa according to the inoculation treatment and propagation methods. Linear discriminant analysis effect size (LEfSe) method to identify differentially abundant bacterial taxa associated to inoculation treatments and propagation methods was carried out (Segata et al., 2011).

Additionally, weighted UniFrac distances were estimated at OTU level taking into account the phylogenetic distance among bacterial communities (Lozupone and Knight, 2005). Principal coordinate analysis (PCoA) of weighted UniFrac distance matrix was used to evaluate similarities among the bacterial communities according to the inoculation treatment or propagation procedure. Additionally, the *adonis* function within the vegan package in R (999 permutations) was performed to test the effects ($P < 0.05$) of the inoculation treatment, the plant propagation method, and their interaction.

RESULTS

Pathogenicity Experiment

Typical Verticillium wilt symptoms consisting of early dropping of green leaves, and necrosis and death of some branches, characteristics of infection by D *Vd*, started to develop in "Ac-15" and "Picual" plants, as expected, by 21 and 27 days after pathogen inoculation, respectively (**Supplementary Figure S1**). The mean incubation period was of 21.5 ± 1.7 and 29.5 ± 1.7 days for "Ac-15" and "Picual," respectively. All "Picual" and "Ac-15" plants were dead by 2 months after inoculation. On the contrary, no symptoms developed on nursery and *in vitro*-adapted propagated "Ac-18" plants as expected (Jiménez-Fernández et al., 2016). Surprisingly, plants of "Ac-18" that underwent *in vitro* propagation under aseptic conditions (i.e., *in vitro*-standard) started to develop disease symptoms 29 days after inoculation (mean incubation period of 33.8 ± 1.8 days), reaching a disease incidence of 100% and a final disease severity of 1.95 ± 0.6 on a 0–4 rating scale at the end of the experiment (**Figure 1** and **Supplementary Figure S1**). *Vd* was not re-isolated from any of the stem zones sampled from "Ac-18" nursery and *in vitro*-adapted propagated plants, but was isolated from all "Ac-18" *in vitro*-standard propagated plants, with a mean SCI value of 80.95 ± 6.55%. No symptoms were developed on non-inoculated control plants (**Supplementary Figure S1**).

In parallel with the *Vd* isolations, the pathogen was detected in DNA samples from all *in vitro*-standard propagated plants, with mean Ct values of 28.84 ± 0.18. In contrast, *Vd* was not detected in DNA samples of any of the nursery propagated plants,

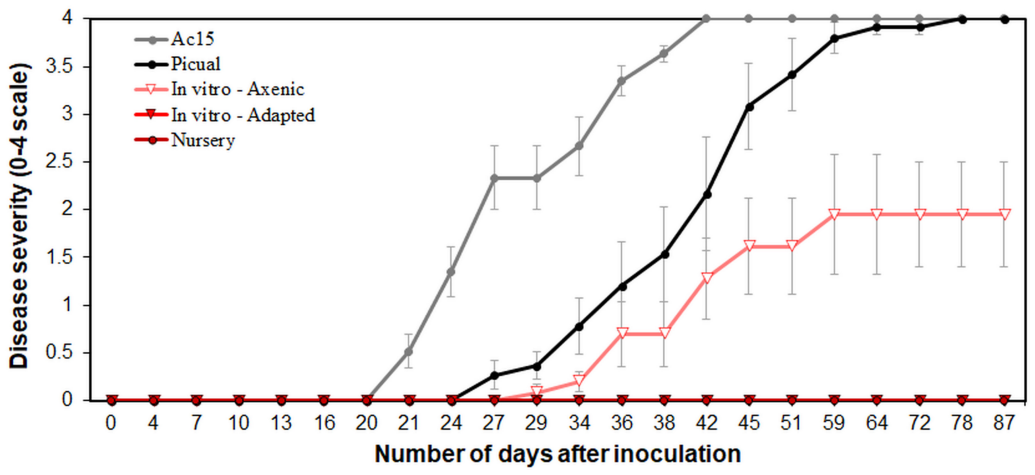

FIGURE 1 | *Verticillium* wilt disease progression in "Ac-18" *in vitro* (standard and adapted) and nursery propagated olive plants inoculated with the defoliating pathotype of *Verticillium dahliae*. "Picual" and "Ac-15" olive genotypes were used as positive controls to determine the inoculation success and development of the disease. Each point represents the mean disease severity (0–4 scale: 0 = healthy, 4 = dead plant) of data and error bars show the standard error from six plants per treatment.

and *Vd* was detected only in three samples from the *in vitro*-adapted propagated plants, which showed Ct values slightly above the detection limit (i.e., 37.0 ± 0.30).

Alpha and Beta Diversity Analysis

Sequencing analysis resulted in a total of 125,685 raw reads. After removal of chimeras, unassigned, or mitochondrial reads, we finished with 19,936 good quality reads assigned to bacteria with a mean length of 333 bp. No chloroplast reads were detected in our samples. A total of 118 OTUs were identified for all treatments, with 18 OTUs being retained for alpha and beta diversity analysis after rarefying all data to the minimum number of reads obtained and singleton removal. High values of Good's coverage (*data not shown*) and rarefaction curves of observed OTUs reaching a plateau (**Supplementary Figure S2**) were obtained for all samples indicating enough sequencing depth.

Rarefaction curves of observed OTUs clearly showed a higher number of OTUs on *Vd*-inoculated plants when compared to that on non-inoculated plants, with lower differences among propagation methods within them (**Supplementary Figure S2**). Similarly, the Scheirer–Ray–Hare test indicated significant differences ($P < 0.05$) for two alpha-diversity indexes (Richness and Shannon) according to the inoculation treatment ($H > 9.94$, $P < 0.002$), with no significant differences ($P \geq 0.05$) among propagation methods ($H < 5.14$, $P > 0.076$) or its interaction with the inoculation treatment ($H < 1.08$, $P > 0.581$) (**Figure 2** and **Supplementary Table S1**). Interestingly, inoculation with *Vd* significantly increased Richness and Shannon alpha diversity indexes in all types of propagation; i.e., inoculated plants presented a higher number of OTUs either analyzed as counts or considering their relative abundances (**Figure 2** and **Supplementary Figure S2**). On the other hand, for the Evenness index, we found a significant effect ($H = 10.79$, $P = 0.004$) for the propagation method, but not for the inoculation treatment ($H = 2.51$, $P = 0.113$) or their interaction ($H = 0.87$, $P = 0.648$), due to a less even distribution of OTUs for the nursery propagated plants (**Figure 2** and **Supplementary Table S1**).

Principal coordinate analysis of weighted UniFrac distances differentiated xylem bacterial communities mainly according to the inoculation treatment. Thus, with a few exceptions, there was a clear trend to group the bacterial communities along Axis 1, which explained 51.3% of the variation, mainly by the presence of *Vd* in the soil mixture, and then along Axis 2, which explained 17.2% of the variation, also according to the inoculation procedure and in a lower extent according to the propagation method, with *in vitro*-adapted and nursery propagated plants being more similar between them (**Figure 3**). Thus, ADONIS analysis supported these results and indicated a main significant effect of the propagation method ($R^2 = 0.268$, $P < 0.001$) followed by the inoculation treatment ($R^2 = 0.112$, $P = 0.004$), with no interaction effect ($R^2 = 0.062$, $P = 0.175$) (**Supplementary Table S2**).

Composition and Abundance of Olive Xylem Bacterial Communities

A total of 10 phyla, 15 classes, 45 orders, 67 families, 103 genera, and 118 OTUs were identified considering all treatments, with four phyla, five classes, 11 orders, 11 families, 10 genera, and five OTUs being shared among all of them (**Supplementary Figure S3**). When analyzing the samples according to the inoculation procedure, a higher number of OTUs were found in "Ac-18" plants inoculated with *Vd* (94), while 61 were found on non-inoculated plants. This trend of detecting higher number of taxa on *Vd*-inoculated plants was observed at all the taxonomic levels. The lowest number of OTUs from all taxonomy ranks was found in non-inoculated plants propagated under *in vitro* conditions. Additionally, *in vitro*-adapted plants shared a higher number of OTUs with nursery instead of with *in vitro*-standard propagated plants (**Supplementary Figure S3**).

At the genus level, a total of 18 genera formed the core microbiome of *Vd*-inoculated plants when considering all propagation methods jointly, whereas 10 genera were shared within non-inoculated plants. Those same 10 genera were shared between *Vd*-inoculated and non-inoculated treatments (*Acidibacter*, *Anoxybacillus, Bradyrhizobium*, *Corynebacterium*, *Cutibacterium*, *Methylobacterium-Methylorubrum*, *Paenibacillus*, *Pseudomonas*, *Sphingomonas*, and *Staphylococcus*), while eight genera (*Acinetobacter*, *Caulobacter*, *Comamonadaceae*, *Dermacoccus*, *Flavisolibacter*, *Massilia*, *Paracoccus*, and *Sericytochromatia*) were detected exclusively in *Vd*-inoculated plants. In addition, a higher number of genera were found in *Vd*-inoculated plants (82) compared to those in non-inoculated plants (53) (**Figure 4A**).

When analyzing only non-inoculated plants, 25 and 27 genera were identified in *in vitro*-standard and *in vitro*-adapted propagated plants, respectively, while 29 genera were identified in nursery propagated plants. Additionally, a high number of unique genera were found in each propagation method. A total of 12 unique genera were identified for *in vitro*-standard propagated plants (*Adhaeribacter*, *Bacillus*, *Bifidobacterium*, *Burkholderia-Caballeronia-Paraburkholderia*, *Cupriavidus*, *Curtobacterium*, *Eremococcus*, *Escherichia-Shigella*, *Frigoribacterium*, *Kocuria*, *Sphingopyxis*, and *Variovorax*), 12 for nursery propagated plants (*Bergeyella*, *Brevibacillus*, *Caulobacteraceae*, *Deinococcus*, *Hymenobacter*, *Nevskia*, *Phreatobacter*, *Propionibacteriaceae*, *Pseudokineococcus*, *Psychrobacter*, *Sediminibacterium*, and *Spirosoma*), and 11 for *in vitro*-adapted plants (*Aeromonas*, *Dialister*, *Haemophilus*, *Lawsonella*, *Leptotrichia*, *Micrococcus*, *Paracoccus*, *Peptoniphilus*, *Prevotella*, *Saccharimonadales*, and *Sphingobium*) (**Figure 4B**). On the other hand, when analyzing each propagation methodology for *Vd*-inoculated plants, 45, 42, and 42 genera were identified in *in vitro*-standard, *in vitro*-adapted, and nursery propagated plants, respectively. Unique genera differed according to the propagation approach. Thus, a total of 19 unique genera were found in *in vitro*-standard propagated plants (*CAG-352*, *Allorhizobium-Neorhizobium-Pararhizobium-Rhizobium*, *Azospirillum*, *Blautia*, *Eremococcus*, *Faecalibacterium*, *Frigoribacterium*, *Leuconostoc*, *Modestobacter*, *Mucilaginibacter*, *Niastella*, *Pediococcus*, *Raoultella*, *Solirubrobacter*, *Stenotrophomonas, unc.-Chitinophagales, unc.-Oxalobacteraceae, unc.-Solimonadaceae*, and *Variovorax*), 16 genera were exclusive from *in vitro*-adapted propagated plants (*Aeromonas, Atopobium, Brevibacillus, Candidatus_Nitrotoga, Carnobacterium, Cloacibacterium, Dialister, Fimbriimonadaceae, Gardnerella,*

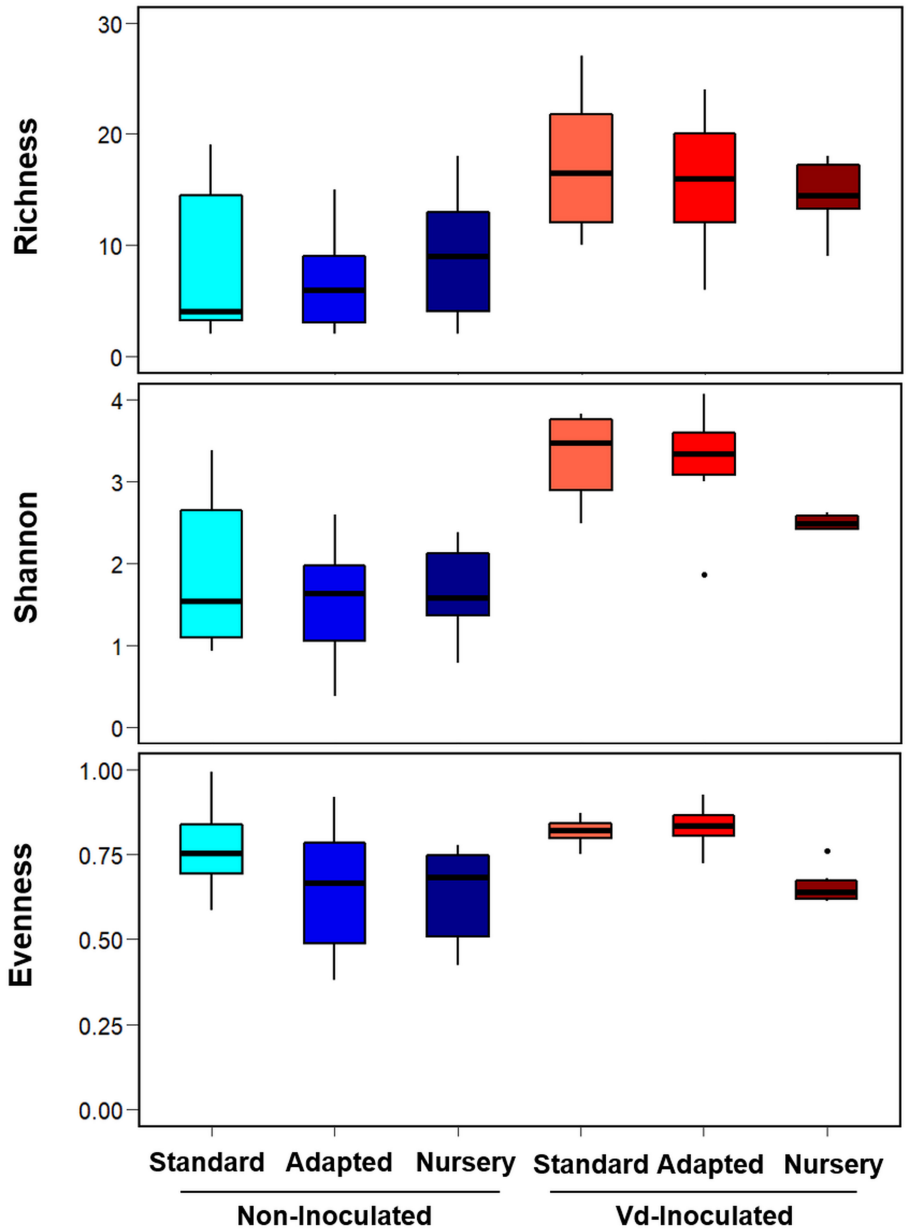

FIGURE 2 | Boxplots of Richness, Shannon, and Evenness alpha diversity indices at OTU taxonomic level in olive xylem from *Verticillium dahliae* (*Vd*)-inoculated and non-inoculated "Ac-18" plants following *in vitro* (standard and adapted) and nursery propagation methods. Boxes represent the interquartile range, while the horizontal line inside the box defines the median and whiskers represent the lowest and highest values of six values for each treatment combination. For all three indexes and propagation methods, values on *Vd*-inoculated plants were significantly higher compared to that on non-inoculated treatments according to the Scheirer–Ray–Hare test at $P < 0.05$.

Gemella, Haemophilus, hgcI_clade, Hyphomicrobium, Pelistega, Streptococcus, and *Vibrio*), and 18 from nursery propagated plants (*Actinomyces, Aeromicrobium, Agromyces, Aureimonas, Bosea, Burkholderia-Caballeronia-Paraburkholderia, Caulobacteraceae, Empedobacter, Enhydrobacter, Exiguobacterium, Fusobacterium, Klenkia, Lachnospiraceae, Marmoricola Neisseria, Pseudokineococcus, Ralstonia,* and *Rothia*) (**Figure 4B**).

At the phylum level, *Actinobacteriota* presented the highest relative abundance considering all experimental treatments together (43.62%), followed by *Proteobacteria* (38.72%), *Firmicutes* (15.24%), and *Bacteroidota* (1.63%). However, these relative abundances varied within each treatment tested. *Actinobacteria* was more abundant in non-inoculated plants, with a proportion of 46.41%, decreasing to 40.82% for *Vd*-inoculated plants. For non-inoculated plants, this phylum was the most abundant for *in vitro*-adapted propagated plants (70.98%), followed by nursery (52.21%) and *in vitro*-standard (16.04%) propagation methods, and it showed the same

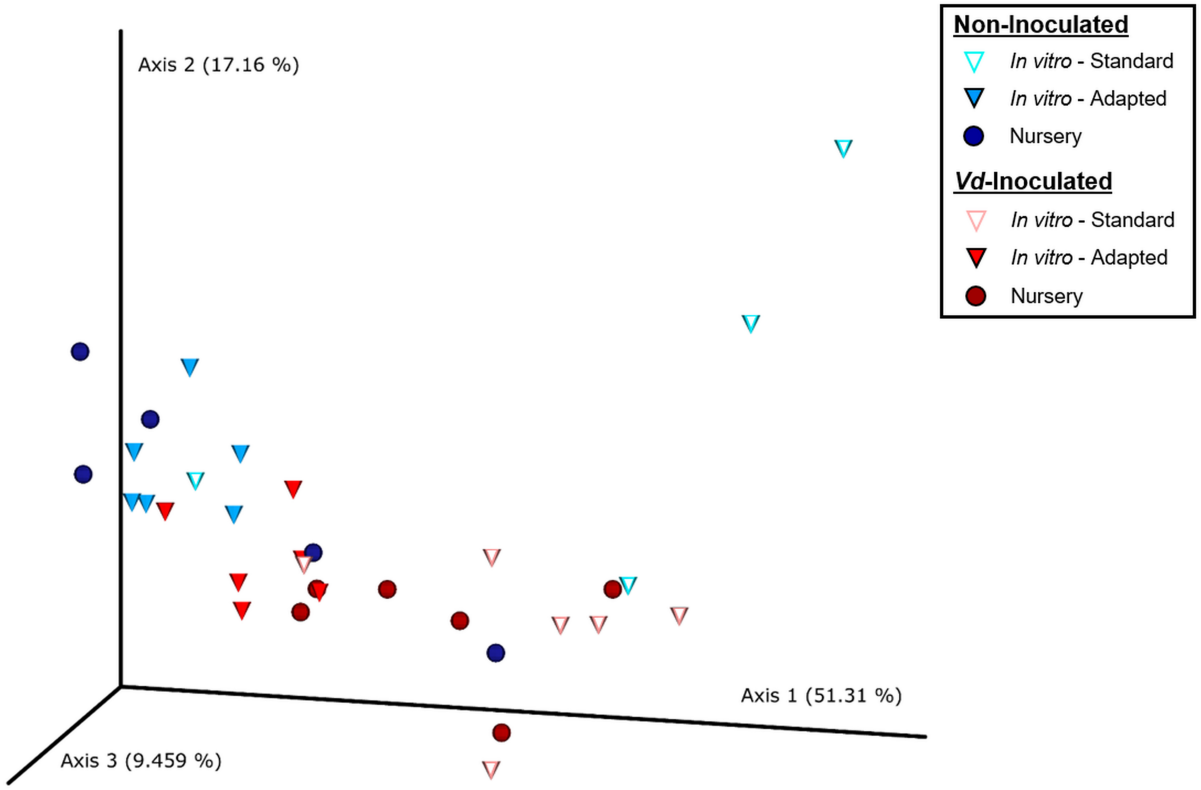

FIGURE 3 | Principal coordinates plot of weighted UniFrac distances of bacterial communities at OTU taxonomic level in olive xylem from *Verticillium dahliae* (*Vd*)-inoculated and non-inoculated "Ac-18" plants following *in vitro* (standard and adapted) and nursery propagation methods. Points are colored by plant inoculation treatment and shaped by propagation methods.

trend in *Vd*-inoculated plants, reaching 58.56, 39.16, and 24.73%, for these same propagation methods, respectively. *Proteobacteria* were present at a similar percentage in non-inoculated (40.77%) and *Vd*-inoculated (36.66%) plants, but varied within propagation methods. Thus, it was the most abundant phylum for the *in vitro*-standard plants for both non-inoculated (75.86%) and *Vd*-inoculated plants (49.94%), finding its lowest relative abundance for *in vitro*-adapted propagated plants with 18.79 and 22.12%, for non- and *Vd*-inoculated plants, respectively. Finally, *Firmicutes* showed a different response to both inoculation treatment and propagation methods compared to the two previous phyla. First, it showed the highest global relative abundance in *Vd*-inoculated plants with 18.92%, which decreased to 11.56% in non-inoculated plants. Second, while in non-inoculated plants, the highest relative abundance was estimated in nursery propagated plants (17.53%), it represented ca. 8.57% in both *in vitro* propagation methods, but similar abundance values were reached in *Vd*-inoculated plants (17.61–20.34%), irrespective of the plant propagation method (**Figure 5A**).

In line with these results, LEfSe was used to identify the key phylotypes that could be differentially associated to the different experimental treatments. When comparing the three propagation methods within each inoculation treatment, *Proteobacteria-Gammaproteobacteria* was the most significant Phylum-Class for non-inoculated *in vitro*-standard propagated plants. For plants growing in *Vd*-infested soils, more diversity was observed among propagation methods, with *Proteobacteria-Gammaproteobacteria* and *Bacteroidota-Bacteroidia*, and *Actinobacteriota-Actinobacteria* being the most prevalent Phylum-Class in *in vitro*-standard and in *in vitro*-adapted propagated plants, respectively, while the Phylum-Class *Proteobacteria-Alphaproteobacteria* was the most prominent in nursery propagated plants. On the other hand, when comparing the effect of the inoculation with the pathogen within each propagation method, *Firmicutes-Bacilli* was a significant Phylum-Class for *Vd*-inoculated plants, in both *in vitro*-standard and *in vitro*-adapted propagated plants, whereas *Proteobacteria-Gammaproteobacteria* and *Bacteroidota-Bacteroidia* were also the prevalent Phyla-Class for *in vitro*-standard and nursery propagated plants, respectively. For non-inoculated plants, only a Phylum-Class (*Actinobacteriota-Actinobacteria*) appeared as the most prominent and only for *in vitro*-adapted plants (**Figure 6**).

The most abundant genera identified among all treatments were *Cutibacterium* (36.85%), *Pseudomonas* (20.93%), *Anoxybacillus* (6.28%), *Staphylococcus* (4.95%), *Methylobacterium-Methylorubrum* (3.91%), *Bradyrhizobium* (3.54%), *Corynebacterium* (2.53%), *Acinetobacter* (1.77%), *Pseudokineococcus* (1.59%), and *Flavisolibacter* (1.07%) (**Figure 5B**). *Cutibacterium* was the genus with the highest

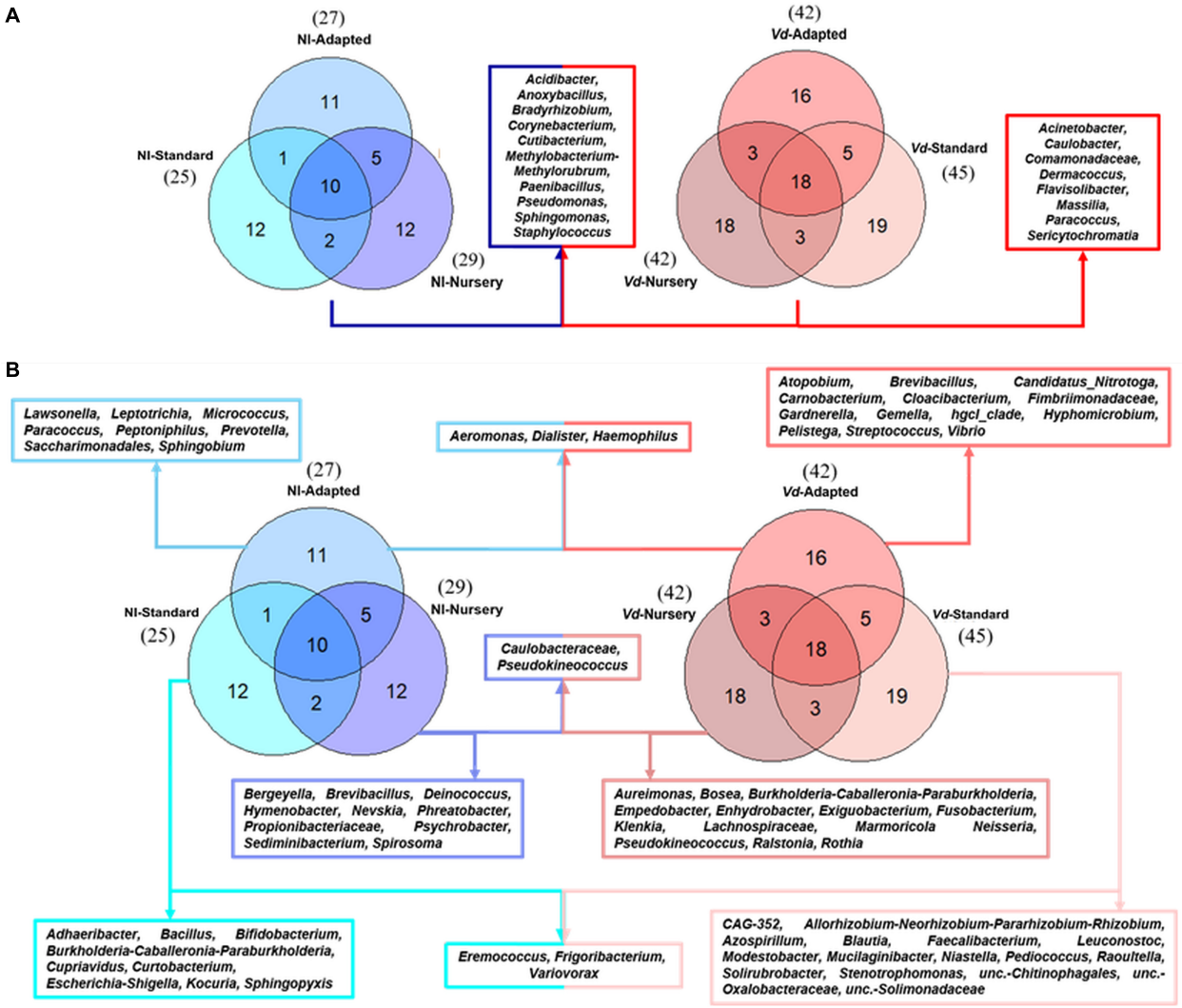

FIGURE 4 | Prevalence Venn diagram showing the unique and shared bacterial genera in olive xylem from the core microbiome **(A)** or by each propagation approach **(B)** obtained from *Verticillium dahliae* (*Vd*)-inoculated and non-inoculated (NI) "Ac-18" plants following *in vitro* (standard and adapted) and nursery propagation methods.

relative abundance, reaching maximum frequencies in *in vitro*-adapted plants [both in non-inoculated (67.81%) and *Vd*-inoculated plants (53.04%)]. Other predominant genera varied in proportion depending on the treatment combination. *Pseudomonas* (54.25%) was the most representative genera in non-inoculated plants, propagated by the *in vitro*-standard method, whereas similar abundances were found for the remaining treatments (14.66%), with the exception of *in vitro*-adapted non-inoculated plants (5.77%). A noticeable lower relative abundance of *Anoxybacillus* was found in non-inoculated plants from both *in vitro* propagation methods (standard and adapted with 2.42 and 1.06%, respectively) compared with the nursery propagation (7.32%). Also, the small proportion of *Staphylococcus* found in *in vitro*-standard propagated and non-inoculated plants (0.52%) compared with the rest of the treatments (5.84%) was remarkable. Also, *Acinetobacter* showed a high proportion (3.34%) in *in vitro* propagated plants, but was much less relatively abundant in nursery propagated plants (0.04%) (**Figure 5B**).

In line with these results, when comparing the propagation methods within each inoculation treatment, LEfSe displayed *Cutibacterium* as the only genus with significant changes in relative abundance for *in vitro*-adapted propagated plants and for both inoculation treatments (**Figures 6A,B**), whereas for *Vd*-inoculated plants, *Pseudomonas* appeared as the most dominant genera for *in vitro*-standard propagated plants (**Figure 6B**). On the other hand, when comparing the effect of pathogen inoculation within each propagation method, *Anoxybacillus* appeared as a genus with significant higher relative abundances in *Vd*-inoculated plants for all propagation methods (**Figures 6C–E**). Furthermore, other distinct genera appeared as the most dominant within each propagation

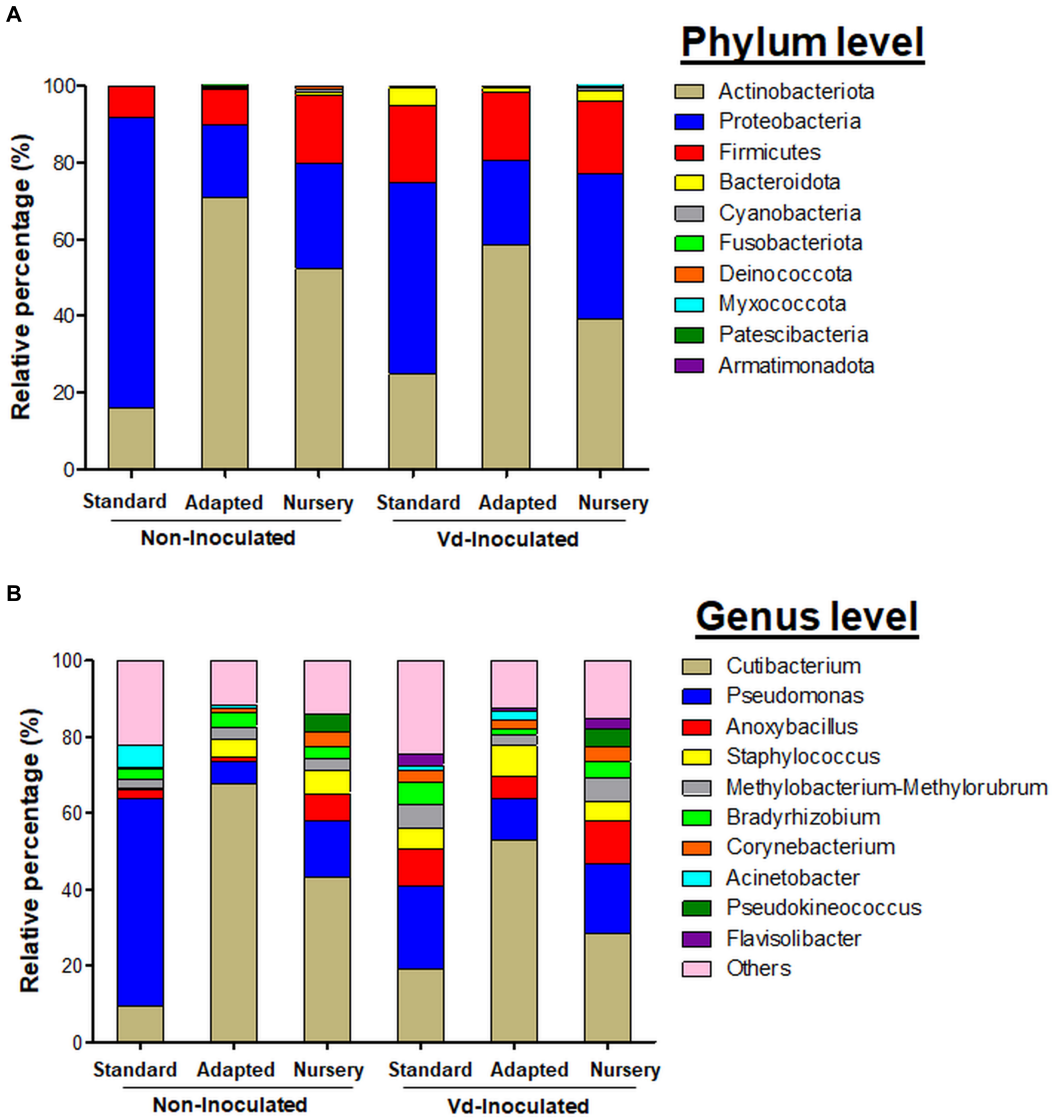

FIGURE 5 | Bar plots showing the relative bacterial abundance taxa at phylum **(A)** and genera **(B)** level present in olive xylem from *Verticillium dahliae* (*Vd*)-inoculated and non-inoculated "Ac-18" plants following *in vitro* (standard and adapted) and nursery propagation methods.

method when *Vd* was infesting the soil including *Flavisolibacter, Methylobacterium-Methylorubrum,* and *Pseudomonas* in nursery propagated plants (**Figure 6E**); *Corynebacterium, Staphylococcus*, and *Methylobacterium-Methylorubrum* in *in vitro*-standard propagated plants (**Figure 6C**); and *Gardnerella* in *in vitro*-adapted plants (**Figure 6D**).

DISCUSSION

The use of host resistance is the most practical, long-term, and economically efficient disease control measure for vascular diseases in olive, and it is at the core of integrated disease strategies that must be practiced for the efficient management of these diseases (Jiménez-Díaz et al., 2011; Schneider et al., 2020). For Verticillium wilt, the use of wild olive rootstocks highly resistant to D *Vd* can provide an improved mean for its management, especially for grafting susceptible olive cultivars that are agronomically adapted, commercially desirable, or used in protected designation of origin extra virgin olive oils (Jiménez-Díaz et al., 2011; Trapero et al., 2012, 2013; Jiménez-Fernández et al., 2016; Ostos et al., 2020). In addition to that, use of endophytic plant-associated microorganisms with a specific beneficial interaction with the host plant could help to improve olive health and productivity providing a potential perspective for sustainable plant protection (Ryan et al., 2008; Berg, 2009; Berg et al., 2014; Müller et al., 2015).

In olive, several defense mechanisms, including both biochemical responses and plant structural characteristics, have been proposed as factors contributing to the resistance

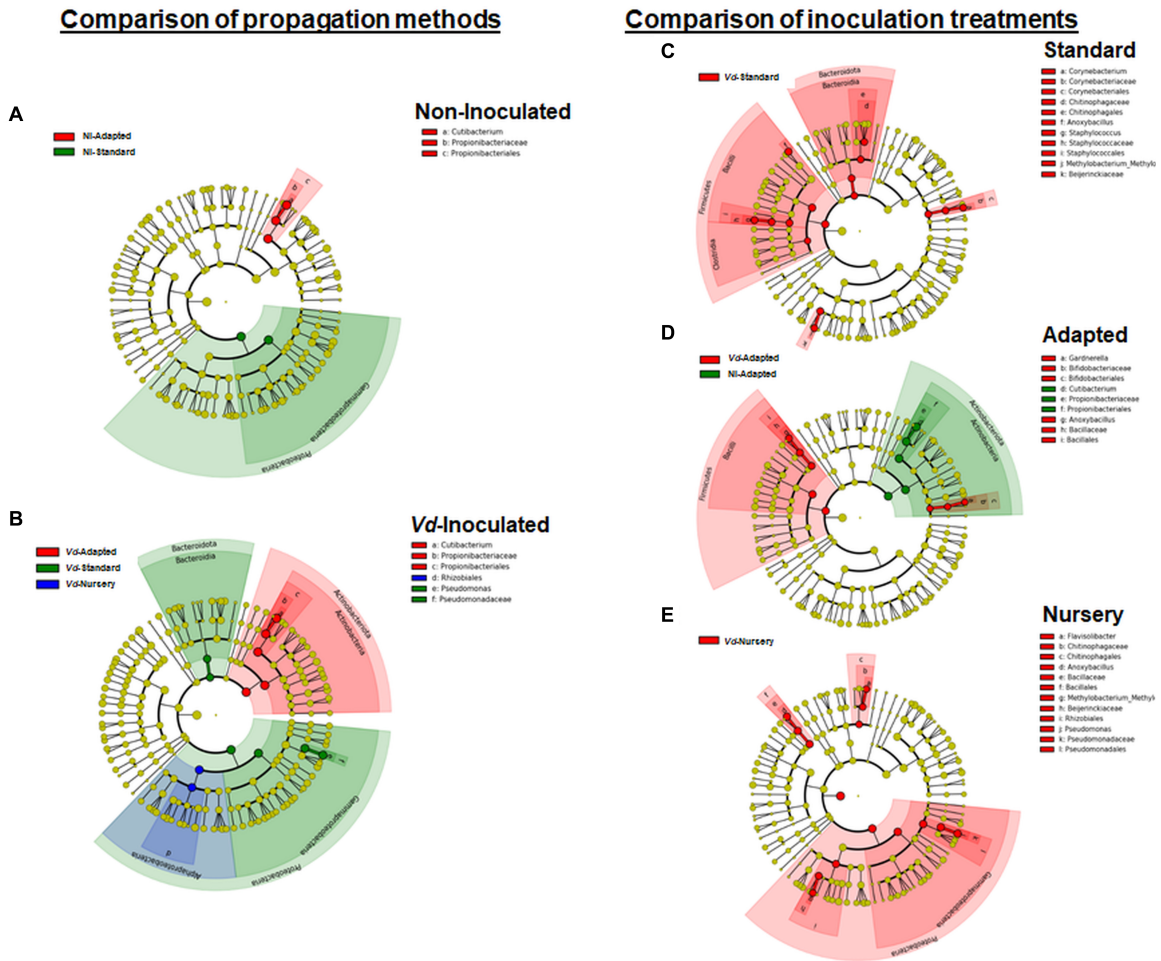

FIGURE 6 | Cladogram representation from LEfSe analysis showing the taxonomic ranks from the innermost phylum ring to the outermost genera ring. Each point is a member within each taxonomic rank. Significant taxa ($P < 0.05$) appearing as dominant for each treatment when comparing each propagation method by inoculation treatment **(A,B)**, or each inoculation treatment by propagation method **(C–E)** are shown in different colors (red, green, or blue) associated to the legend of each individual cladogram.

shown by different genotypes against its vascular pathogens *Vd* and/or Xylella *fastidiosa*. Those mechanisms should operate within the xylem tissues contributing to reduce systemic colonization by the pathogen and may include buildup of vessel occlusions by gums, gels or tyloses, phenolics and lignin content or their accumulation, induction of pathogenesis-related proteins, antioxidant-related enzymes, and ionome content (Báidez et al., 2007; Markakis et al., 2010; Jiménez-Fernández et al., 2016; de la O Leyva-Pérez et al., 2017; Gharbi et al., 2017a,b; Luvisi et al., 2017; Sabella et al., 2017, 2019; D'Attoma et al., 2019).

Although some cultivated and wild olive clones, including the "Ac-18" used in this study, have been described as resistant to D *Vd* based on asymptomatic pathogen colonization; for most of them, the pathogen could be detected by molecular methods or re-isolated from roots and stem tissues (Colella et al., 2008; Bubici and Cirulli, 2012; Gramaje et al., 2013; Jiménez-Fernández et al., 2016), indicating the plant's ability to reduce the extent of stem colonization or other pathogenesis mechanisms that result in the absence of visible disease symptoms. However, the role that xylem microbial communities may play in that resistant response to *Vd* has been overlooked and remains unexplored to date. In this study, we tested the hypothesis that xylem microbiome may have a functional role on plant resistance. With that purpose, we explored whether or not *in vitro* propagation of "Ac-18" plants can alter the diversity and composition of xylem-inhabiting bacteria, and to which extent this could result in a modification of the high resistance response of that wild olive clone to the highly virulent D pathotype of *Vd*. Surprisingly, plants that underwent *in vitro* propagation under aseptic conditions lost the high resistance phenotype characteristic of the "Ac-18" clone. Actually, those plants developed wilting symptoms similar to those reported for other olive cultivars with a moderate-susceptible reaction to D *Vd* such as "Frantoio," "Oblonga," "Koroneiki," "Empeltre," or "Leccino" in similar inoculation experiments using olive plants of age similar to that in our

study (López-Escudero et al., 2004; Martos-Moreno et al., 2006; Trapero et al., 2013).

Some authors found that disease severity in olive cultivars susceptible to *Vd* decreases with host age (López-Escudero et al., 2010; Trapero et al., 2013). In this present study, the loss of resistance shown by "Ac-18" *in vitro*-standard propagated plants cannot be associated to a more juvenile stage since those plants were of the same age as that of nursery-propagated plants and showed a similar growth (i.e., similar bark lignification and root development). However, "Ac-18" plants showed distinct xylem microbiome profiles according to the propagation procedure. The most significant change associated to *in vitro*-standard propagation was a decrease in the total number of OTUs detected, and a significantly higher number of Gammaproteobacteria (mainly *Pseudomonas*) and a lower number of Actinobacteria (mainly *Cutibacterium*). In parallel, beta-diversity indexes of xylem microbiome differed among propagation procedures, with plants that were initially propagated under *in vitro*-standard conditions and then challenged to less restricted aseptic environmental conditions (i.e., *in vitro*-adapted plants) showing a xylem microbiome more similar with the commercial nursery propagated plants. The olive explants from tissue culture may contain a genotype-specific core xylem microbiome that is transmitted from shoot tips of last generation (Liu et al., 2019). In our study, the explants grew under aseptic conditions and roots that differentiated from *in vitro*-standard plants did not get into contact with outside microbes, at least until the challenge with the pathogen. Thus, most of the differences found with *in vitro*-adapted or nursery propagated plants may be attributed to bacteria that were present at very low level, below the detection limit, at the beginning of micropropagation procedure and could not be detected by NGS. Alternatively, those bacteria might have been acquired by roots after recruitment when plants grew on a non-sterile substrate under less aseptic environmental conditions as proposed for other woody crops, including olive (Antoniou et al., 2017; Fausto et al., 2018; Deyett and Rolshausen, 2019).

Plant core microorganisms are considered to be consistently established in plants not being influenced by differences in space, time, or plant organs (Vorholt et al., 2017). In our study, 10 keystone bacterial genera could be considered the core microbiome being transmitted from generation to generation in olive, since they were detected in all samples regardless of plant propagation procedure (*in vitro* vs nursery) or inoculation with the pathogen, with *Cutibacterium, Pseudomonas, Anoxybacillus, Staphylococcus, Methylobacterium-Methylorubrum*, and *Bradyrhizobium* being the most abundant, in that order. These bacterial taxa have also been identified in olive xylem in other works in which olive trees of different ages, belonging to different cultivars or growing under different environments, were evaluated (Müller et al., 2015; Fausto et al., 2018; Sofo et al., 2019; Anguita-Maeso et al., 2020; Giampetruzzi et al., 2020), which strengthens the hypothesis that those genera may represent keystone olive xylem bacteria. More interestingly, some of these genera have already been reported or used as plant growth-promoting bacteria (Otieno et al., 2015; Subramanian et al., 2015) or proposed as biological control agents against *V. dahliae* (Berg et al., 2006; Aranda et al., 2011; Gómez-Lama Cabanás et al., 2018; Tao et al., 2020). Interestingly, the ratio *Cutibacterium/Pseudomonas* seemed to be an important factor associated to the plant propagation procedure. Thus, *Pseudomonas* spp. and *Cutibacterium* were present at high and low relative abundance, respectively, for *in vitro*-standard propagated plants, that lost resistance to D *Vd*, whereas for *in vitro*-adapted and nursery propagated plants, the opposite trend occurred. However, whereas little is known about the role of the genus *Cutibacterium* as a component of plant microbiomes, the beneficial functions of *Pseudomonas* spp. in plants have been widely reported for several crops, including olive (Mercado-Blanco et al., 2004; Weller, 2007; Loper et al., 2012).

The role of microorganisms in the biocontrol of Verticillium wilt diseases has been reported mostly on non-woody plant species such as cotton, potato, strawberry, or tomato (Azad et al., 1985; Nallanchakravarthula et al., 2014; Cao et al., 2016; Wei et al., 2019; Snelders et al., 2020) with few studies focused on woody hosts including olive (Mercado-Blanco et al., 2004; Aranda et al., 2011; Gómez-Lama Cabanás et al., 2018). However, the characterization of microbial communities inhabiting xylem vessels colonized by *Vd* has not been studied to date, despite some work done on other tree species or other vascular pathogens (Martín et al., 2015; Pérez-Martínez et al., 2018; Giampetruzzi et al., 2020; Vergine et al., 2020). To the best of our knowledge, the present study is the first to address this knowledge gap, by determining changes in xylem bacterial communities of a resistant olive clone after challenge inoculation with D *Vd*. Our results indicated that a significantly higher diversity and number of OTUs occur in *Vd*-inoculated plants regardless of the plant propagation method and success of stem vascular infection by the pathogen. Additionally, several genera appeared as unique in *Vd*-inoculated plants and *Anoxybacillus* represented keystone bacterial taxa that significantly increased its frequency after challenge inoculation with *Vd* in all treatments.

The significantly higher xylem microbial diversity in *Vd*-inoculated plants is in line with results from other studies involving vascular pathogens on woody crops. For instance, Deyett and Rolshausen (2019) found higher diversity in *X. fastidiosa*-infected vines as compared to healthier ones. Several of the unique or keystone bacterial genera (e.g., *Acinetobacter, Comamonas, Caulobacter, Massilia,* and *Methylobacterium*) detected in our study in the xylem of *Vd*-inoculated plants were also found in the xylem of other plant species such as banana, citrus, grapevine, and olive, so that those bacteria may be biomarkers of plant infection by vascular-plant pathogens (Araújo et al., 2002; Deyett and Rolshausen, 2019; Liu et al., 2019). The role of these bacterial genera in *Vd*-infected olive plants remains unknown, although several studies suggested that antifungal activity, induction of plant resistance, production of diverse cellulose-, hemicellulose-, lignin-, and starch-degrading enzymes might be involved in pathogen suppression and reduction of disease symptoms (Liu et al., 2007; Rajendran et al., 2009; Ardanov et al., 2012; Goh et al., 2013; Azevedo et al., 2016; AlMatar et al., 2017).

The general increase in alpha diversity and abundance of specific bacterial taxa observed in our study after challenging with D *Vd* may be explained by several hypotheses, including (i) a passive entry or direct recruitment of new bacterial species from the plant rhizosphere or soil, taking advantage of injuries

caused during root infection and colonization by the pathogen (Ardanov et al., 2012; Liu et al., 2019); (ii) the secretion of specific molecules by the pathogen (such as effector proteins) with antimicrobial activity that modify host microbiome to facilitate host colonization (Snelders et al., 2020); and/or (iii) the pathogen provokes a series of host physiological responses that trigger multiplication and increase in abundance of a specific microbiome to cope with the pathogen infection in order to mitigate its effect (Ardanov et al., 2012; Hassani et al., 2018; Carrión et al., 2019). These hypotheses emphasize the need for better understanding of the changes occurring in xylem microbial communities in response to vascular infection by pathogens, in order to determine specifically activated disease-suppressive and/or plant-protecting microbiome-mediated activities in olive.

Our study provides new insights for the characterization of changes occurring in the xylem microbial communities of a wild olive genotype following inoculation with the vascular plant pathogen *Vd.* Also, it provides a quantitative and qualitative assessment of the effect of specific propagation methods where the attenuation and reduction of olive xylem microbiome is a unique approach described to date. We are aware of limitations in our study, in part because only some propagation methods and a single host genotype were evaluated. However, this present research is relevant for future studies on the olive xylem microbiome that may lead to identification of xylem-inhabiting bacteria potentially involved in host resistance and plant defense by acting as biocontrol agents against xylem-inhabiting pathogens. Deciphering the core olive xylem microbiome and their correlation with the host plant and its pathogens is a first critical step for exploiting the microbiome in order to enhance olive growth and health.

DATA AVAILABILITY STATEMENT

Raw sequence data have been deposited in the Sequence Read Archive (SRA) database at the NCBI under BioProject accession number PRJNA679263.

AUTHOR CONTRIBUTIONS

MA-M and BL conceived the research, performed statistical and bioinformatics analyses, interpreted the results, and wrote the manuscript. MA-M, JT-C, CO-G, DR-R, and EP-R prepared materials and equipment and performed the experiments. JN-C and RJ-D contributed to reviewing the manuscript and interpreted the results. All authors viewed the draft of the manuscript.

FUNDING

This study was funded by project AGL2016-75606-R (Programa Estatal de I+D Orientado a los Retos de la Sociedad from the Spanish Government, the Spanish State Research Agency, and FEDER-EU). MA-M is a recipient of a research fellowship BES-2017-082361 from the Spanish Ministry of Economy and Competitiveness.

SUPPLEMENTARY MATERIAL

The Supplementary Material for this article can be found online at: https://www.frontiersin.org/articles/10.3389/fpls.2021.632689/full#supplementary-material

REFERENCES

AlMatar, M., Eldeeb, M., Makky, E. A., Köksal, F., Var, I., and Kayar, B. (2017). Are there any other compounds isolated from *Dermacoccus* spp at all? *Curr. Microbiol.* 74, 132–144. doi: 10.1007/s00284-016-1152-3

Anguita-Maeso, M., Olivares-García, C., Haro, C., Imperial, J., Navas-Cortés, J. A., and Landa, B. B. (2020). Culture-dependent and culture-independent characterization of the olive xylem microbiota: effect of sap extraction methods. *Front. Plant Sci.* 10:1708. doi: 10.3389/fpls.2019.01708

Antoniou, A., Tsolakidou, M.-D., Stringlis, I. A., and Pantelides, I. S. (2017). Rhizosphere microbiome recruited from a suppressive compost improves plant fitness and increases protection against vascular wilt pathogens of tomato. *Front. Plant Sci.* 8:2022. doi: 10.3389/fpls.2017.02022

Aranda, S., Montes-Borrego, M., Jiménez-Díaz, R. M., and Landa, B. B. (2011). Microbial communities associated with the root system of wild olives (*Olea europaea* L. subsp. europaea var. sylvestris) are good reservoirs of bacteria with antagonistic potential against *Verticillium dahliae*. *Plant Soil* 343, 329–345. doi: 10.1007/s11104-011-0721-2

Araújo, W. L., Marcon, J., Maccheroni, W. J., Van Elsas, J. D., Van Vuurde, J. W. L., and Azevedo, J. L. (2002). Diversity of endophytic bacterial populations and their interaction with *Xylella fastidiosa* in citrus plants. *Appl. Environ. Microbiol.* 68, 4906–4914. doi: 10.1128/aem.68.10.4906-4914.2002

Ardanov, P., Sessitsch, A., Häggman, H., Kozyrovska, N., and Pirttilä, A. M. (2012). Methylobacterium-induced endophyte community changes correspond with protection of plants against pathogen attack. *PLoS One* 7:e46802. doi: 10.1371/journal.pone.0046802

Azad, H. R., Davis, J. R., Schnathorst, W. C., and Kado, C. I. (1985). Relationships between rhizoplane and rhizosphere bacteria and verticillium wilt resistance in potato. *Arch. Microbiol.* 140, 347–351. doi: 10.1007/BF00446976

Azevedo, J., Araújo, W. L., and Lacava, P. T. (2016). The diversity of citrus endophytic bacteria and their interactions with *Xylella fastidiosa* and host plants. *Genet. Mol. Biol.* 39, 476–491. doi: 10.1590/1678-4685-GMB-2016-0056

Azevedo, J. L., Maccheroni Junior, W., Pereira, J. O., and Araújo, W. L. (2000). Endophytic microorganisms: a review on insect control and recent advances on tropical plants. *Electron. J. Biotechnol.* 3, 40–65. doi: 10.2225/vol3-issue1-fulltext-4

Báidez, A. G., Gómez, P., Del Río, J. A., and Ortuño, A. (2007). Dysfunctionality of the xylem in *Olea europaea* L. Plants associated with the infection process by *Verticillium dahliae* Kleb. Role of phenolic compounds in plant defense mechanism. *J. Agric. Food Chem.* 55, 3373–3377. doi: 10.1021/jf063166d

Benjamini, Y., and Hochberg, Y. (1995). Controlling the false discovery rate: a practical and powerful approach to multiple testing. *J. R. Stat. Soc. Ser. B* 57, 289–300. doi: 10.1111/j.2517-6161.1995.tb02031.x

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., 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., Opelt, K., Zachow, C., Lottmann, J., Götz, M., Costa, R., et al. (2006). The rhizosphere effect on bacteria antagonistic towards the pathogenic fungus *Verticillium* differs depending on plant species and site. *FEMS Microbiol. Ecol.* 56, 250–261. doi: 10.1111/j.1574-6941.2005.00025.x

Bilodeau, G. J., Koike, S. T., Uribe, P., and Martin, F. N. (2012). Development of an assay for rapid detection and quantification of *Verticillium dahliae* in soil. *Phytopathology* 102, 331–343. doi: 10.1094/PHYTO-05-11-0130

Bolyen, E., Rideout, J. R., Dillon, M. R., Bokulich, N. A., Abnet, C., Al-Ghalith, G. A., et al. (2019). Reproducible, interactive, scalable and extensible microbiome data science using QIIME 2. *Nat. Biotechnol.* 37, 852–857. doi: 10.1038/s41587-019-0209-9

Bubici, G., and Cirulli, M. (2012). Control of *Verticillium* wilt of olive by resistant rootstocks. *Plant Soil* 352, 363–376. doi: 10.1007/s11104-011-1002-9

Caballero, J., and Del Río, C. (2010). "Propagation methods. Olive grow," in *Pendle Hill*, eds D. Barranco, R. Fernández-Escobar, and L. Rallo (Australia: RIRDC), 83–112. doi: 10.1353/syl.2003.0004

Calderon, R., Lucena, C., Trapero-Casas, J. L., Zarco-Tejada, P. J., and Navas-Cortes, J. A. (2014). Soil temperature determines the reaction of olive cultivars to *Verticillium dahliae* pathotypes. *PLoS One* 9:e110664. doi: 10.1371/journal.pone.0110664

Cao, P., Liu, C., Sun, P., Fu, X., Wang, S., Wu, F., et al. (2016). An endophytic *Streptomyces* sp. strain DHV3-2 from diseased root as a potential biocontrol agent against *Verticillium dahliae* and growth elicitor in tomato (*Solanum lycopersicum*). *Antonie Van Leeuwenhoek* 109, 1573–1582. doi: 10.1007/s10482-016-0758-6

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. doi: 10.1038/nmeth.f.303

Carrión, V. J., Perez-Jaramillo, J., Cordovez, V., Tracanna, V., de Hollander, M., Ruiz-Buck, D., et al. (2019). Pathogen-induced activation of disease-suppressive functions in the endophytic root microbiome. *Science* 366, 606–612. doi: 10.1126/science.aaw9285

Colella, C., Miacola, C., Amenduni, M., D'Amico, M., Bubici, G., and Cirulli, M. (2008). Sources of verticillium wilt resistance in wild olive germplasm from the Mediterranean region. *Plant Pathol.* 57, 533–539. doi: 10.1111/j.1365-3059.2007.01785.x

Compant, S., Saikkonen, K., Mitter, B., Campisano, A., and Mercado-Blanco, J. (2016). Editorial special issue: soil, plants and endophytes. *Plant Soil* 405, 1–11. doi: 10.1007/s11104-016-2927-9

D'Attoma, G., Morelli, M., Saldarelli, P., Saponari, M., Giampetruzzi, A., Boscia, D., et al. (2019). Ionomic differences between susceptible and resistant olive cultivars infected by *Xylella fastidiosa* in the outbreak area of Salento. *Italy. Pathogens* 8:272. doi: 10.3390/pathogens8040272

de la O Leyva-Pérez, M., Jiménez-Ruiz, J., Gómez-Lama Cabanás, C., Valverde-Corredor, A., Barroso, J. B., Luque, F., et al. (2017). Tolerance of olive (*Olea europaea*) cv Frantoio to *Verticillium dahliae* relies on both basal and pathogen-induced differential transcriptomic responses. *New Phytol.* 217, 671–686. doi: 10.1111/nph.14833

Deyett, E., and Rolshausen, P. E. (2019). Temporal dynamics of the sap microbiome of grapevine under high Pierce's disease pressure. *Front. Plant Sci.* 10:1246. doi: 10.3389/fpls.2019.01246

Dusa, A. (2018). *Package 'venn'. Version 1.9*.

Fabbri, A., Lambardi, M., and Ozden-Tokatli, Y. (2009). *Olive Breeding BT - Breeding Plantation Tree Crops: Tropical Species*. New York, NY: Springer, 423–465. doi: 10.1007/978-0-387-71201-7_12

Fausto, C., Mininni, A. N., Sofo, A., Crecchio, C., Scagliola, M., Dichio, B., et al. (2018). Olive orchard microbiome: characterisation of bacterial communities in soil-plant compartments and their comparison between sustainable and conventional soil management systems. *Plant Ecol. Divers.* 11, 597–610. doi: 10.1080/17550874.2019.1596172

Gharbi, Y., Barkallah, M., Bouazizi, E., Gdoura, R., and Triki, M. A. (2017a). 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

Gharbi, Y., Barkallah, M., Bouazizi, E., Hibar, K., Gdoura, R., and Triki, M. A. (2017b). Lignification, phenols accumulation, induction of PR proteins and antioxidant-related enzymes are key factors in the resistance of *Olea europaea* to *Verticillium* wilt of olive. *Acta Physiol. Plant.* 39:43. doi: 10.1007/s11738-016-2343-z

Giampetruzzi, A., Baptista, P., Morelli, M., Cameirão, C., Lino Neto, T., Costa, D., et al. (2020). Differences in the endophytic microbiome of olive cultivars infected by *Xylella fastidiosa* across seasons. *Pathogens* 9:732. doi: 10.3390/pathogens9090723

Goh, K. M., Kahar, U. M., Chai, Y. Y., Chong, C. S., Chai, K. P., Ranjani, V., et al. (2013). Recent discoveries and applications of *Anoxybacillus*. *Appl. Microbiol. Biotechnol.* 97, 1475–1488. doi: 10.1007/s00253-012-4663-2

Gómez-Lama Cabanás, C., Legarda, G., Ruano-Rosa, D., Pizarro-Tobías, P., Valverde-Corredor, A., Niqui, J. L., et al. (2018). Indigenous *Pseudomonas* spp. strains from the olive (*Olea europaea* L.) rhizosphere as effective biocontrol agents against *Verticillium dahliae*: from the host roots to the bacterial genomes. *Front. Microbiol.* 9:277. doi: 10.3389/fmicb.2018.00277

Gramaje, D., Pérez-Serrano, V., Montes-Borrego, M., Navas-Cortés, J. A., Jiménez-Díaz, R. M., and Landa, B. B. (2013). A comparison of Real-Time PCR protocols for the quantitative monitoring of asymptomatic olive infections by *Verticillium dahliae* pathotypes. *Phytopathology* 103, 1058–1068. doi: 10.1094/PHYTO-11-12-0312-R

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

Hassani, M. A., Durán, P., and Hacquard, S. (2018). Microbial interactions within the plant holobiont. *Microbiome* 6:58. doi: 10.1186/s40168-018-0445-0

Hoagland, D. R., and Arnon, D. I. (1950). The water-culture method for growing plants without soil. *Circ. Calif. Agric. Exp. Stn.* 347:32.

Hong, C. E., and Park, J. M. (2016). Endophytic bacteria as biocontrol agents against plant pathogens: current state-of-the-art. *Plant Biotechnol. Rep.* 10, 353–357. doi: 10.1007/s11816-016-0423-6

Jiménez-Díaz, R. M., Cirulli, M., Bubici, G., del Mar Jiménez-Gasco, M., Antoniou, P. P., and Tjamos, E. C. (2011). Verticillium Wilt, a major threat to olive production: current status and future prospects for its management. *Plant Dis.* 96, 304–329. doi: 10.1094/PDIS-06-11-0496

Jiménez-Fernández, D., Trapero-Casas, J. L., Landa, B. B., Navas-Cortés, J. A., Bubici, G., Cirulli, M., et al. (2016). Characterization of resistance against the olive-defoliating *Verticillium dahliae* pathotype in selected clones of wild olive. *Plant Pathol.* 65, 1279–1291. doi: 10.1111/ppa.12516

Landa, B. B., Pérez, A. G., Luaces, P., Montes-Borrego, M., Navas-Cortés, J. A., and Sanz, C. (2019). Insights into the effect of *Verticillium dahliae* defoliating-pathotype infection on the content of phenolic and volatile compounds related to the sensory properties of virgin olive oil. *Front. Plant Sci.* 10:232. doi: 10.3389/fpls.2019.00232

Liu, C. H., Chen, X., Liu, T. T., Lian, B., Gu, Y., Caer, V., et al. (2007). Study of the antifungal activity of *Acinetobacter baumannii* LCH001 in vitro and identification of its antifungal components. *Appl. Microbiol. Biotechnol.* 76, 459–466. doi: 10.1007/s00253-007-1010-0

Liu, Y., Zhu, A., Tan, H., Cao, L., and Zhang, R. (2019). Engineering banana endosphere microbiome to improve Fusarium wilt resistance in banana. *Microbiome* 7:74. doi: 10.1186/s40168-019-0690-x

Loper, J. E., Hassan, K. A., Mavrodi, D. V., Davis, E. W. II, Lim, C. K., Shaffer, B. T., et al. (2012). Comparative genomics of plant-associated *Pseudomonas* spp.: insights into diversity and inheritance of traits involved in multitrophic interactions. *PLoS Genet.* 8:e1002784. doi: 10.1371/journal.pgen.1002784

López-Escudero, F. J., del Río, C., Caballero, J. M., and Blanco-López, M. A. (2004). Evaluation of olive cultivars for resistance to *Verticillium dahliae*. *Eur. J. Plant Pathol.* 110, 79–85. doi: 10.1023/B:EJPP.0000010150.08098.2d

López-Escudero, F. J., Mercado-Blanco, J., Roca, J. M., Valverde-Corredor, A., and Blanco-López, M. (2010). *Verticillium* wilt of olive in the Guadalquivir Valley (southern Spain): relations with some agronomical factors and spread of *Verticillium dahliae*. *Phytopathol. Mediterr.* 49, 370–380. doi: 10.14601/Phytopathol_Mediterr-3154

Lozupone, C., and Knight, R. (2005). UniFrac: a new phylogenetic method for comparing microbial communities. *Appl. Environ. Microbiol.* 71, 8228–8235. doi: 10.1128/AEM.71.12.8228-8235.2005

Luvisi, A., Aprile, A., Sabella, E., Vergine, M., Nicolì, F., Nutricati, E., et al. (2017). *Xylella fastidiosa* subsp. pauca (CoDiRO strain) infection in four olive (*Olea europaea* L.) cultivars: profile of phenolic compounds in leaves and progression of leaf scorch symptoms. *Phytopathol. Mediterr.* 56, 259–273. doi: 10.14601/PHYTOPATHOL_MEDITERR-20578

Mangiafico, S. (2020). *rcompanion: Functions to Support Extension Education Program Evaluation. R Package Version 2.3.25*.

Markakis, E. A., Tjamos, S. E., Antoniou, P. P., Roussos, P. A., Paplomatas, E. J., and Tjamos, E. C. (2010). Phenolic responses of resistant and susceptible olive cultivars induced by defoliating and nondefoliating *Verticillium*

dahliae pathotypes. *Plant Dis.* 94, 1156–1162. doi: 10.1094/PDIS-94-9-1156

Martín, J. A., Macaya-Sanz, D., Witzell, J., Blumenstein, K., and Gil, L. (2015). Strong in vitro antagonism by elm xylem endophytes is not accompanied by temporally stable in planta protection against a vascular pathogen under field conditions. *Eur. J. Plant Pathol.* 142, 185–196. doi: 10.1007/s10658-015-0602-2

Martos-Moreno, C., Lòpez-Escudero, F. J., and Blanco-Lòpez, M. A. (2006). Resistance of olive cultivars to the defoliating pathotype of *Verticillium dahliae*. *HortScience* 41, 1313–1316. doi: 10.21273/HORTSCI.41.5.1313

Mercado-Blanco, J., Rodrìguez-Jurado, D., Hervás, A., and Jiménez-Dìaz, R. M. (2004). Suppression of *Verticillium* wilt in olive planting stocks by root-associated fluorescent *Pseudomonas* spp. *Biol. Control* 30, 474–486. doi: 10.1016/j.biocontrol.2004.02.002

Müller, H., Berg, C., Landa, B. B., Auerbach, A., Moissl-Eichinger, C., and Berg, G. (2015). Plant genotype-specific archaeal and bacterial endophytes but similar Bacillus antagonists colonize Mediterranean olive trees. *Front. Microbiol.* 6:138. doi: 10.3389/fmicb.2015.00138

Nallanchakravarthula, S., Mahmood, S., Alström, S., and Finlay, R. D. (2014). Influence of soil type, cultivar and *Verticillium dahliae* on the structure of the root and rhizosphere soil fungal microbiome of strawberry. *PLoS One* 9:e111455. doi: 10.1371/journal.pone.0111455

Narváez, I., Martín, C., Jiménez-Díaz, R. M., Mercado, J. A., and Pliego-Alfaro, F. (2019). Plant regeneration via somatic embryogenesis in mature wild olive genotypes resistant to the defoliating pathotype of *Verticillium dahliae*. *Front. Plant Sci.* 10:1471. doi: 10.3389/fpls.2019.01471

Narváez, I., Pliego Prieto, C., Palomo-Ríos, E., Fresta, L., Jiménez-Díaz, R. M., Trapero-Casas, J. L., et al. (2020). Heterologous expression of the AtNPR1 gene in olive and its effects on fungal tolerance. *Front. Plant Sci.* 11:308. doi: 10.3389/fpls.2020.00308

Navas-Cortés, J. A., Landa, B. B., Mercado-Blanco, J., Trapero-Casas, J. L., Rodríguez-Jurado, D., and Jiménez-Díaz, R. M. (2008). Spatiotemporal analysis of spread of infections by *Verticillium dahliae* pathotypes within a high tree density olive orchard in southern Spain. *Phytopathology* 98, 167–180. doi: 10.1094/PHYTO-98-2-0167

Ostos, E., Garcia-Lopez, M. T., Porras, R., Lopez-Escudero, F. J., Trapero-Casas, A., Michailides, T. J., et al. (2020). Effect of cultivar resistance and soil management on spatial–temporal development of *Verticillium* wilt of olive: a long-term study. *Front. Plant Sci.* 11:1595. doi: 10.3389/fpls.2020.584496

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

Pérez-Martínez, J., Ploetz, R. C., and Konkol, J. L. (2018). Significant in vitro antagonism of the laurel wilt pathogen by endophytic fungi from the xylem of avocado does not predict their ability to control the disease. *Plant Pathol.* 67, 1768–1776. doi: 10.1111/ppa.12878

Rajendran, P., Sundaram, S. P., and Kumutha, K. (2009). In vitro biocontrol activity of *Methylobacterium Extorquens* against fungal pathogens. *Int. J. Plant Prot.* 2, 59–62. doi: 10.13140/2.1.3086.0163

Rognes, T., Flouri, T., Nichols, B., Quince, C., and Mahe, F. (2016). VSEARCH: a versatile open source tool for metagenomics. *PeerJ* 4:e2584. doi: 10.7717/peerj.2584

Roussos, P. A., and Pontikis, C. A. (2002). In vitro propagation of olive (*Olea europaea* L.) cv. *Koroneiki*. *Plant Growth Regul.* 37, 295–304. doi: 10.1023/A:1020824330589

Ryan, R. P., Germaine, K., Franks, A., Ryan, D. J., and Dowling, D. N. (2008). Bacterial endophytes: recent developments and applications. *FEMS Microbiol. Lett.* 278, 1–9. doi: 10.1111/j.1574-6968.2007.00918.x

Sabella, E., Aprile, A., Genga, A., Siciliano, T., Nutricati, E., Nicolì, F., et al. (2019). Xylem cavitation susceptibility and refilling mechanisms in olive trees infected by *Xylella fastidiosa*. *Sci. Rep.* 9:9602. doi: 10.1038/s41598-019-46092-0

Sabella, E., Luvisi, A., Aprile, A., Negro, C., Vergine, M., Nicolì, F., et al. (2017). *Xylella fastidiosa* induces differential expression of lignification related-genes and lignin accumulation in tolerant olive trees cv. Leccino. *J. Plant Physiol.* 220, 60–68. doi: 10.1016/j.jplph.2017.10.007

Schneider, K., van der Werf, W., Cendoya, M., Mourits, M., Navas-Cortés, J. A., Vicent, A., et al. (2020). Impact of *Xylella fastidiosa* subspecies Pauca in European olives. *Proc. Natl. Acad. Sci. U.S.A.* 117, 9250–9259. doi: 10.1073/pnas.1912206117

Segata, N., Izard, J., Waldron, L., Gevers, D., Miropolsky, L., Garrett, W. S., et al. (2011). Metagenomic biomarker discovery and explanation. *Genome Biol.* 12, R60–R60. doi: 10.1186/gb-2011-12-6-r60

Snelders, N. C., Rovenich, H., Petti, G. C., Rocafort, M., van den Berg, G. C. M., Vorholt, J. A., et al. (2020). Microbiome manipulation by a soil-borne fungal plant pathogen using effector proteins. *Nat. Plants* 6, 1365–1374. doi: 10.1038/s41477-020-00799-5

Sofo, A., Mininni, A. N., Fausto, C., Scagliola, M., Crecchio, C., Xiloyannis, C., et al. (2019). Evaluation of the possible persistence of potential human pathogenic bacteria in olive orchards irrigated with treated urban wastewater. *Sci. Total Environ.* 658, 763–767. doi: 10.1016/j.scitotenv.2018.12.264

Subramanian, P., Kim, K., Krishnamoorthy, R., Sundaram, S., and Sa, T. (2015). Endophytic bacteria improve nodule function and plant nitrogen in soybean on co-inoculation with *Bradyrhizobium japonicum* MN110. *Plant Growth Regul.* 76, 327–332. doi: 10.1007/s10725-014-9993-x

Tao, X., Zhang, H., Gao, M., Li, M., Zhao, T., and Guan, X. (2020). *Pseudomonas* species isolated via high-throughput screening significantly protect cotton plants against *Verticillium* wilt. *AMB Express* 10:193. doi: 10.1186/s13568-020-01132-1

Trapero, C., Díez, C. M., Rallo, L., Barranco, D., and López-Escudero, F. J. (2013). Effective inoculation methods to screen for resistance to *Verticillium* wilt in olive. *Sci. Hortic.* 162, 252–259. doi: 10.1016/j.scienta.2013.08.036

Trapero, C., Serrano, N., Arquero, O., Del Río, C., Trapero, A., and López-Escudero, F. J. (2012). Field resistance to *Verticillium* wilt in selected olive cultivars grown in two naturally infested soils. *Plant Dis.* 97, 668–674. doi: 10.1094/PDIS-07-12-0654-RE

Vannier, N., Mony, C., Bittebiere, A.-K., Michon-Coudouel, S., Biget, M., and Vandenkoornhuyse, P. (2018). A microorganisms' journey between plant generations. *Microbiome* 6:79. doi: 10.1186/s40168-018-0459-7

Vergine, M., Meyer, J. B., Cardinale, M., Sabella, E., Hartmann, M., Cherubini, P., et al. (2020). The *Xylella fastidiosa*-resistant olive cultivar "Leccino" has stable endophytic microbiota during the olive quick decline syndrome (OQDS). *Pathogens* 9:35. doi: 10.3390/pathogens9010035

Vidoy-Mercado, I., Imbroda-Solano, I., Barceló-Muñoz, A., and Pliego-Alfaro, F. (2012). Differential in vitro behaviour of the Spanish olive (*Olea europaea* L.) cultivars 'Arbequina' and 'Picual.'. *Acta Hortic.* 949, 27–30. doi: 10.17660/ActaHortic.2012.949.1

Vorholt, J. A., Vogel, C., Carlström, C. I., and Müller, D. B. (2017). Establishing causality: opportunities of synthetic communities for plant microbiome research. *Cell Host Microbe* 22, 142–155. doi: 10.1016/j.chom.2017.07.004

Wei, F., Zhao, L., Xu, X., Feng, H., Shi, Y., Deakin, G., et al. (2019). Cultivar-dependent variation of the cotton rhizosphere and endosphere microbiome under field conditions. *Front. Plant Sci.* 10:1659. doi: 10.3389/fpls.2019.01659

Weller, D. M. (2007). *Pseudomonas* biocontrol agents of soilborne pathogens: looking back over 30 years. *Phytopathology* 97, 250–256. doi: 10.1094/PHYTO-97-2-0250

Zicca, S., De Bellis, P., Masiello, M., Saponari, M., Saldarelli, P., Boscia, D., et al. (2020). Antagonistic activity of olive endophytic bacteria and of *Bacillus* spp. strains against *Xylella fastidiosa*. *Microbiol. Res.* 236:126467. doi: 10.1016/j.micres.2020.126467

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 Microbiology

ORIGINAL RESEARCH
published: 15 March 2021
doi: 10.3389/fmicb.2021.579920

Antagonistic Activity of *Trichoderma* spp. Against *Fusarium oxysporum* in Rhizosphere of *Radix pseudostellariae* Triggers the Expression of Host Defense Genes and Improves Its Growth Under Long-Term Monoculture System

Jun Chen[1,2,3], Liuting Zhou[1,2], Israr Ud Din[4], Yasir Arafat[1,3,5], Qian Li[1,2,3], Juanying Wang[1,2], Tingting Wu[1,2,6], Linkun Wu[1,2,3], Hongmiao Wu[1,2,3], Xianjin Qin[2,3], Ganga Raj Pokhrel[7], Sheng Lin[1,2] and Wenxiong Lin[1,2,3]**

[1] College of Life Sciences, Fujian Agriculture and Forestry University, Fuzhou, China, [2] Fujian Provincial Key Laboratory of Agroecological Processing and Safety Monitoring, Fujian Agriculture and Forestry University, Fuzhou, China, [3] Key Laboratory of Crop Genetic Breeding and Comprehensive Utilization, Ministry of Education, Fujian Agriculture and Forestry University, Fuzhou, China, [4] Institute of Biotechnology and Genetic Engineering, The University of Agriculture, Peshawar, Pakistan, [5] Department of Wildlife and Ecology, Faculty of Life Sciences, University of Okara, Okara, Pakistan, [6] Fujian Provincial Academy of Environmental Science, Fujian Provincial Technology Center of Emission Storage and Management, Fujian, China, [7] Department of Chemistry, Tribhuvan University, Kirtipur, Nepal

Under consecutive monoculture, the abundance of pathogenic fungi, such as *Fusarium oxysporum* in the rhizosphere of *Radix pseudostellariae*, negatively affects the yield and quality of the plant. Therefore, it is pertinent to explore the role of antagonistic fungi for the management of fungal pathogens such as *F. oxysporum.* Our PCR-denatured gradient gel electrophoresis (DGGE) results revealed that the diversity of *Trichoderma* spp. was significantly declined due to extended monoculture. Similarly, quantitative PCR analysis showed a decline in *Trichoderma* spp., whereas a significant increase was observed in *F. oxysporum*. Furthermore, seven *Trichoderma* isolates from the *R. pseudostellariae* rhizosphere were identified and evaluated *in vitro* for their potentiality to antagonize *F. oxysporum*. The highest and lowest percentage of inhibition (PI) observed among these isolates were 47.91 and 16.67%, respectively. In *in vivo* assays, the *R. pseudostellariae* treated with four *Trichoderma* isolates, having PI > 30%, was used to evaluate the biocontrol efficiency against *F. oxysporum* in which *T. harzianum* ZC51 enhanced the growth of the plant without displaying any disease symptoms. Furthermore, the expression of eight defense-related genes of *R. pseudostellariae* in response to a combination of *F. oxysporum* and *T. harzianum* ZC51 treatment was checked, and most of these defense genes were found to be upregulated. In conclusion, this study reveals that the extended monoculture of *R. pseudostellariae* could alter the *Trichoderma* communities in the plant rhizosphere leading to relatively low level of

OPEN ACCESS

Edited by:
Paulo José Pereira Lima Teixeira, University of São Paulo, Brazil

Reviewed by:
Rosa Hermosa, University of Salamanca, Spain
Alberto Pascale, Utrecht University, Netherlands

***Correspondence:**
Wenxiong Lin
lwx@fafu.edu.cn
Sheng Lin
linsh@fafu.edu.cn

Specialty section:
This article was submitted to Microbe and Virus Interactions with Plants, a section of the journal Frontiers in Microbiology

Received: *03 July 2020*
Accepted: *11 February 2021*
Published: *15 March 2021*

Citation:
Chen J, Zhou L, Din IU, Arafat Y, Li Q, Wang J, Wu T, Wu L, Wu H, Qin X, Pokhrel GR, Lin S and Lin W (2021) Antagonistic Activity of Trichoderma spp. Against Fusarium oxysporum in Rhizosphere of Radix pseudostellariae Triggers the Expression of Host Defense Genes and Improves Its Growth Under Long-Term Monoculture System. Front. Microbiol. 12:579920. doi: 10.3389/fmicb.2021.579920

antagonistic microorganisms. However, *T. harzianum* ZC51 could inhibit the pathogenic *F. oxysporum* and induce the expression of *R. pseudostellariae* defense genes. Hence, *T. harzianum* ZC51 improves the plant resistance and reduces the growth inhibitory effect of consecutive monoculture problem.

Keywords: defense genes, *Trichoderma*, PCR-DGGE, monoculture, *Radix pseudostellariae*

INTRODUCTION

Due to the allelopathy and the dysbiosis of microorganisms, continuous planting of many Chinese medicinal herbs in the same land results in a significant decrease in yield and quality, which is known as continuous monoculture problem or soil sickness (Zhang and Lin, 2009; Zhao et al., 2015; Wu et al., 2016). *Radix pseudostellariae* is a perennial herb of the *Caryophllaceae* family, and its tuberous roots are used for medicinal purposes, which has very high economic value (Zhao et al., 2015). However, successive cultivation of *R. pseudostellariae* on the same piece of land leads to a decline in both the quality and yield owing to poor plant performance and insufficient biotic stress resistance (Lin et al., 2015). In consecutive monocultures, previous studies have reported the imbalance in the rhizosphere microbial community of *R. pseudostellariae*, especially the abundance of the pathogenic fungi (*Fusarium oxysporum*) increased significantly under consecutive monoculture (Zhao et al., 2014; Wu et al., 2016a,b,c; Chen et al., 2017). Moreover, most of researches in continuous monoculture problem of *R. pseudostellariae* and their potential biological microorganisms are focused on prokaryotes (i.e., *Pseudomonas* spp. and *Burkholderia* spp.) (Wu et al., 2016a; Chen et al., 2017). Therefore, to develop a reliable system of biological control against plant pathogens, we need to explore the antagonizing role of potentially important eukaryotic microorganisms like fungi as well.

The importance of the beneficial microbes in improving nutrient availability and promoting plant growth, antagonizing soil-borne pathogens, and priming the plant's immune system is well established and abundantly used in biocontrol strategies (Cotxarrera et al., 2002; Howell, 2006; Lorito et al., 2010; Mendes et al., 2011; Matarese et al., 2012; Walters et al., 2013). *Trichoderma* spp. is a fungal genus in the family *Hypocreaceae*, which is found in the soil, rotting wood, plants, and the ocean. Many species are characterized as opportunistic avirulent, symbiotic and can be used as biological control agents against important plant pathogenic fungi (Harman et al., 2004). For example, *T. harzianum* (SQR-T307) and *T. asperellum* (T-34) are effective biological control agents against *F. oxysporum* (Corrales et al., 2010; Yang et al., 2011). *T. asperellum* isolates could significantly reduce the incidence of tomato wilt when used to suppress *Fusarium* wilt of tomato (Cotxarrera et al., 2002). *T. gamsii* 6085 was used in a competitive test against *F. subtilis* and *F. graminearum*, which confirmed that *T. gamsii* has the ability to antagonize the pathogens of rice (Matarese et al., 2012).

These root-associated mutualistic microbes, besides impacting on plant nutrition and growth, can further boost plant defenses, rendering the entire plant more resistant to pathogens (Romera et al., 2019). To cope with biotic stresses incited by biological agents, like insects and pathogens, plants develop responses, and some of these responses systemically spread far from the infected tissue into the whole plant. These responses include the systemic acquired resistance (SAR) and the induced systemic resistance (ISR) (Shoresh et al., 2010; Pieterse et al., 2014). SAR is induced by insects and pathogens, while ISR is mediated by beneficial microbes present in the rhizosphere, like bacteria and fungi (Mukherjee et al., 2013). Studies have shown that many species of *Trichoderma* could colonize on the root surface that interacts with the first cell layer of the root bark and the epidermis. This symbiotic relationship can effectively protect the plant from pathogens (Mukherjee et al., 2018; Galletti et al., 2020). When *Trichoderma* interacts with plants, it induces the expression of genes involved in the defense responses of plants (Brotman et al., 2013; Mayo et al., 2016; Manganiello et al., 2018; De Palma et al., 2019; Pimentel et al., 2020) and promotes plant growth and root development (Hermosa et al., 2012). However, the role of root-associated mutualistic plant symbiont, *Trichoderma* spp., in activation of *R. pseudostellariae* immunity by triggering the expression of defense-related genes is never explored.

The objectives of this present study are as follows: (1) to analyze the changes of *Trichoderma* communities in rhizosphere soil under *R. pseudostellariae* monoculture using denatured gradient gel electrophoresis (DGGE) combined with quantitative PCR (qPCR) technique and (2) further, to study the effect of different *Trichoderma* strains on the growth and defense response of *R. pseudostellariae* against the *F. oxysporum* and also to assess the expression level of defense-related genes in *R. pseudostellariae* treated with the selected *Trichoderma* isolate.

MATERIALS AND METHODS

Site Overview and Experimental Design

The study was conducted at Ningde City, Fujian Province (27°26′ N, 120°04′ E). This station has a subtropical monsoon climate with an annual mean air temperature of 18.4°C and precipitation of 1,668.3 mm. The root tuber propagation materials of *Radix pseudostellariae* variety "Zhenshen 2" were used as the experimental plant, which was planted on 20th November and harvested on 10th July of the following year. A loam soil was used in the experiments. Physical–chemical characterization of soil used for the experiments was performed, using the protocol described by Jackson (1958) and Watanabe and Olsen (1965). To keep the soil and climatic conditions during the experimental period uniform and subjected to the same field and fertilization management, four types of plots were established within a single experimental field: (1) unplanted soil (CK), (2) containing

R. pseudostellariae cultivated in fresh soil (FP), (3) plot under cultivation of *R. pseudostellariae* for two consecutive years (SP), and (4) plot under cultivation of *R. pseudostellariae* for three consecutive years (TP). Each type carried three replicate plots with a completely randomized design. The samples were taken in three biological replicates.

Soil Sampling and DNA Extraction

According to our previous study (Chen et al., 2017), after 5 months of planting *R. pseudostellariae*, its above-ground and underground biomass was significantly different (expanding period of root tubers). Therefore, we randomly collected soil samples from five different locations within each plot on 25th April, 2018 (**Figure 1A**). Moreover, for yield determination, we harvested the plants on 10th July, 2018 (**Figure 1B**). While taking soil samples, the rhizosphere soil clung to the root system of *R. pseudostellariae* was collected. DNA of soil (0.5 g) was extracted with BioFast Soil Genomic DNA Extraction Kit (BioFlux, Hangzhou, China) following the instructions. Furthermore, DNA concentration was measured using NanoDrop 2000C Spectrophotometer (Thermo Scientific, United States) and diluted to 20 ngμl^{-1}.

PCR-DGGE Analysis

To evaluate the changes of *Trichoderma* community in the rhizosphere, the nested PCR strategy was designed and applied. In the first round of PCR, we used ITS1F and ITS4, taxon-selective ITS primers. PCR amplification protocol is described in **Supplementary Table 1**. The 50-μl PCR reaction contains 1 μl of each primer (10 mM), 2 μl template DNA (20 ngμl^{-1}), and 25 μl 2X EasyTaq PCR SuperMix (TransGen Biotech, Beijing, China). The products of PCR were subsequently diluted (1:5) for the second PCR reaction via DG-GC (a 40-bp GC-clamp at its 5′ end) and DT primers (**Supplementary Table 1**). PCR reaction followed for the second round was similar to the first round.

We performed DGGE using the Junyi JY-TD331A system (JUNYI, Beijing, China) using an 8% (*w*/*v*) polyacrylamide gel with a denaturation gradient of 30–60%. The gels were electrophoresed in 1X TAE buffer at 80 V and 60°C for 12 h. After electrophoresis, gels were immediately stained with silver stain.

Quantitative PCR for *F. oxysporum* and *Trichoderma* Spp.

We performed real-time PCR quantifications of *F. oxysporum* (ITS1F and AFP308R) and *Trichoderma* spp. (DG and DT, as mentioned above) in four soil samples (CK, FP, SP, and TP), and amplification protocol is described in **Supplementary Table 1**. PCR reaction of 15 μl contains 7.5 μl 2X TransStart Green qPCR SuperMix (TransGen Biotech, Beijing, China), 1 μl of each primer (10 μM), 2 μl of template DNA (20 ngμl^{-1}), and 3.5 μl H_2O. Meanwhile, serial dilutions of plasmid DNA were set as standard curves. The standard curve was generated by log10 value against the threshold cycle (Ct) value. Four independent quantitative PCR assays were performed for each treatment.

Isolation of *Trichoderma* spp. With Antagonistic Activity Toward *F. oxysporum*

To isolate *Trichoderma* spp., soil suspensions were made by adding 10 g of fresh soil into a flask containing 90 ml of sterile water (10^{-1} gl^{-1}). After dilution (10^{-2} gl^{-1}), a total of 100 μl suspensions were plated onto Thayer-Martin agar medium (containing 0.25 gl^{-1} pentachloronitrobenzene and 30 mgl^{-1} streptomycin sulfate). The plates were placed in an incubator at 28°C for 4 days and then each single colony was separated and purified.

After purification, we used the CTAB-based method (Rogers and Bendich, 1985) for DNA extraction from different isolates. For sequencing, three primer sets (ITS1F and ITS4, EF1 and EF2, and fRPB2-5f and fRPB2-7cr) were used for amplification (protocol as described in **Supplementary Table 1**). PCR products were cloned into the pEASY-T1 Cloning vector and sent to BoShang (Fuzhou, China) for sequencing. We further used the BlastN search method to screen for similar sequences in the NCBI and Tricho-BLAST at the website of the International Subcommission on *Trichoderma* and *Hypocrea* Taxonomy. For phylogram, published ITS1, *rpb*2, and *tef*1 sequences for biocontrol isolates were obtained from GenBank. ClustalX2 programs were used for sequence alignment. Gaps/missing data treatment was set to complete deletion. Phylogenetic analysis was carried out with the MEGA6 software. Maximum likelihood was used for statistical method. Neighbor-joining (NJ) trees were constructed for each data set (ITS1, *rpb*2, and *tef*1) using the Tamura–Nei distance measure. The robustness of the internal branches was assessed with 1,000 bootstrap replications (Saitou and Nei, 1987).

Evaluation of the Biocontrol Effects of *Trichoderma* spp.

For *in vitro* antagonism assays, *Trichoderma* isolates were used to evaluate the biocontrol effects against *F. oxysporum* (pathogenic fungi of *R. pseudostellariae*), which was part of the microbial collection of our lab (Chen et al., 2017). The strains (*F. oxysporum* and *Trichoderma* isolates) were inoculated in potato dextrose agar (PDA) slant culture medium at 4°C. We inoculated each *Trichoderma* isolates in dual culture with *F. oxysporum*. Two different isolates were placed 5.5 cm apart on the same PDA plate with three replicates. After incubation at 30°C for 5 days, the parameters of the antagonistic activity of *Trichoderma* isolates against *F. oxysporum* were recorded. Thus, the percentage of inhibition (PI) was calculated by the following formula:%PI = [(r1 - r2)/r1] × 100, where r1 is the distance between the furthest point and sowing point of the *F. oxysporum* and r2 represents the distance between the sowing point and the edge of the *F. oxysporum* from where *F. oxysporum* and *Trichoderma* mycelia came into contact (**Supplementary Figure 1**).

Unplanted soil (CK) was used for pot assays. *R. pseudostellariae* were planted in plastic pots and placed in a greenhouse on December 15, 2017. After 5 months of planting, a 2-ml spore suspension (a spore suspension of *F. oxysporum* with a concentration of 10^6 spores·ml^{-1} was made

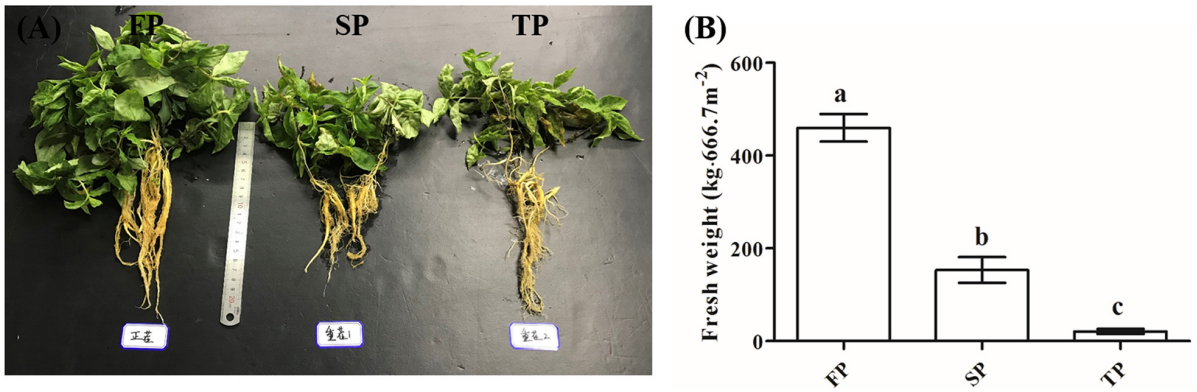

FIGURE 1 | (A) Photographs of *R. pseudostellariae* under FP, SP, and TP treatments. **(B)** Yield of *R. pseudostellariae* under FP, SP, and TP treatments. FP, plot with *R. pseudostellariae* cultivated in fresh soil; SP, plot with *R. pseudostellariae*, monocultured for two consecutive years; TP, plot with cultivation of *R. pseudostellariae* for three consecutive years. Different letters show significant differences according to least significant difference (LSD) ($P \leq 0.05$), $n = 3$.

by rinsing mycelia with sterile water) of isolated *F. oxysporum* was added to the soil for observing the effects of *Fusarium* wilt on *R. pseudostellariae*. In addition, 7 days after inoculation with *F. oxysporum*, spore suspensions of four isolated strains (ZC4, ZC5, ZC51, and CC2-7) were added to pots to evaluate the biocontrol potential of *Trichoderma* spp. Each treatment was three replicates. After 16 days, rhizospheric soil was collected from each treatment (FOX, ZC4, ZC5, ZC51, and CC2-7), and then, soil DNA was immediately extracted for qPCR assays (as mentioned above) of *F. oxysporum* and *Trichoderma* spp.

Expression Analysis of Defense-Related Genes in *R. pseudostellariae*

To further determine the effect of *Trichoderma* treatment and/or *F. oxysporum* infection on the expression of defense-related genes in *R. pseudostellariae*, we prepared MS medium for *in vitro* culture of *R. pseudostellariae*. Seedlings of *R. pseudostellariae* were transferred in the medium with tweezer and placed in a culture room at 26°C. After 60 days of incubation in the culture room, four treatments were set up: (1) inoculated with *F. oxysporum* into tissue-cultured seedlings of *R. pseudostellariae* (F); (2) inoculated with *T. harzianum* ZC51 into tissue-cultured seedlings of *R. pseudostellariae* (T); (3) simultaneously inoculated with *F. oxysporum* and *T. harzianum* ZC51 into the tissue-cultured seedlings of *R. pseudostellariae* (TF); and (4) tissue-cultured seedlings of *R. pseudostellariae* without any treatment (NTF). Seven days after the inoculation, the tissue culture seedlings of *R. pseudostellariae* were taken out; the plants were washed with sterile water, quickly treated with liquid nitrogen, and frozen in a refrigerator at -80°C for later extraction of RNA.

Plant RNA Isolation and Real-Time PCR Analysis

Plants were ground into powder with liquid nitrogen, and plant RNA was extracted with TransZol Up Plus RNA Kit (TransGen Biotech, Beijing, China) in accordance with the instructions. Furthermore, RNA concentration was measured using NanoDrop 2000C Spectrophotometer (Thermo Scientific, United States). According to the kit's instructions, the first-strand cDNA was synthesized using TransScript® miRNA First-Strand cDNA Synthesis SuperMix (TransGen Biotech, Beijing, China). Each sample used 1 μg of total RNA, and the products were immediately diluted to 80 μl with DEPC water as a template.

Based on the previous transcriptome data of *R. pseudostellariae* in our laboratory (Qin et al., 2017), nine primer pairs were used (**Supplementary Table 2**) to analyze the expression of defense-related genes in *R. pseudostellariae* as a result of *Trichoderma* and/or *F. oxysporum* infection. The actin gene (**Supplementary Table 2**) was used as an internal reference gene. The 15 μl of the PCR reaction contains 7.5 μl of 2 × SYBR Green qPCR Master Mix (TransGen Biotech, Beijing, China), 1 μl of each primer (10 μM), 0.6 μl of cDNA template, and 5.9 μl H_2O. The PCR program was as follows: 94°C for 30 s, followed by 40 cycles of 94 C for 5 s and 60°C for 30 s. After RT-PCR, the $2^{-\Delta\Delta CT}$ method (Livak and Schmittgen, 2001) was used to calculate the relative gene expression levels.

Statistical Analysis

Grayscale of DGGE bands was performed with the Quantity One v4.6.2 software to detect the band of gel. Principal component analysis (PCA) of DGGE was performed by SPSS 20.0 software. Diversity analysis of DGGE was performed by DPS 7.05. For RT-PCR, comparison between two groups was done with independent sample *T*-test by Excel 2013 software. Multiple comparison was carried out by one-way analysis of variance (ANOVA) followed by least significant difference (LSD) test ($P \leq 0.05$) using DPS 7.05 software for all parameters.

RESULTS

The Yield of *R. pseudostellariae* Under Consecutive Monoculture

The yield of *R. pseudostellariae* in the FP was significantly higher ($P < 0.05$) than SP and TP (**Figure 1B**). The fresh weights of

roots in FP were 459.8 kg·per 666.7 m^2, while it was 153.4 kg and 20.9 kg·per 666.7 m^2 in SP and TP, respectively (**Figure 1B**).

Soil Nutritional Status

The chemical composition of the soil evaluated was as follows: total nitrogen 1.65 $g{\cdot}kg^{-1}$, available nitrogen 36.42 $mg{\cdot}kg^{-1}$, total phosphorus 0.51 $g{\cdot}kg^{-1}$, effective phosphorus 100.31 $mg{\cdot}kg^{-1}$, total K 7.66 $g{\cdot}kg^{-1}$, and effective potassium 322.52 $mg{\cdot}kg^{-1}$.

Trichoderma-Specific DGGE

Trichoderma-specific DGGE analysis indicated that the shifts of the *Trichoderma* community in the rhizosphere changed with increasing period of monoculture (**Supplementary Figure 2**). Based on DGGE profiles, we performed principal component analysis (PCA) to explore *Trichoderma* rhizosphere community structure between the four different soil conditions. Among them, the first principal component revealed 49.80% of the total variance, and the second principal component indicated 17.10% of the total variance. The results of PCA also showed that the *Trichoderma* community in TP was separated from CK, FP, and SP by the first principal component, and CK was separated from FP and SP by the second principal component (**Figure 2**).

We also analyzed the diversity of *Trichoderma*-specific DGGE. The results showed that the Simpson, Shannon, and Brillouin's indices decreased significantly with increasing period of monoculture ($P \leq 0.05$). However, there was no significant difference in evenness index among the four samples (**Table 1**).

Abundance of *Trichoderma* spp. and *F. oxysporum* by Quantitative PCR Under Different Continuous Years

Quantitative PCR was used to analyze changes in *Trichoderma* spp. and *F. oxysporum* abundance in four soil samples (**Figure 3**). For *Trichoderma* spp. and *F. oxysporum* qPCR analyses, standard curves of $y = -0.2271x + 10.763$ ($R^2 = 0.9944$) and $y = -0.2673x + 10.607$ ($R^2 = 0.9986$), respectively, were developed. Abundance of *Trichoderma* spp. was significantly decreased with prolonged monoculture (**Figure 3**). These results were consistent with the *Trichoderma*-specific DGGE analysis (**Supplementary Figure 2**). However, the quantitative PCR results for *F. oxysporum* were the opposite (**Figure 3**).

Screening for *Trichoderma* Isolates With Antagonistic Activity Toward *F. oxysporum*

For *in vitro* antagonism assays, we screened seven isolates of *Trichoderma* from four different soils. The results of sequencing showed that the seven isolates belonged to three species of *Trichoderma* (**Table 2**). The accession number (ITS, *tef*1, and *rpb*2) of seven isolates are in **Table 2**. Among these, ZC5 isolate showed the highest antagonistic activity (72.77%) (**Table 2**) toward *F. oxysporum*, whereas ZC13 showed the lowest antagonistic activity.

The phylogenetic analysis (ITS) using neighbor-joining method generated a dendrogram with three main branches, where the first branch included *T. harzianum* ZC5 and ZC51; the second branch comprised *T. rugulosum* CC2-7; and the third branch comprised *T. asperelloides* ESK2, ZC13, ZC11, and ZC4 (**Figure 4A**). For *rpb*2, a dendrogram contained three main branches, where the first branch included *T. asperelloides* ZC13, ESK2, ZC14, and ZC11; the second branch comprised *T. rugulosum* CC2-7; and the third branch comprised *T. harzianum* ZC5 and ZC51 (**Figure 4B**). For *tef*1, a dendrogram contained three main branches, where the first branch included *T. asperelloides* ZC4, ESK2, ZC11, and ZC13; the second branch comprised *T. harzianum* ZC5

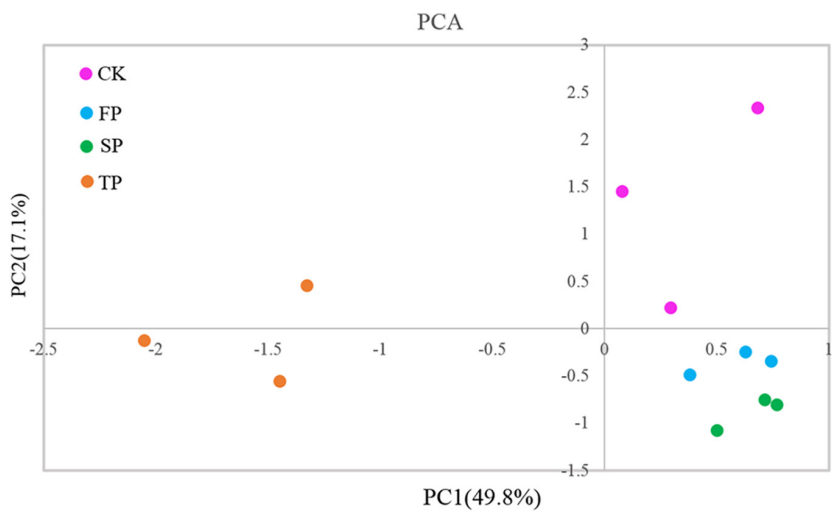

FIGURE 2 | Principal component analysis of *Trichoderma* DGGE. CK, unplanted soil; FP, plot with *R. pseudostellariae* cultivated in fresh soil; SP, plot with *R. pseudostellariae*, monocultured for two consecutive years; TP, plot with cultivation of *R. pseudostellariae* for three consecutive years; DGGE, denatured gradient gel electrophoresis.

TABLE 1 | Estimated Simpson, Shannon, evenness, and Brillouin's indices for all the samples using *Trichoderma*-specific DGGE.

Treatments	Simpson	Shannon	Evenness	Brillouin
CK	0.9094 + 0.0082a	3.5867 + 0.0571a	0.9693 + 0.0154a	3.5603 + 0.054a
FP	0.9147 + 0.0015a	3.6275 + 0.011a	0.9803 + 0.003a	3.6006 + 0.0094a
SP	0.907 + 0.0016a	3.5073 + 0.0093b	0.9783 + 0.0026a	3.4811 + 0.0084b
TP	0.8835 + 0.0025b	3.1317 + 0.0144c	0.9879 + 0.0046a	3.104 + 0.0136c

Different letters within a column show significant differences according to LSD ($P \leq 0.05$), n = 3.
CK, unplanted soil; FP, planting of R. pseudostellariae in the newly planted soil; SP, planting of R. pseudostellariae in two consecutive years; TP, planting of R. pseudostellariae in three consecutive years.

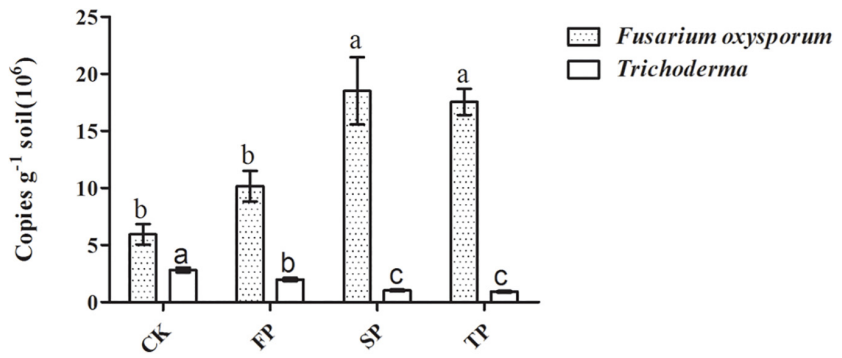

FIGURE 3 | Quantification of *Trichoderma* spp. and *F. oxysporum* in the different plots. CK, control with unplanted soil; FP, plot with *R. pseudostellariae* cultivated in fresh soil; SP, plot with *R. pseudostellariae*, monocultured for two consecutive years; TP, plot with cultivation of *R. pseudostellariae* for three consecutive years. Different letters in the same color show significant differences according to least significant difference (LSD) ($P \leq 0.05$); data are means ± standard errors (one-way analysis of variance, $n = 4$).

TABLE 2 | *In vitro* antifungal activity of *Trichoderma* strains against *F. oxysporum*.

Lab. Code	Inhibition in growth assay	Identify	Accession number (ITS)	Accession number (*rpb2*)	Accession number (*tef1*)
ZC5	47.91 ± 3.41a	*T. harzianum*	MW376900.1	MW407164	MW415424
ZC51	47.66 ± 3.21a	*T. harzianum*	MW376903.1	MW407167	MW415425
ZC4	30.66 ± 1.36b	*T. asperelloides*	MW376899.1	MW407163	WM588808
CC2-7	30.16 ± 11.31b	*T. rugulosum*	MW376897.1	MW407161	MW588806
ESK2	24.41 ± 4.44bc	*T. asperelloides*	MW376898.1	MW407162	MW588807
ZC11	23.62 ± 1.67bc	*T. asperelloides*	MW376901.1	MW407165	MW588809
ZC13	16.67 ± 2.67c	*T. asperelloides*	MW376902.1	MW588811	MW588810

Different letters within a column show significant differences according to LSD ($P \leq 0.05$), n = 3.

and ZC51; and the third branch comprised *T. rugulosum* CC2-7 (**Figure 4C**).

Biocontrol Effects of *Trichoderma* spp.

Based on the *in vitro* antagonism assays, four isolates of *Trichoderma* (ZC4, ZC5, ZC51, and CC2-7) that showed inhibition activity against *F. oxysporum* higher than 30% were selected for further *in vivo* biocontrol assay (**Figure 5**). In the pot experiment, compared with the control (FOX), we found that *T. harzianum* ZC51 significantly inhibited the growth of *F. oxysporum* and enhanced the growth of *R. pseudostellariae*, whereas no disease symptoms developed during the period of the experiment (**Figure 5D**).

Moreover, quantitative PCR was used to analyze changes in *Trichoderma* spp. and *F. oxysporum* abundance in the pot experiments. Compared with the control (FOX), the abundance of *Trichoderma* spp. in the soil treated with *Trichoderma* strains ZC4, ZC5, and ZC51 increased significantly, with *T. harzianu*m ZC51 showing the highest abundance (**Figure 6**). Finally, the quantitative PCR results for *F. oxysporum* in the pot experiments showed that all treatments significantly decreased as compared to control (**Figure 6**). The results showed that *T. harzianum* ZC51 strain, potentially, could be used as a biological control agent against *F. oxysporum*.

Effect of *Trichoderma* Treatment and/or *F. oxysporum* in the Expression of *R. pseudostellariae* Defense-Related Genes

The *T. harzianum* ZC51 strain was selected, based on its positive effect on *R. pseudostellariae* phenotype without

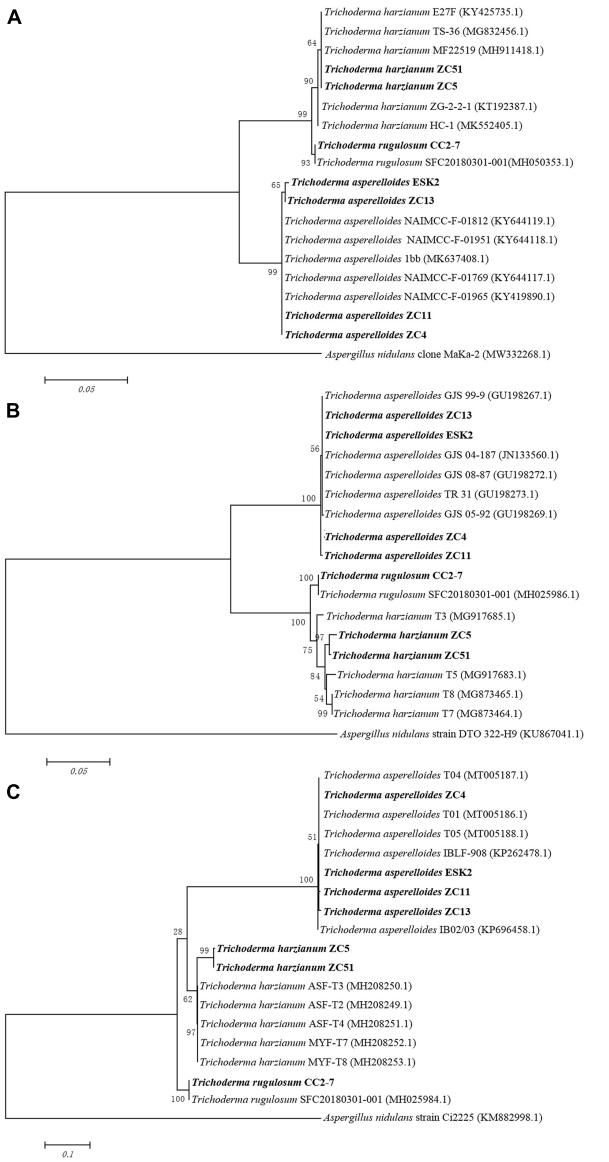

FIGURE 4 | Neighbor-joining tree based on sequence analysis of *Trichoderma* isolates used in this study. Values of the bootstrap analysis (1,000 repetitions) are given at the nodes. **(A)** ITS; **(B)** *rpb2*; **(C)** *tef1*. *Aspergillus nidulans* was the outgroup. Sequences of biocontrol isolates used for this comparison were obtained from GenBank.

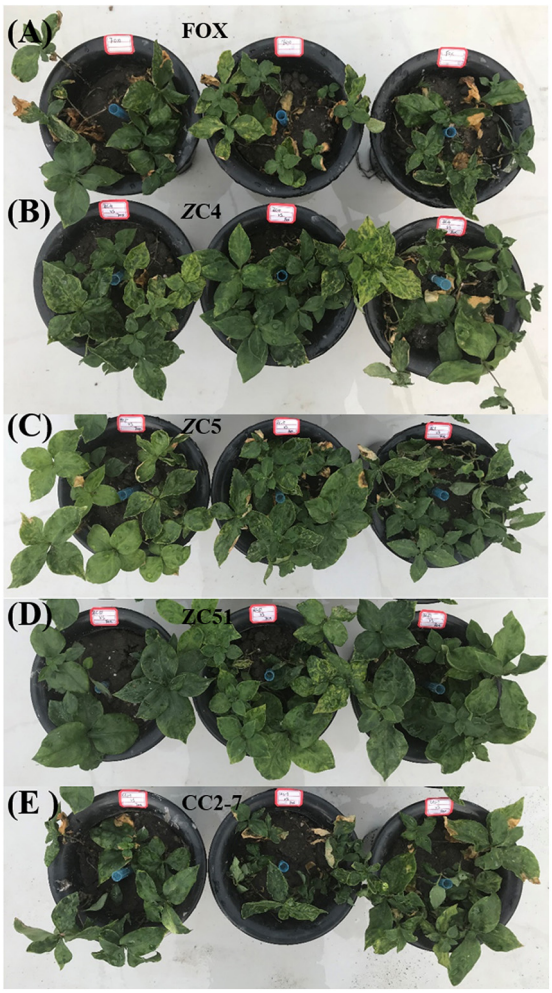

FIGURE 5 | Biocontrol potential of *Trichoderma* against *F. oxysporum*. **(A)** *R. pseudostellariae* was treated with *F. oxysporum* (FOX); **(B)** *R. pseudostellariae* was treated with *F. oxysporum* and *T. asperellum* ZC4 (ZC4); **(C)** *R. pseudostellariae* was treated with *F. oxysporum* and *T. harzianum* ZC5 (ZC5); **(D)** *R. pseudostellariae* was treated with *F. oxysporum* and *T. harzianum* ZC51 (ZC51); **(E)** *R. pseudostellariae* was treated with *F. oxysporum* and *T. hamatum* CC2-7 (CC2-7).

infection (**Figure 5D**) and the highest abundance among all treatments (**Figure 6**). The expression of defense-related genes was examined in these plants, i.e., *Trichoderma* ZC51-non-inoculated and *F. oxysporum*-infected plants (F), *Trichoderma* ZC51-inoculated and *F. oxysporum*-non-infected plants (T), *Trichoderma* ZC51-inoculated and *F. oxysporum*-infected (TF) plants, or *Trichoderma* ZC51-non-inoculated and *F. oxysporum*-non-infected plants (NTF).

To analyze the expression of defense-related genes, we used actin as a housekeeping gene to determine the relative expression levels of other genes. Expressions of *PAL1* and *PAL3* were studied to determine the involvement of phenylalanine ammonia lyase in *R. pseudostellariae* response to *T. harzianum* ZC51 treatment and/or *F. oxysporum*. There was no significant difference of the *PAL1* and *PAL3* expression in the three treatments (**Figures 7A–C**). We also examined the expression of *CH1*, *CH4*, and *CH5* of chitinase by treated plants. Expression of *CH4* and *CH5* increased in plants inoculated with *Trichoderma* ZC51 (T) (**Figure 7B**) and Trichoderma ZC51-inoculated and *F. oxysporum*-infected (TF) (**Figure 7C**). In contrast, the expression of *CH1* and *CH5* decreased in plants only infected with *F. oxysporum* (F) (**Figure 7A**). In addition to these genes, we further analyzed the expression of *PRSTH-21*, *PR1a*, *PR4*, and *PR10* involved in the process of plant disease resistance. The expression of *PRSTH-21*, *PR1a*, *PR4*, and *PR10* increased in *Trichoderma* ZC51-inoculated (T) (**Figure 7B**) and *F. oxysporum*-infected (TF) (**Figure 7C**)

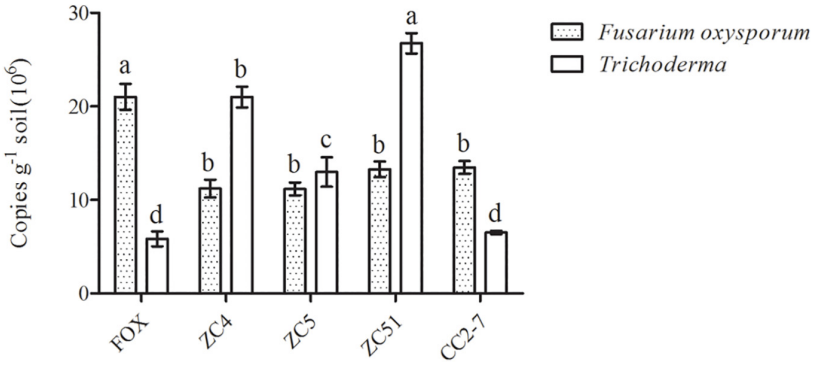

FIGURE 6 | Quantification of *Trichoderma* spp. and *F. oxysporum* in the pot experiment. FOX, *R. pseudostellariae* was treated with *F. oxysporum*; ZC4, *R. pseudostellariae* was treated with *F. oxysporum* and *T. asperellum* ZC4; ZC5, *R. pseudostellariae* was treated with *F. oxysporum* and *T. harzianum* ZC5; ZC51, *R. pseudostellariae* was treated with *F. oxysporum* and *T. harzianum* ZC51; CC2-7, *R. pseudostellariae* was treated with *F. oxysporum* and *T. hamatum* CC2-7. Different letters in the same color show significant differences according to least significant difference (LSD) ($P \leq 0.05$); data are means ± standard errors (one-way analysis of variance, $n = 4$).

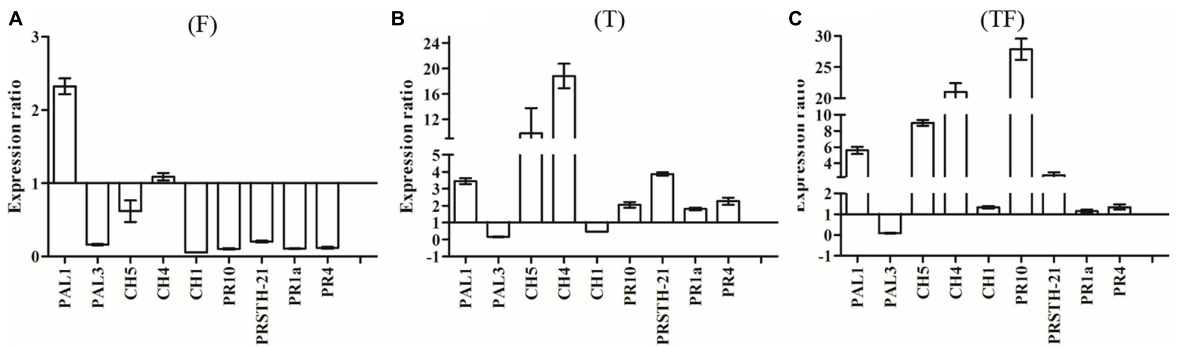

FIGURE 7 | Expression of *PAL1*, *PAL3*, *CH5*, *CH4*, *CH1*, *PR10*, *PRSTH-21*, *PR1a*, and *PR4* genes in comparison with α-actin reference genes. **(A)** Inoculated with *F. oxysporum* into tissue-cultured seedlings of *R. pseudostellariae*; **(B)** inoculated with *T. harzianum* ZC51 into tissue-cultured seedlings of *R. pseudostellariae*; **(C)** simultaneously inoculated with *F. oxysporum* and *T. harzianum* ZC51 into the tissue-cultured seedlings of *R. pseudostellariae*. Data are means ± standard errors (one-way analysis of variance, $n = 4$).

plants. However, the opposite was true for the plants only infected with *F. oxysporum* (F) (**Figure 7A**).

Also, in order to understand the effect of *Trichoderma* ZC51 (T) on gene expression of *F. oxysporum* (F)-infected *R. pseudostellariae*, we compared the expression of the same gene in different treatments (**Table 3**). Among these genes, the expression of *PAL1*, *CH5*, *CH4*, *CH1*, *PR10*, *PRSTH-21*, *PR1a*, and *PR4* significantly increased in plants which were treated with T and TF treatments (**Table 3**).

In general, the results demonstrated that *Trichoderma* ZC51 interaction with *R. pseudostellariae* affected the expression of plant defense-related genes related to the chitinase and pathogenesis-related proteins, but does not involve phenylalanine ammonia lyase.

DISCUSSION

The low quality and reduced yield of Chinese medicinal herbs are commonly observed due to recurrent cultivation on the same land for many years. This phenomenon of low yield, compromised medicinal quality, poor growth of plants, and high disease susceptibility is owing to consecutive monoculture problems or soil sickness (Zhang and Lin, 2009; Wu et al., 2016c). Our study revealed the facts for typical growth inhibition effects under consecutive monoculture of *R. pseudostellariae*, with poor plant performance and insufficient resistance to disease. Soil physical and chemical properties, accumulation of root exudates, and shift in the soil microbial community are some factors responsible for the consecutive monoculture problem of *R. Pseudostellariae* (Zhang and Lin, 2009; Wu et al., 2016a). The biological relationships between plants and microorganisms in the rhizosphere play a crucial role for the health and growth of a plant, which has been paid much attention in recent days (Haney and Ausubel, 2015; Lebeis et al., 2015).

Trichoderma spp. have been studied commonly because of its ability to inhibit soil-borne pathogens and have good plant defense responses (Papavizas, 1985; Hermosa et al., 2012; Pimentel et al., 2020). In this study, PCR-DGGE results showed significant shifts in *Trichoderma* community in the

rhizosphere of *R. pseudostellariae* after extended monoculture (**Supplementary Figure 2** and **Figure 2**). Based on PCR-DGGE of *Trichoderma*, results of diversity showed that the extended monoculture of *R. pseudostellariae* significantly decreased the *Trichoderma* spp. diversity (**Table 1**). Quantitative PCR assay confirmed the decrease in *Trichoderma* with the increasing years of monoculture (**Figure 3A**), whereas the abundance of *F. oxysporum* was significantly increased (**Figure 3B**). A previous study has also reported the changes in the composition and diversity of *Fusarium* spp. and increase in the abundance of *F. oxysporum* with the increasing years of monoculture (Chen et al., 2017). This selective change in the microbial community is due to the difference in response of these microorganisms to the root exudates in the rhizosphere (Huang et al., 2014; Zhalnina et al., 2018).

A negative shift in the composition of the soil microbial community is a consequence of the development of soil-borne diseases (Mazzola, 2004). Therefore, maintaining the biodiversity of beneficial soil microbes is crucial to soil health. Biological control with exploitation of the rhizosphere microorganisms that can directly antagonize with plant pathogens is considered to be the most promising method for preventing plant diseases (Qiu et al., 2012; Shen et al., 2014). These species mostly include antagonistic fungi such as *Trichoderma* spp. and *Penicillium* spp. (Cotxarrera et al., 2002; Howell, 2002; Siddiqui and Akhtar, 2009). In this study, most of the isolated strains of *Trichoderma* can inhibit the growth of *F. oxysporum* (**Table 2**). The difference in antagonistic abilities may be due to genotype variability (Debbi et al., 2018). The *in vivo* assays revealed that *R. pseudostellariae* treated with *T. harzianum* ZC51 has the best growth phenotype without displaying any disease symptom. Yedidia et al. (2001) reported that *T. harzianum* T-203 increased the root length, aerial parts, dry weight, and size of the blade by 75, 95, 80, and 45%, respectively, in cucumber plants. Other studies have shown that *Trichoderma* spp. could promote plant growth, increase nutrient utilization, and improve crop production (Harman et al., 2004).

TABLE 3 | Expression of *PAL1*, *PAL3*, *CH5*, *CH4*, *CH1*, *PR10*, *PRSTH-21*, *PR1a*, and *PR4* genes in comparison with α-actin reference genes.

Treatments	*F*	*T*	TF
PAL1	2.3231 ± 0.1857c	3.4471 ± 0.3011b	5.5851 ± 0.7776a
PAL3	0.1622 ± 0.0157a	0.1699 ± 0.0257a	0.0929 ± 0.026b
CH5	0.6188 ± 0.2544b	9.8098 ± 6.8382a	8.9884 ± 0.6374a
CH4	1.0905 ± 0.0894b	18.8246 ± 3.3248a	21.0104 ± 2.4285a
CH1	0.0565 ± 0.0014c	0.471 ± 0.0095b	1.3374 ± 0.1003a
PR10	0.1049 ± 0.0136b	2.0501 ± 0.2914b	27.8775 ± 2.9425a
PRSTH-21	0.2035 ± 0.0206c	3.872 ± 0.1593a	2.5438 ± 0.5835b
PR1a	0.1075 ± 0.005c	1.8026 ± 0.1541a	1.1476 ± 0.1243b
PR4	0.1188 ± 0.021c	2.2681 ± 0.3655a	1.3469 ± 0.2162b

Different letters within a row show significant differences according to LSD ($P \leq 0.05$), n = 4.

F, inoculated with F. oxysporum into tissue-cultured seedlings of R. pseudostellariae; T, inoculated with T. harzianum ZC51 into tissue-cultured seedlings of R. pseudostellariae; TF, simultaneously inoculated with F. oxysporum and T. harzianum ZC51 into the tissue-cultured seedlings of R. pseudostellariae.

There is ample evidence that *Trichoderma* species could induce plant defense responses (Yedidia et al., 1999; Gallou et al., 2009; Singh et al., 2011). However, little is known about the effect of *Trichoderma* treatment on the expression of defense-related genes in *R. Pseudostellariae*. PR proteins are well-known proteins that is induced by pathogens and play an important role in the process of plant disease resistance (Linthorst and Van Loon, 1991; Edreva, 2005). Moreover, chitinase proteins a pathogenesis-related proteins that are induced by pathogens; thus, chitinase constitute a crucial part of the plant's defense against fungal pathogens (Punja and Zhang, 1993; El Hadrami et al., 2010).

As described, the interaction of *R. pseudostellariae* with *F. oxysporum* caused the repression of the seven defense-related genes (*PAL3, CH5, CH1, PR10, PRSTH-21, PR1a*, and *PR4*), via a mechanism to overcome plant defense responses and thereby promoting the process of infection in plants (Peix et al., 2001). Zhao et al. (2003) also reported similar results in experiments with tomato plants infected with *Pseudomonas syringae*, where tomatoes showed repression of *PR1* and *PR4*, suggesting that infection with pathogen would reduce the plant self-defense mechanism, hence promoting the development of the disease.

In this study, when the interaction of *R. pseudostellariae* with *T. harzianum* ZC51 was analyzed, *PR10*, *PRSTH-21*, *PR1a, PR4*, *CH4*, and *CH5b* were up-regulated. Others have shown that *T. harzianum* T39 reduces the incidence of downy mildew of grapes by directly regulating the expression of defense-related genes (Perazzolli et al., 2011). Similarly, a study has reported the increase in the expression level of several defense-related genes in olive trees only when *T. harzianum* (Ths97) was applied together with the root rot pathogen *F. solani* (Amira et al., 2017). Other studies have shown that *Trichoderma* spp. may also trigger ISR in plants, mainly related to the expression of pathogenesis-related proteins (i.e., *PR1*, *PR2*, and *PR5*) (Hermosa et al., 2012; Mathys et al., 2012). Phenylalanine ammonia lyase (*PAL*) is one of the most widely studied enzymes involved in the process of plant disease resistance (Kim and Hwang, 2014). In this study, *T. harzianum* ZC51 did not change the expression of the *PAL* genes.

Compared with the "simple" two-partner systems (i.e., plant–pathogen or plant–antagonist), the complex three-way interactions involving *Trichoderma*, plant, and pathogen has received less attention, and this model can better simulate the natural interactions occurring in soil (Vinale et al., 2008). In our study, when *T. harzianum* ZC51 and *F. oxysporum* were applied together on *R. pseudostellariae*, we observed an upregulation of all the analyzed genes with the exception of PAL3. Similar results in the experiments to bean (*Phaseolus vulgaris* L.) infected with *R. solani* and/or *Trichoderma* were observed. The level of expression of defense-related genes (*CH5b*, *CH1*, *PR1*, *PR2*, *PR3*, and *PR4*) were up-regulated. Marra et al. (2006) studied the three-way interaction of *Trichoderma* with plant and fungal pathogens using proteomics methods, and the results show that antagonistic fungi will reduce the production of some defense proteins but will lead to the accumulation of others (i.e., PR proteins). This suggests that even in the presence of pathogens, several mechanisms are induced in *Trichoderma* that potentiates its ability to elicit plant defense responses (Mayo et al., 2016).

Our results indicate that *Trichoderma* activate plants' defense responses and so could be an optimized defense strategy against different plant stress, including plant pathogens and monocropping disease.

CONCLUSION

To sum up, this study revealed that the continuous monocropping of *R. pseudostellariae* favored the growth of pathogenic *F. oxysporum* but decreased the antagonistic fungi (*Trichoderma* spp.), which resulted in poor yield of *R. pseudostellariae.* The exogenous application of *T. harzianum* ZC51 increased the expression levels of genes (*PR10*, *PRSTH-21*, *PR1a*, *PR4*, *CH4*, and *CH5b*) previously involved in plant defense, leading to enhanced defense response and improved growth of the host plant. These findings can be useful to develop locally customized and innovative approaches to address major threats facing medicinal plant cultivation.

DATA AVAILABILITY STATEMENT

The raw data supporting the conclusions of this article will be made available by the authors, without undue reservation.

AUTHOR CONTRIBUTIONS

WL, JC, and SL conceived the study. JC, LZ, and ID wrote the manuscript. JC, QL, and JW performed the experiments. TW, LW, LZ, and HW performed the statistical analyses. XQ and YA were involved in field management and soil sampling. GP assisted in English correction. All the authors discussed the results and commented on the manuscript.

FUNDING

This work was supported by the National Key Research and Development Plan 2017YFE0121800 and grants from the National Natural Science Foundation of China (Nos. 81973412 and 81573530), the Scientific Research Foundation of Graduate School of Fujian Agriculture and Forestry University (YB2018002), the Project of Key Laboratory of Ministry of Education (GBMUC-2018-006), and the Science and Technology Development Fund of Fujian Agriculture and Forestry University (KF2015043).

ACKNOWLEDGMENTS

We are thankful to Professor Irina Druzhinina (the chair of International Commission on Trichoderma Taxonomy) and Dr. Feng Cai (Nanjing Agricultural University) for assistance in the identification of *Trichoderma* strains.

SUPPLEMENTARY MATERIAL

The Supplementary Material for this article can be found online at: https://www.frontiersin.org/articles/10.3389/fmicb.2021.579920/full#supplementary-material

REFERENCES

Amira, M. B., Lopez, D., Mohamed, A. T., Khouaja, A., Chaar, H., Fumanal, B., et al. (2017). Beneficial effect of *Trichoderma harzianum* strain Ths97 in biocontrolling *Fusarium solani* causal agent of root rot disease in olive trees. *Biol. Control* 110, 70–78. doi: 10.1016/j.biocontrol.2017.04.008

Brotman, Y., Landau, U., Cuadros-Inostroza, A., 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

Chen, J., Wu, L. K., Xiao, Z. G., Wu, Y. H., Wu, H. M., Qin, X. J., et al. (2017).). Assessment of the diversity of *Pseudomonas* spp. and *Fusarium* spp. in *Radix pseudostellariae* rhizosphere under monoculture by combining DGGE and quantitative PCR. *Front. Microbiol.* 8:1748. doi: 10.3389/fmicb.2017.01748

Corrales, M., Fernandez, A., Pinto, M. G. V., Butz, P., Franz, C. M., Schuele, E., et al. (2010). Characterization of phenolic content, *in vitro* biological activity, and pesticide loads of extracts from white grape skins from organic and conventional cultivars. *Food Chem. Toxicol.* 48, 3471–3476.doi: 10.1016/j.fct.2010.09.025

Cotxarrera, L., Trillas-Gay, M. I., Steinberg, C., and Alabouvette, C. (2002). Use of sewage sludge compost and *Trichoderma asperellum* isolates to suppress *Fusarium* wilt of tomato. *Soil Biol. Biochem.* 34, 467–476.doi: 10.1016/S0038-0717(01)00205-X

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*. *Horticul. Res.* 6:5. doi: 10.1038/s41438-018-0079-1

Debbi, A., Boureghda, H., Monte, E., and Hermosa, R. (2018). Distribution and genetic variability of *Fusarium oxysporum* associated with tomato diseases in Algeria and a biocontrol strategy with indigenous *Trichoderma* spp. *Front. Microbiol.* 9:282. doi: 10.3389/fmicb.2018.00282

Edreva, A. (2005). Pathogenesis-related proteins: research progress in the last 15 years. *Gen. Appl. Plant Physiol.* 2005, 105–124.

El Hadrami, A., Adam, L. R., El Hadrami, I., and Daayf, F. (2010). Chitosan in plant protection. *Mar. Drugs* 8, 968–987. doi: 10.3390/md8040968

Galletti, S., Paris, R., and Cianchetta, S. (2020). Selected isolates of *Trichoderma gamsii* induce different pathways of systemic resistance in maize upon *Fusarium verticillioides* challenge. *Microbiol. Res.* 233:126406.doi: 10.1016/j.micres.2019.126406

Gallou, A., Cranenbrouck, S., and Declerck, S. (2009). Trichoderma harzianum elicits defence response genes in roots of potato plantlets challenged by *Rhizoctonia solani*. *Eur. J. Plant Pathol.* 124, 219–230.doi: 10.1007/s10658-008-9407-x

Haney, C. H., and Ausubel, F. M. (2015). Plant microbiome blueprints. *Science* 349, 788–789.doi: 10.1126/science.aad0092

Harman, G. E., Howell, C. R., Viterbo, A., Chel, 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. (2002). Cotton seedling preemergence damping-off incited by *Rhizopus oryzae* and *Pythium* spp. and its biological control with

Trichoderma spp. *Phytopathology* 92, 177–180.doi: 10.1094/PHYTO.2002.92.2.177

Howell, C. R. (2006). Understanding the mechanisms employed by *Trichoderma virens* to effect biological control of cotton diseases. *Phytopathology* 96, 178–180.doi: 10.1094/PHYTO-96-0178

Huang, X., Chaparro, J. M., Reardon, K. F., Zhang, R., Shen, Q., and Vivanco, J. M. (2014). Rhizosphere interactions: root exudates, microbes, and microbial communities. *Botany* 92, 267–275.doi: 10.1139/cjb-2013-0225

Jackson, M. L. (1958). *Soil Chemical Analysis*, Vol. 498. Englewood Cliffs. NJ: Prentice Hall. Inc., 183–204.

Kim, D. S., and Hwang, B. K. (2014). An important role of the pepper phenylalanine ammonia-lyase gene (*PAL1*) in salicylic acid-dependent signalling of the defence response to microbial pathogens. *J. Exp. Bot.* 65, 2295–2306.doi: 10.1093/jxb/eru109

Lebeis, S. L., Paredes, S. H., Lundberg, D. S., Breakfield, N., Gehring, J., McDonald, M., et al. (2015). Salicylic acid modulates colonization of the root microbiome by specific bacterial taxa. *Science* 349, 860–864. doi: 10.1126/science.aaa8764

Lin, S., Huangpu, J. J., Chen, T., Wu, L. K., Zhang, Z. Y., and Lin, W. X. (2015). Analysis of soil microbial community structure and enzyme activities associated with negative effects of *Pseudostellaria heterophylla* consecutive monoculture on yield. *Pak. J. Bot.* 47, 761–769.

Linthorst, H. J., and Van Loon, L. C. (1991). Pathogenesis-related proteins of plants. *Crit. Rev. Plant Sci.* 10, 123–150. doi: 10.1080/07352689109382309

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

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

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

Marra, R., Ambrosino, P., Carbone, V., Vinale, F., Woo, S. L., Ruocco, M., et al. (2006). Study of the three-way interaction between Trichoderma atroviride, plant and fungal pathogens by using a proteomic approach. *Curr. Genet.* 50, 307–321.doi: 10.1007/s00294-006-0091-0

Matarese, F., Sarrocco, S., and Gruber, S. (2012). Biocontrol of *Fusarium* head blight: interactions between *Trichoderma* and mycotoxigenic *Fusarium*. *Microbiology* 158, 98–106.doi: 10.1099/mic.0.052639-0

Mathys, J., De Cremer, K., Timmermans, P., Van Kerkhove, S., Lievens, B., Vanhaecke, M., et al. (2012). Genome-wide characterization of ISR induced in *Arabidopsis thaliana* by *Trichoderma hamatum* T382 against *Botrytis cinerea* infection. *Front. Plant Sci.* 3:108. doi: 10.3389/fpls.2012.00108

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

Mazzola, M. (2004). Assessment and management of soil microbial community structure for disease suppression. *Annu. Rev. Phytopathol.* 42, 35–59. doi: 10.1146/annurev.phyto.42.040803.140408

Mendes, R., Kruijt, M., de Bruijn, I., Dekkers, E., van der Voort, M., Schneider, J. H., et al. (2011). Deciphering the rhizosphere microbiome for disease-suppressive bacteria. *Science* 332, 1097–1100.doi: 10.1126/science.1203980

Mukherjee, P. K., Horwitz, B. A., Herrera-Estrella, A., Schmoll, M., and Kenerley, C. M. (2013). *Trichoderma* research in the genome era. *Annu. Rev. Phytopathol.* 51, 105–129. doi: 10.1146/annurev-phyto-082712-102353

Mukherjee, P. K., Hurley, J. F., Taylor, J. T., Puckhaber, L., Lehner, S., Druzhinina, I., et al. (2018). Ferricrocin, the intracellular siderophore of *Trichoderma virens*, is involved in growth, conidiation, gliotoxin biosynthesis and induction of systemic resistance in maize. *Biochem. Bioph. Res. Commun.* 505, 606–611.doi: 10.1016/j.bbrc.2018.09.170

Papavizas, G. C. (1985). *Trichoderma* and *Gliocladium*: biology, ecology, and potential for biocontrol. *Annu. Rev. Phytopathol.* 23, 23–54.doi: 10.1146/annurev.py.23.090185.000323

Peix, A., Mateos, P. F., Martinez-Molina, E., Rodriguez-Barrueco, C., and Velazquez, E. (2001). Growth promotion of common bean (*Phaseolus vulgaris* L.) by a strain of *Burkholderia cepacia* under growth chamber conditions. *Soil. Biol. Biochem.* 33, 1927–1935.doi: 10.1016/s0038-0717(01)00119-5

Perazzolli, M., Roatti, B., Bozza, E., and Pertot, I. (2011). *Trichoderma harzianum* T39 induces resistance against downy mildew by priming for defense without costs for grapevine. *Biol Control* 58, 74–82.doi: 10.1016/j.biocontrol.2011.04.006

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

Pimentel, M. F., Arnao, E., Warner, A. J., Subedi, A., Rocha, L. F., Srour, A. Y., et al. (2020). *Trichoderma* isolates inhibit *Fusarium virguliforme* growth, reduce root rot, and induce defense-related genes on soybean seedlings. *Plant Dis.* 104, 1949–1959.doi: 10.1094/PDIS-08-19-1676-RE

Punja, Z. K., and Zhang, Y. (1993). Plant chitinases and their roles in resistance to fungal diseases. *J. Nematol.* 25, 526–540. doi: 10.1016/0022-1910(93)90133-C

Qin, X. J., Wu, H. M., Chen, J., Wu, L. K., Lin, S., Khan, M. U., et al. (2017). Transcriptome analysis of *Pseudostellaria heterophylla* in response to the infection of pathogenic *fusarium oxysporum*. *BMC Plant Biol.* 17:155. doi: 10.1186/s12870-017-1106-3

Qiu, M. H., Zhang, R. F., Xue, C., Zhang, S. S., Li, S. Q., Zhang, N., et al. (2012). Application of bio-organic fertilizer can control *Fusarium wilt* of cucumber plants by regulating microbial community of rhizosphere soil. *Biol. Fert. Soils* 48, 807–816.doi: 10.1007/s00374-012-0675-4

Rogers, S. O., and Bendich, A. J. (1985). Extraction of DNA from milligram amounts of fresh, herbarium and mummified plant tissues. *Plant Mol Biol.* 5, 69–76.doi: 10.1007/BF00020088

Romera, F. J., García, M. J., Lucena, C., Martínez-Medina, A., Aparicio, M. A., Ramos, J., et al. (2019). Induced systemic resistance (ISR) and Fe deficiency responses in dicot plants. *Front. Plant Sci.* 10:287. doi: 10.3389/fpls.2019.00287

Saitou, N. N. M., 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

Shen, Z. Z., Wang, D. S., Ruan, Y., Xue, C., Zhang, J., Li, R., et al. (2014). Deep 16S rRNA pyrosequencing reveals a bacterial community associated with banana *Fusarium wilt* disease suppression induced by bio-organic fertilizer application. *PLoS One* 9:e98420.doi: 10.1371/journal.pone.0098420

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

Siddiqui, Z. A., and Akhtar, M. S. (2009). Effects of antagonistic fungi, plant growth-promoting rhizobacteria, and arbuscular mycorrhizal fungi alone and in combination on the reproduction of *Meloidogyne* incognita and growth of tomato. *J. Gen. Plant Pathol.* 75, 144–153.doi: 10.1007/s10327-009-0154-4

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

Vinale, F., Sivasithamparam, K., Ghisalberti, E. L., Marra, R., Woo, S. L., and Lorito, M. (2008). *Trichoderma*–plant–pathogen interactions. *Soil Biol. Biochem.* 40, 1–10.doi: 10.1016/j.soilbio.2007.07.002

Walters, D., Ratsep, J., and Havis, N. (2013). Controlling crop diseases using induced resistance: challenges for the future. *J. Exp. Bot.* 64, 1263–1280.doi: 10.1093/jxb/ert026

Watanabe, F. S., and Olsen, S. R. (1965). Test of an ascorbic acid method for determining phosphorus in water and NaHCO3 extracts from soil. *Soil Sci. Soc. Am. J.* 29, 677–680. doi: 10.2136/sssaj1965.03615995002900060025x

Wu, H. M., Wu, L. K., Wang, J. Y., Zhu, Q., Lin, S., Xu, J. J., et al. (2016). Mixed phenolic acids mediated proliferation of pathogens *Talaromyces helicus* and *Kosakonia sacchari* in continuously monocultured *Radix pseudostellariae* rhizosphere soil. *Front Microbiol.* 7:335. doi: 10.3389/fmicb.2016.00335

Wu, L. K., Chen, J., Wu, H. M., Qin, X. J., Wang, J. Y., Wu, Y. H., et al. (2016a). Insights into the regulation of rhizosphere bacterial communities by application of bio-organic fertilizer in *Pseudostellaria heterophylla* monoculture regime. *Front. Microbiol.* 7:1788. doi: 10.3389/fmicb.2016.01788

Wu, L. K., Chen, J., Wu, H. M., Wang, J. Y., Wu, Y. H., Lin, S., et al. (2016b). Effects of consecutive monoculture of *Pseudostellaria heterophylla* on soil fungal community as determined by pyrosequencing. *Sci. Rep. U. K.* 6:26601. doi: 10.1038/srep26601

Wu, L. K., Wang, J. Y., Huang, W. M., Wu, H. M., Chen, J., Yang, Y. Q., et al. (2016c). Plant-microbe rhizosphere interactions mediated by *Rehmannia glutinosa* root exudates under consecutive monoculture. *Sci. Rep. U. K.* 6:19101. doi: 10.1038/srep15871

Yang, X., Chen, L., Yong, X., and Shen, Q. (2011). Formulations can affect rhizosphere colonization and biocontrol efficiency of *Trichoderma harzianum* SQR-T037 against *Fusarium* wilt of cucumbers. *Biol. Fert. Soils* 47, 239–248. doi: 10.1007/s00374-010-0527-z

Yedidia, I., Benhamou, N., and Chet, I. (1999). Induction of defense responses in cucumber plants (*Cucumis sativus* L.) by the biocontrol agent *Trichoderma harzianum*. *Appl. Environ. Microb.* 65, 1061–1070. doi: 10.1128/AEM.65.3.1061-1070.1999

Yedidia, I., Srivastva, A. K., Kapulnik, Y., and Chet, I. (2001). Effect of *Trichoderma harzianum* on microelement concentrations and increased growth of cucumber plants. *Plant Soil* 235, 235–242. doi: 10.1023/a:1011990013955

Zhalnina, K., Louie, K. B., Hao, Z., Mansoori, N., Da Rocha, U. N., Shi, S., et al. (2018). Dynamic root exudate chemistry and microbial substrate preferences drive patterns in rhizosphere microbial community assembly. *Nat. Microbiol.* 3, 470–480. doi: 10.1038/s41564-018-0129-3

Zhang, Z. Y., and Lin, W. X. (2009). Continuous cropping obstacle and allelopathic autotoxicity of medicinal plants. *Chin. J. Eco Agric.* 17, 189–196. doi: 10.3724/sp.j.1011.2009.00189

Zhao, W. O., Pang, L., Dong, N., and Yang, S. (2015). LC-ESI-MS/MS analysis and pharmacokinetics of heterophyllin B, a cyclic octapeptide from *Pseudostellaria heterophylla* in rat plasma. *Biomed. Chromatog.* 29, 1693–1699. doi: 10.1002/bmc.3481

Zhao, Y. F., Thilmony, R., Bender, C. L., Schaller, A., He, S. Y., and Howe, G. A. (2003). Virulence systems of *Pseudomonas syringae* pv. tomato promote bacterial speck disease in tomato by targeting the jasmonate signaling pathway. *Plant J.* 36, 485–499. doi: 10.1046/j.1365-313X.2003.01895.x

Zhao, Y. P., Wu, L. K., Chu, L. X., Yang, Y. Q., Li, Z. F., Azeem, S., et al. (2014). Interaction of *Pseudostellaria heterophylla* with *Fusarium oxysporum* f.sp. *heterophylla* mediated by its root exudates in a consecutive monoculture system. *Sci. Rep. U. K.* 5:8197. doi: 10.1038/srep08197

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.

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: frontiersin.org/about/contact

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