

MICROBIAL MEDIATION OF CROP ABIOTIC STRESS TOLERANCE

EDITED BY: Alvaro Sanz-Saez, Mariangela Hungria, Maite Lacuesta
and Juan Manuel Ruiz-Lozano

PUBLISHED IN: Frontiers in Plant Science





frontiers

Frontiers eBook Copyright Statement

The copyright in the text of individual articles in this eBook is the property of their respective authors or their respective institutions or funders. The copyright in graphics and images within each article may be subject to copyright of other parties. In both cases this is subject to a license granted to Frontiers.

The compilation of articles constituting this eBook is the property of Frontiers.

Each article within this eBook, and the eBook itself, are published under the most recent version of the Creative Commons CC-BY licence.

The version current at the date of publication of this eBook is CC-BY 4.0. If the CC-BY licence is updated, the licence granted by Frontiers is automatically updated to the new version.

When exercising any right under the CC-BY licence, Frontiers must be attributed as the original publisher of the article or eBook, as applicable.

Authors have the responsibility of ensuring that any graphics or other materials which are the property of others may be included in the CC-BY licence, but this should be checked before relying on the CC-BY licence to reproduce those materials. Any copyright notices relating to those materials must be complied with.

Copyright and source acknowledgement notices may not be removed and must be displayed in any copy, derivative work or partial copy which includes the elements in question.

All copyright, and all rights therein, are protected by national and international copyright laws. The above represents a summary only. For further information please read Frontiers' Conditions for Website Use and Copyright Statement, and the applicable CC-BY licence.

ISSN 1664-8714

ISBN 978-2-88971-720-0

DOI 10.3389/978-2-88971-720-0

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

MICROBIAL MEDIATION OF CROP ABIOTIC STRESS TOLERANCE

Topic Editors:

Alvaro Sanz-Saez, Auburn University, United States

Mariangela Hungria, Embrapa Soja, Brazil

Maite Lacuesta, University of the Basque Country, Spain

Juan Manuel Ruiz-Lozano, Spanish National Research Council (CSIC), Spain

Citation: Sanz-Saez, A., Hungria, M., Lacuesta, M., Ruiz-Lozano, J. M., eds. (2021). Microbial Mediation of Crop Abiotic Stress Tolerance. Lausanne: Frontiers Media SA. doi: 10.3389/978-2-88971-720-0

Table of Contents

- 04 Responses of Arbuscular Mycorrhizal Symbiosis to Abiotic Stress: A Lipid-Centric Perspective**
Zengwei Feng, Xiaodi Liu, Honghui Zhu and Qing Yao
- 15 Plant Salinity Tolerance Conferred by Arbuscular Mycorrhizal Fungi and Associated Mechanisms: A Meta-Analysis**
Khondoker M. G. Dastogeer, Mst Ishrat Zahan, Md. Tahjib-Ul-Arif, Mst Arjina Akter and Shin Okazaki
- 37 Arbuscular Mycorrhizal Fungi Regulate Polyamine Homeostasis in Roots of Trifoliate Orange for Improved Adaptation to Soil Moisture Deficit Stress**
Ying-Ning Zou, Fei Zhang, Anoop K. Srivastava, Qiang-Sheng Wu and Kamil Kuča
- 48 Arbuscular Mycorrhiza Improves Photosynthesis and Restores Alteration in Sugar Metabolism in *Triticum aestivum* L. Grown in Arsenic Contaminated Soil**
Samta Gupta, Sarda Devi Thokchom and Rupam Kapoor
- 64 Soybean Inoculated With One *Bradyrhizobium* Strain Isolated at Elevated [CO₂] Show an Impaired C and N Metabolism When Grown at Ambient [CO₂]**
David Soba, Iker Aranjuelo, Bertrand Gakière, Françoise Gilard, Usue Pérez-López, Amaia Mena-Petite, Alberto Muñoz-Rueda, Maite Lacuesta and Alvaro Sanz-Saez
- 80 Rhizospheric *Bacillus amyloliquefaciens* Protects *Capsicum annuum* cv. *Geumsugangsan* From Multiple Abiotic Stresses via Multifarious Plant Growth-Promoting Attributes**
Elham Ahmed Kazerooni, Sajeewa S. N. Maharachchikumbura, Arjun Adhikari, Abdullah Mohammed Al-Sadi, Sang-Mo Kang, Lee-Rang Kim and In-Jung Lee
- 99 Plant Growth Promotion and Induction of Systemic Tolerance to Drought and Salt Stress of Plants by Quorum Sensing Auto-Inducers of the N-acyl-homoserine Lactone Type: Recent Developments**
Anton Hartmann, Sophia Klink and Michael Rothballer
- 105 Arbuscular Mycorrhiza-Mediated Regulation of Polyamines and Aquaporins During Abiotic Stress: Deep Insights on the Recondite Players**
Karuna Sharma, Samta Gupta, Sarda Devi Thokchom, Pooja Jangir and Rupam Kapoor
- 129 Mycorrhizal-Assisted Phytoremediation and Intercropping Strategies Improved the Health of Contaminated Soil in a Peri-Urban Area**
María Teresa Gómez-Sagasti, Carlos Garbisu, Julen Urrea, Fátima Míguez, Unai Artetxe, Antonio Hernández, Juan Vilela, Itziar Alkorta and José M. Becerril
- 147 Characterization of Arsenic-Resistant Endophytic Bacteria From Alfalfa and Chickpea Plants**
Hazhir Tashan, Behrouz Harighi, Jalal Rostamzadeh and Abdolbaset Azizi



Responses of Arbuscular Mycorrhizal Symbiosis to Abiotic Stress: A Lipid-Centric Perspective

Zengwei Feng^{1,2}, Xiaodi Liu^{1,2}, Honghui Zhu^{2*} and Qing Yao^{1*}

¹College of Horticulture, Guangdong Province Key Laboratory of Microbial Signals and Disease Control, South China Agricultural University, Guangzhou, China, ²State Key Laboratory of Applied Microbiology Southern China, Guangdong Provincial Key Laboratory of Microbial Culture Collection and Application, Guangdong Open Laboratory of Applied Microbiology, Guangdong Microbial Culture Collection Center (GDMCC), Guangdong Institute of Microbiology, Guangdong Academy of Sciences, Guangzhou, China

OPEN ACCESS

Edited by:

Juan Manuel Ruiz-Lozano,
Consejo Superior de Investigaciones
Científicas (CSIC), Spain

Reviewed by:

Alberto Bago,
Consejo Superior de Investigaciones
Científicas (CSIC), Spain
Rosa Porcel,
Universitat Politècnica de València,
Spain

*Correspondence:

Honghui Zhu
zhuhh@gdim.cn
Qing Yao
yaoqsc@scu.edu.cn

Specialty section:

This article was submitted to
Plant Abiotic Stress,
a section of the journal
Frontiers in Plant Science

Received: 17 July 2020

Accepted: 19 October 2020

Published: 12 November 2020

Citation:

Feng Z, Liu X, Zhu H and
Yao Q (2020) Responses of
Arbuscular Mycorrhizal Symbiosis to
Abiotic Stress: A
Lipid-Centric Perspective.
Front. Plant Sci. 11:578919.
doi: 10.3389/fpls.2020.578919

Arbuscular mycorrhizal (AM) fungi are one of the most important soil microbial resources that help host plants cope with various abiotic stresses. Although a tremendous number of studies have revealed the responses of AM fungi to abiotic stress and their beneficial effects transferred to host plants, little work has focused on the role of lipid metabolism in AM fungi under abiotic stress conditions. AM fungi contain a large amount of lipids in their biomass, including phospholipids (PLs) in their hyphal membranes and neutral lipids (NLs) in their storage structures (e.g., vesicles and spores). Recently, lipid transfer from plants to AM fungi has been suggested to be indispensable for the establishment of AM symbiosis, and extraradical hyphae are capable of directly taking up lipids from the environment. This experimental evidence highlights the importance of lipids in AM symbiosis. Moreover, abiotic stress reduces lipid transfer to AM fungi and promotes arbuscule collapse as well as the hydrolysis and conversion of PLs to NLs in collapsed arbuscules. Overall, this knowledge encourages us to rethink the responses of AM symbiosis to abiotic stress from a lipid-centric perspective. The present review provides current and comprehensive knowledge on lipid metabolism in AM fungi, especially in response to various abiotic stresses. A regulatory role of abscisic acid (ABA), which is considered a “stress hormone,” in lipid metabolism and in the resulting consequences is also proposed.

Keywords: abscisic acid, arbuscular mycorrhizae, abiotic stress, lipid metabolism, neutral lipids, phospholipids

INTRODUCTION

Arbuscular mycorrhizal (AM) fungi, which phylogenetically belong to the subphylum Glomeromycotina, can form mutualistic symbiotic associations with more than 80% of terrestrial plant species (Smith and Read, 2008; Spatafora et al., 2016). This extremely ancient (>450 million years) and coevolutionary relationship is considered the key factor in early plant colonization of land and has also been verified to be generally beneficial to both partners (Remy et al., 1994; Smith and Read, 2008). AM fungi are obligate biotrophic fungi that rely exclusively on carbon in the form of lipids and sugars from their host plants to sustain their growth, development, and function (Bago et al., 2003; Helber et al., 2011; Jiang et al., 2017; Luginbuehl et al., 2017). In return, AM fungi are capable of helping their host plants grow vigorously under a variety of abiotic stress conditions by mediating a series of complex signal communications and

enhancing the exchange of multiple substances between partners, which leads to enhanced physiological-biochemical traits and increased uptake of nutrients and water (Bitterlich et al., 2018; Begum et al., 2019; Evelin et al., 2019).

Abiotic stresses are widespread in terrestrial ecosystems and are becoming increasingly severe because of dramatic changes in the global climate, environmental pollution, and excessive human activities during the past several decades. In agricultural ecosystems, numerous studies have highlighted that AM fungi are capable of improving the tolerance of their host plants to drought (Grümbert et al., 2015; Li et al., 2019b), salinity (Augé et al., 2014; Chandrasekaran et al., 2019), heavy metals (Wu et al., 2018; Zhang et al., 2018, 2019; Rask et al., 2019), low nutrient availability (Tanaka and Yano, 2005; Garcia and Zimmermann, 2014; Koegel et al., 2015; Chu et al., 2020), extreme temperature (heat and cold; Zhu et al., 2011; Chen et al., 2013; Cabral et al., 2016; Mathur et al., 2018), acidic soils (low pH; Huang et al., 2017a; Wang et al., 2017; Feng et al., 2020), aluminum (Al) toxicity (Seguela et al., 2016; Aguilera et al., 2018), and pollutants (As and polycyclic aromatic hydrocarbons; Aranda et al., 2013; Calonne et al., 2014) to varying degrees. The mechanisms underlying the improved tolerance afforded by AM fungi involve increased nutrient levels, optimized water balance, enhanced photosynthesis, and increased reactive oxygen species (ROS) scavenging activity in plants. In contrast, however, the responses of AM fungi to abiotic stresses are largely neglected, hindering our understanding of AM symbiosis under stress conditions. Recently, lipids have been suggested to be the indispensable carbon forms delivered from plant cells to AM fungi (Jiang et al., 2017; Luginbuehl et al., 2017), highlighting the central role of lipids in regulating AM symbiosis. More recently, Feng et al. (2020) demonstrated that decreased transfer of lipids from root cells to AM fungi contributed to the inhibition of colonization and functionality of AM fungi in response to low-pH stress. Therefore, now is the right time to evaluate the role of lipids in AM symbiosis under various abiotic stress conditions. To address this topic, we discuss the cytobiochemical changes in plants and physiological changes in AM fungi in response to abiotic stress and focus on lipid metabolism in symbiosis.

CYTOBIOCHEMICAL CHANGES IN PLANTS IN RESPONSE TO ABIOTIC STRESS

The optimal growth status of plants requires precise cellular homeostasis achieved by a delicate balance between multiple pathways in various cellular compartments (Miller et al., 2010).

Abbreviations: ABA, Absciscic acid; AM, Arbuscular mycorrhiza; DAGs, Diacylglycerols; DGAT, Acyl-CoA:diacylglycerol acyltransferase; DGDG, Digalactosyldiacylglycerol; EH, Extraradical hyphae; G3P, Glycerol-3-phosphate; IH, Intraradical hyphae; NLs, Neutral lipids; PA, Phosphatidic acid; PC, Phosphatidylcholine; PDAT, Phospholipid:diacylglycerol acyltransferase; PE, Phosphatidylethanolamine; PLs, Phospholipids; ROS, Reactive oxygen species; SEs, Sterol esters; TAGs, Triacylglycerols.

This coordination may, however, be disrupted rapidly when plants are exposed to a series of abiotic stresses. As a consequence of adverse conditions, different cytobiochemical changes in plants emerge concomitantly. These changes mainly include ROS generation, membrane lipid peroxidation, and increases in abscisic acid (ABA), all of which exert dramatic and regulatory effects on AM symbiosis.

ROS generation is the most significant event in plant cells subjected to abiotic stress; this generation occurs in several main organelles, e.g., chloroplasts, mitochondria, and peroxisomes, as well as in the plasma membrane and apoplast. ROS at high doses (ROS bursts) are capable of causing oxidative damage to many biomacromolecules (e.g., membrane lipids, proteins, RNA, and DNA), which ultimately results in cellular damage and even death (Apel and Hirt, 2004; Miller et al., 2010; You and Chan, 2015). In addition, at low doses, ROS function as signaling molecules in the induction of pathogen resistance by AM fungi (Zhang et al., 2013; Zhu et al., 2015). The membrane structures of plant cells and subcellular organelles are mainly composed of lipids, e.g., polyunsaturated fatty acids, which are highly sensitive to ROS (Tsikas, 2017). Under abiotic stress conditions, excessive lipid peroxidation can alter the assembly, composition, structure, and dynamics (fluidity) of membranes, further leading to membrane damage. Polyunsaturated fatty acids, which are long-chain fatty acids with more than one double bond, are preferentially oxidized to the final form malondialdehyde, either by chemical reactions with ROS or by enzymatic reactions catalyzed by lipoxygenase in the lipid peroxidation process (Gaschler and Stockwell, 2017; Tsikas, 2017). In roots colonized by AM fungi, the plasma membrane encapsulating arbuscules is highly specialized and is referred to as the periarbuscular membrane. However, the lipid peroxidation of the periarbuscular membrane has not yet been explored, let alone that of the arbuscular membrane. ABA is commonly known as a “stress hormone” and plays a crucial role in the plant response to abiotic stress (Fujii et al., 2009; Kim et al., 2016; Huang et al., 2017b). Under weakly stressing conditions, elevated ABA induces a mild increase in ROS (Jiang and Zhang, 2002). Intriguingly, AM fungal colonization can also trigger an increase in ROS, which further induces localized and systemic resistance to pathogens (Zhu et al., 2015). However, long-term and severe stress conditions can result in ROS bursts and cell damage.

ARBUSCULAR MYCORRHIZAL FUNGAL RESPONSES TO ABIOTIC STRESS

Similar to their host plants, AM fungi also undergo various changes to acclimate abiotic stress. The adaptation of AM fungi to stressed conditions is mainly reflected by several different aspects, such as colonization, arbuscule formation, spore germination, and sporulation. In AM fungi, the hyphal membrane is composed of phospholipids (PLs), while spores contain a large amount of neutral lipids (NLs; Olsson and Johansen, 2000). Consequently, any changes in these structures are linked to the dynamics of lipid metabolism.

Many studies have shown that abiotic stress has an overall negative effect on mycorrhizal colonization (**Figure 1**). Salinity-alkalinity stress significantly inhibits the quantities of entry points on roots and vesicles inside roots (Ye et al., 2019). Mycorrhizal frequency (F%) and intensity (M%) significantly decreases with increasing salinity (Krishnamoorthy et al., 2014). Drought, low temperature, and heavy metals were found to suppress mycorrhizal colonization (Chen et al., 2013; He et al., 2017; Zhang et al., 2019). Acidic soil decreased F% by 4.39% and M% by 20.30% in pot experiments (Liu et al., 2020), which is in accordance with previous results in axenic culture (Wang et al., 2017). Vesicle abundance at a pH of 4.5 was shown to be approximately half of that at a pH of 6.5 (Feng et al., 2020). Even so, Wang et al. (2017) indicated that extraradical hyphae (EH) of *Rhizophagus irregularis* DAOM 197198 were more tolerant to low pH than were tomato roots in axenic culture. However, several studies showed that AM fungal colonization was unaffected or was promoted by abiotic stress (Nakatani et al., 2011; Li et al., 2016; Mo et al., 2016). Further analysis is needed because the results will vary depending on the timing of the observation.

Arbuscules are the central sites for nutrient exchange between two symbiotic partners and are therefore considered the core structures of AM symbiosis (Harrison, 2012; Luginbuehl and Oldroyd, 2017). The life span and functional lifetime of arbuscules are only 7.5–8.5 and 2–3 days, respectively (Alexander et al., 1989; Kobae and Hata, 2010), and abiotic stress greatly impedes arbuscule formation (**Figure 1**; Feng et al., 2020; Liu et al., 2020). A dramatic decrease in arbuscule abundance has been reported under an array of abiotic stresses, such as heavy metal pollution (Zhang et al., 2019), salt stress (Krishnamoorthy et al., 2014;

Ye et al., 2019), aluminum toxicity (Göransson et al., 2008), and low pH (Zhu et al., 2007). It is worth noting that the decrease in arbuscule abundance is much greater than that in the abundance of other fungal structures. For instance, arbuscule abundance decreased by 93.27% in contrast to 20.30% for mycorrhizal intensity when the symbiosis was exposed to low pH (Liu et al., 2020). The authors classified arbuscule development into five stages and demonstrated that fewer juvenile arbuscules could develop fully and reach mature status, while mature arbuscules were promoted to become senescent and collapsing in response to low-pH or acidic soil conditions (Feng et al., 2020; Liu et al., 2020). Gutjahr et al. (2012) demonstrated that the half-size ABC transporters STR1 and STR2 were indispensable for arbuscule formation in rice. Intriguingly, arbuscules stopped development but did not collapse in a *str/str2* mutant of *Medicago truncatula*, in which the transfer of lipids from host plants to AM fungi was greatly inhibited (Zhang et al., 2010). This different behavior of arbuscules can be explained by the presence of abiotic stresses or not. In *str* mutant experiment without stress, only the transfer of lipids to AM fungi is inhibited; however, low pH (abiotic stress) not only can inhibit the transfer of lipids to AM fungi but also promote the sporulation (Wang et al., 2017), which requires a huge amount of neutral lipids as storage substance and might drive the collapse of senescent arbuscules to release lipids.

Sporulation determines the spore density in the soil; however, AM fungal sporulation has been less investigated than colonization thus far. Krishnamoorthy et al. (2014) found that the spore density of *Glomus*, *Paraglomus*, *Acaulospora*, *Entrophospora*, *Gigaspora*, and *Scutellospora* exhibited a significantly negative correlation with soil salinity. Yang et al.

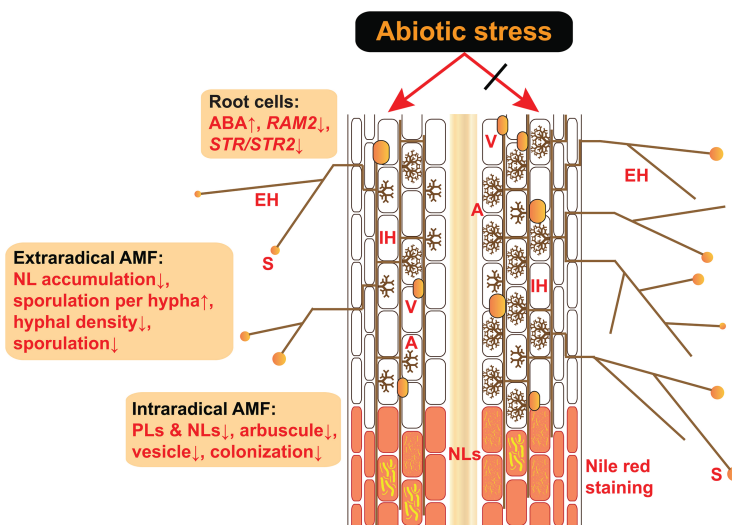


FIGURE 1 | Effects of abiotic stress on the intraradical and extraradical structures of AM fungi and their host plant root cells. Abiotic stress induces an ABA increase in host plants as well as decreased expression of *RAM2* and *STR/STR2*, both of which are induced specifically by AM fungi and encode enzymes responsible for lipid biosynthesis and transfer from the roots to AM fungi (Bravo et al., 2017; Feng et al., 2020). Under abiotic stress, the accumulations of PLs and NLs are reduced in the intraradical phase, in parallel with decreased mycorrhizal colonization (e.g., arbuscule abundance, vesicle abundance, and colonization rate). In the extraradical phase, the accumulation of NLs in the hyphae and hyphal density is reduced in response to abiotic stress. Although the sporulation per hypha is accelerated by increased ABA (Liu et al., 2020), the total sporulation is inhibited due to the reduction in hyphal density. A, arbuscules; V, vesicles; IH, intraradical hyphae; EH, extraradical hyphae; S, spores; NLs, neutral lipids; PLs, phospholipids; ABA, abscisic acid; AM, arbuscular mycorrhiza.

(2015) reported an overall trend that AM fungal spore density was higher in heavy metal-contaminated soils than in noncontaminated soils. Moreover, elevated temperature was shown to decrease spore density and diameter (Zhang et al., 2016). More intriguingly, a period of drought stress before harvest is frequently practiced to promote sporulation in AM fungal propagation systems (Selvakumar et al., 2018).

LIPIDS AS KEY NUTRIENTS FOR ARBUSCULAR MYCORRHIZAL FUNGI IN RESPONSE TO ABIOTIC STRESS

Lipids are the most abundant compounds in AM fungi; these lipids mainly include PLs and NLs, but there are also small amounts of other lipids (Bago et al., 2000; Olsson and Johansen, 2000; Wewer et al., 2014). Moreover, AM fungi do not synthesize lipids *de novo* but receive lipids from their hosts (Jiang et al., 2017), indicating the significance of lipids as key nutrients in AM symbiosis.

Phospholipids in the Membrane (Intraradical and Extraradical Hyphae, and Arbuscules)

The EH and intraradical hyphae (IH), particularly arbuscules, are characterized by large amounts of membrane area, which consists of PLs and phosphate-free lipids called glycolipids (Van Aarle and Olsson, 2003; Wewer et al., 2014). As polar lipid fractions in AM fungi, PLs comprise phosphatidylcholine (PC), phosphatidylethanolamine (PE), phosphatidylinositol, phosphatidylserine, and phosphatidic acid (PA), while glycolipids comprise acylated sterol glucoside, glucosylceramide, and sterol glucoside (Wewer et al., 2014). PLs account for approximately 1–2% of the total lipids in AM fungal biomass, but glycolipids are even more negligible (Jabaji-Hare, 1988; Gaspar et al., 1994; Wewer et al., 2014). PC is the most abundant component of PLs in EH (>60%) and colonized roots (>40%), and furthermore, lyso-PC is considered a signaling molecule during mycorrhization (Drissner et al., 2007; Wewer et al., 2014). However, Gaspar et al. (1997) found that the total contents of PC and PE continuously increased and that the relative contents of PC and PE were dynamic in the roots with AM fungal colonization. Compared with that of PE, the content of PC was greater in the first 3 months, whereas the content of PE was greater in colonized roots after 4 months of AM fungal inoculation (Gaspar et al., 1997). At the molecular level, the expression of monomethyl-PE/dimethyl-PE methyltransferase, which is involved in PC synthesis from PE, and lysophospholipid acyltransferase, which is involved in the interconversion of PC (or PE) and lyso-PC (or lyso-PE), were detected both in colonized roots and in EH (Wewer et al., 2014).

The conversion of PLs to glycolipids is vital in the membranes of both plants and AM during P deprivation to conserve P (Härtel et al., 2000; Wewer et al., 2011, 2014; Pant et al., 2015). Digalactosyldiacylglycerol (DGDG) decreases in parallel

with an increase in PC and PE (Wewer et al., 2014). Moreover, relatively low expression of the two DGDGs that encode DGDG synthases was observed in colonized roots and noncolonized roots under high-phosphate levels in *Lotus japonicus* due to an increased phosphate supply for PL synthesis (Wewer et al., 2014). Recently, Feng et al. (2020) reported a significant decrease in AM fungal PLs and an accumulation of NLs in colonized roots under low-pH stress (pH 4.5 vs. 6.5), highlighting the interconversion of lipid fractions in AM fungi in response to abiotic stress.

Neutral Lipids in Storage Structures (Vesicles and Spores)

AM fungi accumulate large amounts of nonpolar storage lipids, i.e., NLs and triacylglycerols (TAGs), mainly in vesicles, extraradical spores, intraradical spores (in some species such as those of *Glomus* and *Rhizophagus*), IH, and EH (Gaspar et al., 1994, 1997; Bago et al., 2000; Wewer et al., 2014). Vesicles are round, elliptical, or irregular in shape and are considered to be the primary storage structures of AM fungi inside roots (Jabaji-Hare et al., 1984; Jabaji-Hare, 1988). Jabaji-Hare (1988) found that NLs in spores and vesicles of several species of *Glomus* and *Rhizophagus* and in spores of *Gigaspora margarita* accounted for 96–98% of total lipids, while polar lipids accounted for 2–4% (Jabaji-Hare, 1988). A continuous decrease in NLs and an increase in PLs were observed in spores during germination (Gaspar et al., 1994). During AM fungal spore germination, lipid synthesis is largely or entirely confined to PL synthesis and, consequently, membrane production (Bago et al., 1999). In AM fungal extraradical structures, spores and hyphae accounted for 90.7 and 9.3% of the total biomass, respectively (Olsson and Johansen, 2000). TAGs are the predominant form of AM fungal lipids; TAGs include 16:0 (palmitic acid) and 16:1 ω 5 (palmitavaccenic acid) acyl groups (Jabaji-Hare, 1988; Gaspar et al., 1997; Bago et al., 2002; Wewer et al., 2014; Roth and Paszkowski, 2017). Other nonpolar lipids, such as free fatty acids (9–19% of lipids), monoacylglycerols (<9% of lipids), sterol esters (SEs; <7% of lipids), and diacylglycerols (DAGs; <3% of lipids) were also detected in these storage structures (Jabaji-Hare, 1988).

Previous isotopic labeling experiments and advanced imaging technologies have shown that NLs are synthesized in IH and then transported to EH to sustain extraradical hyphal growth, the formation of new spores, and their subsequent germination (Pfeffer et al., 1999; Bago et al., 2002; Kobae et al., 2014). A continuous mycorrhizal colonization experiment showed a higher accumulation of TAGs than of PLs in EH during the first 3 months after AM fungal inoculation, which accounted for more than 90% of the total lipids in the EH (Gaspar et al., 1997); however, other nonpolar lipids, such as DAGs, free sterols, SEs and PLs, were less abundant (Wewer et al., 2014). In these processes, DAGs serve as the immediate precursor of TAGs, and SEs are involved in the regulation of membrane free sterol homeostasis (Bouvier-Navé et al., 2010). In contrast, the front fragment of growing EH contains fewer NLs than do the rear fragments, probably due to the consumption of

NLs for hyphal elongation and sporulation, which implies that continuous NL delivery is probably essential to maintain AM fungal growth (Bago et al., 2002; Kobae et al., 2014). In general, the accumulation of NLs and their conversion to PLs showed different patterns in the growing hyphae and in senescent and sporulating hyphae (Figure 2; Bago et al., 2002), with the latter probably accelerated by increased ABA in response to abiotic stress.

Potential Conversion of Phospholipids to Neutral Lipids in Arbuscular Mycorrhizal Fungi Under Abiotic Stress

Arbuscules are specialized IHs that account for a large proportion of IHs in terms of membrane surface area in colonized roots. Arbuscule abundance is strongly correlated with the accumulation of PLs across different AM fungal species (Van Aarle and Olsson, 2003; Feng et al., 2020). However, arbuscules are short lived, with a life span of 7.5–8.5 days (Alexander et al., 1989; Kobae and Hata, 2010). This poses a critical issue concerning the fate of PLs after the collapse of arbuscules. A double-staining experiment with vital staining of succinate dehydrogenase and lipid staining (Nile red) revealed that a large number of NLs mainly occurred in senescent arbuscules accompanied by waning vitality and function (Kobae et al., 2014). In line with this result, Feng et al. (2020) observed that there was a distinct negative relationship between arbuscule intactness and NL accumulation, as revealed by fungal cell wall staining (WGA-488) and lipid staining. Furthermore, more dynamic and direct evidence through a live imaging technique indicated that NLs were

transported subsequently from IH to EH (Kobae et al., 2014). These observations support that PLs in IH, especially in arbuscules, are hydrolyzed and converted to NLs for recycling. Li et al. (2019a) suggested that a purple acid phosphatase of plant origin was involved in the degradation of arbuscules; however, this was not supported by other experiments (Feng et al., 2020). Therefore, additional work is needed to elucidate the enzymatic mechanism involving the hydrolysis of PLs in collapsing arbuscules.

Arbuscules have a short life span, and furthermore, abiotic stress is capable of accelerating the senescence and collapse of arbuscules. Liu et al. (2020) found that acidic soil resulted in more senescent and collapsed arbuscules in colonized roots. Consistent with this result, Feng et al. (2020) visualized the lipid dynamics in root cells and found that NLs occurred only in senescent and collapsed arbuscule-containing cells. In this scenario, it is highly important to link lipid metabolism in AM fungi with abiotic stress. Molecular analysis indicated that genes encoding a glycerol-3-phosphate acyltransferase (RAM2, involved in lipid biosynthesis) and ATP-binding cassette transporters (STR/STR2, involved in lipid transfer) in colonized roots are greatly inhibited by low pH (Feng et al., 2020). At the physiological level, abiotic stress has been shown to induce ROS generation (Benabdellah et al., 2009) and the visual accumulation of ROS in different structures of AM fungi (Fester and Hause, 2005), leading to membrane lipid peroxidation (malondialdehyde accumulation; González-Guerrero et al., 2007, 2010; Debiante et al., 2011) and a decrease in the accumulation of PLs/PC in AM fungi (Debiante et al., 2011; Calonne et al., 2014; Feng et al., 2020).

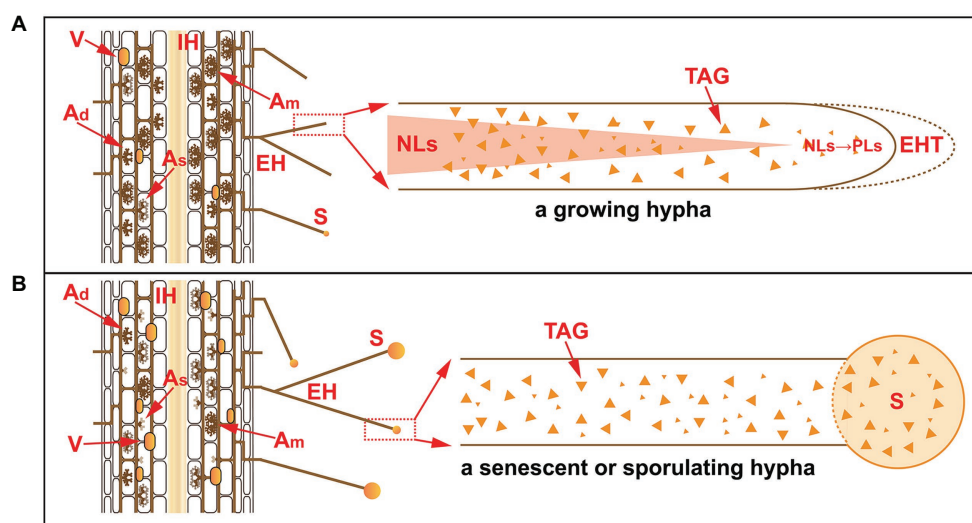


FIGURE 2 | The accumulation of NLs in extraradical hyphae in a growing hypha (A) and in a senescent or sporulating hypha (B). Under normal conditions, the life span of arbuscules is 7.5–8.5 days, during which time different stages occur, e.g., developing arbuscules (A_d), mature arbuscules (A_m), and senescent arbuscules (A_s). The PLs in senescent arbuscules are subjected to hydrolysis and converted to NLs for recycling. In the extraradical phase, NLs accumulate but are subsequently consumed. In growing hyphae, NLs are converted to PLs at the front fragment of the hyphae for the synthesis of hyphal tips. In senescent or sporulating hyphae, NLs are transported to developing spores as storage substances. V, vesicles; IH, intraradical hyphae; EH, extraradical hyphae; EHT, extraradical hyphal tip; S, spore; TAG, triacylglycerol; NLs, neutral lipids; PLs, phospholipids.

Evidence of PL dynamics in response to abiotic stress is limited; however, the decrease in PLs is supported by experiments in other organisms. In green algae and *Arabidopsis thaliana*, for example, PLs decrease and are replaced by glycolipids in the membranes, in parallel with the dramatic accumulation in TAGs under P deficiency (Gong et al., 2013; Iwai et al., 2014; Pant et al., 2015). Moreover, abiotic stress impedes P acquisition by AM fungal EH (Wang et al., 2017), which may reduce the P source for PL synthesis in AM fungi. Therefore, abiotic stress can accelerate PL degradation and TAG accumulation, namely, the conversion of PLs to NLs in AM fungi (Calonne et al., 2014; Feng et al., 2020). This conversion can provide at least two benefits: the release of P for recycling under P starvation induced by abiotic stress and an increase in NLs for sporulation in response to abiotic stress.

How NLs are produced in collapsed arbuscules remains unknown. It is well acknowledged that a gene encoding cytosolic multidomain fatty acid synthase responsible for the *de novo* synthesis of the bulk of fatty acids is lost in the genome of *Rhizophagus irregularis* (Tisserant et al., 2013; Wewer et al., 2014). Therefore, the transfer of lipids from plant cells to arbuscules is indispensable for AM symbiosis (Jiang et al., 2017). Recently, axenic culture of *R. irregularis* indicated that EH can take up lipids directly from the media and that myristate is the most effective fatty acid to promote hyphal growth, while (S)-12-methyltetradecanoic acid promotes both hyphal growth and sporulation (Kameoka et al., 2019; Sugiura et al., 2019).

These results highlight the significance of lipids in AM symbiosis. Under stress conditions, PLs in the hyphal membranes (including arbuscules) undergo hydrolysis and give rise to glycerol-3-phosphate. Given the conversion of PLs to NLs and the accumulation of NLs in AM fungi, as well as the synthesis of NLs in plants and other microbes (Dahlqvist et al., 2000; Oelkers et al., 2000; Arabolaza et al., 2008; Banaś et al., 2013), we speculate that AM fungi employ two main pathways to produce NLs by utilizing PLs (PC, lyso-PC, PE, PA, and lyso-PA) as substrates in the endoplasmic reticulum. In the partial Kennedy pathway, acyl-CoA is utilized for PA synthesis by esterification to the sn-2 positions of lyso-PA by acyl-CoA:lyso-PA acyltransferase. DAGs are subsequently synthesized by the dephosphorylation of PA phosphatase. Finally, DAGs are utilized for TAG synthesis with the participation of acyl-CoA by acyl-CoA:diacylglycerol acyltransferase (DGAT; **Figure 3A**; Bates and Browse, 2012). In the acyl editing pathway, FAs provided by PC or PE and DAGs are utilized for TAG and lyso-PC synthesis by phospholipid:diacylglycerol acyltransferase (PDAT; Dahlqvist et al., 2000; Oelkers et al., 2000; Bates and Browse, 2012). However, reesterification of lyso-PC by acyl-CoA:lyso-PC acyltransferase regenerates PC (**Figure 3B**; Millar et al., 2000; Bates and Browse, 2012). Considering that PC and PE are the two most abundant species of PLs, we infer that the acyl editing pathway is likely the key pathway involved in the conversion of PLs to NLs in AM fungi. The key genes encoding these proteins mentioned above are present in the genome of

Endoplasmic reticulum

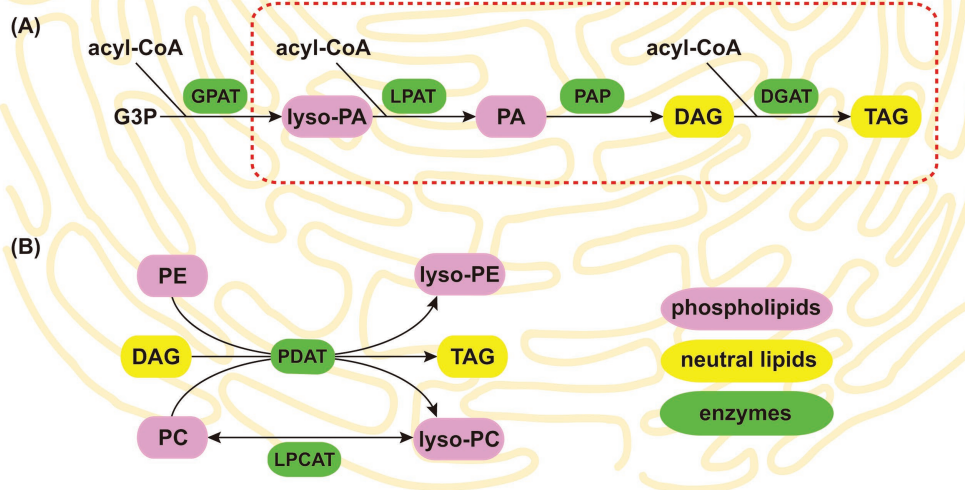


FIGURE 3 | Putative conversion of PLs to NLs in the AM fungal endoplasmic reticulum. Two main pathways include the partial Kennedy pathway, shown as the red dotted line (A), and the acyl editing pathway (B). Considering that PC and PE are the two most abundant species of PLs, the acyl editing pathway is likely the key pathway for converting PLs to NLs in AM fungi. The background represents the AM fungal endoplasmic reticulum. The purple, yellow, and green rectangles represent PLs, NLs, and enzymes, respectively. PA, phosphatidic acid; lyso-PA, lyso-phosphatidic acid; PC, phosphatidylcholine; lyso-PC, lyso-phosphatidylcholine; PE, phosphatidylethanolamine; lyso-PE, lyso-phosphatidylethanolamine; DAG, diacylglycerol; TAG, triacylglycerol; G3P, glycerol-3-phosphate; GPAT, acyl-CoA: glycerol-3-phosphate acyltransferase; LPAT, acyl-CoA:lyso-phosphatidic acid acyltransferase; PAP, phosphatidic acid phosphatase; DGAT, acyl-CoA:diacylglycerol acyltransferase; PDAT, phospholipid:diacylglycerol acyltransferase; LPCAT, acyl-CoA:lyso-phosphatidylcholine acyltransferase; PL, phospholipid; NL, neutral lipid; AM, arbuscular mycorrhizal.

R. irregularis (Tisserant et al., 2013); however, more work is needed to explore this hypothesis. Although few studies have focused on the effects of abiotic stresses on these genes in AM fungi, several of them are upregulated in plants and other microbes, which occurs simultaneously with the accumulation in NLs under abiotic stress (Mus et al., 2013; Zienkiewicz et al., 2016; Yuan et al., 2017; Tan et al., 2018). However, the functional characterization of these genes in AM fungi under various abiotic stresses remains unexplored.

ABSCISIC ACID REGULATION

As a key “stress hormone,” ABA dramatically accumulates in plants to cope with abiotic stress (Fujii et al., 2009; Kim et al., 2016; Huang et al., 2017b). Apart from its role in plant growth and development, ABA accumulation also directly or indirectly affects plant-associated microbes, such as phytopathogens (Asselbergh et al., 2008) and symbiotic partners, including AM fungi (Liu et al., 2019). Fungal sporulation is a crucial reproductive process and one of the mechanisms by which fungi respond to various adverse conditions, in many cases producing chlamydospores (Spraker et al., 2016; Dijksterhuis 2018). Liu et al. (2019) demonstrated for the first time that exogenous ABA was able to directly improve AM fungal sporulation during both asymbiotic and presymbiotic statuses, highlighting the direct effect of ABA on spores or germ tubes. In AM fungal propagation systems, several weeks of drought stress before harvest is generally believed to induce sporulation (Selvakumar et al., 2016, 2018). ABA accumulation is one of the main responses in plants to drought stress. Therefore, it is likely that there is a positive relationship between ABA and AM fungal sporulation. Nevertheless, it is worth noting that the total spore number decreases under abiotic stress, whereas the sporulation per hypha increases (Debiane et al., 2011), which is supported by the study by Wang et al. (2017). This implies that ABA induces more efficient reproductive growth of AM fungi under abiotic stress. Sporulation involves a large consumption of NLs, which partly originate from the hydrolysis of PLs in arbuscules in response to abiotic stress.

However, in terms of different AM fungal structures, the contents of ABA and ABA-GE as the storage form of ABA in EH were approximately 3-fold and 2.5-fold higher than those in AM fungal spores, respectively (Esch et al., 1994). Remarkably, the content of ABA in AM fungi is more than one order of magnitude higher than that in plant roots (Esch et al., 1994). Pons et al. (2020) provided direct evidence that AM fungi can synthesize cytokinins, auxin, gibberellins, and ethylene but not ABA, which indicates that ABA in AM fungi is derived from plants. Therefore, functional genes responsible for ABA biosynthesis in AM fungi or transporters responsible for transporting ABA from the roots to AM fungi remain unexplored. Additionally, the allocation of ABA among different AM fungal structures (e.g., intraradical vs. extraradical structures) under abiotic stress has not yet been investigated.

To date, no studies have focused on the relationship between ABA accumulation and the conversion of PLs to NLs in AM fungi under abiotic stress. However, the biosynthesis of NLs induced by ABA has been reported in plants, with the plant-AM fungal interaction not considered. In the ABA signaling pathway, the transcription factors *ABSCISIC ACID INSENSITIVE 4 (ABI4)* and *ABI5* can synergistically trigger stress-induced *DGAT1* expression and TAG accumulation (Yang et al., 2011; Kong et al., 2013). Under nitrogen limitation, *ABI4* promotes TAG accumulation by upregulating the expression of *DGAT1* in *Arabidopsis* (Yang et al., 2011). Recently, Tan et al. (2018) demonstrated that *DGAT1* is critical for freezing tolerance of plants, acting by balancing TAG and PA production in *Arabidopsis*. Additionally, the application of exogenous ABA resulted in an increase in *DGAT1* expression and accumulation of NLs in *Arabidopsis*, which occurred simultaneously with the effects of salt and sorbitol stress (Kong et al., 2013). Various abiotic stresses, including cold, drought, salt, and osmotic stress, increased *PDAT* member expression and TAG accumulation in *Camelina sativa* (Yuan et al., 2017). Multiple analyses and experiments have also shown that *DGAT1*- and *PDAT1*-mediated conversion of membrane lipids into TAGs was enhanced by abiotic stresses in both microalgae and *Arabidopsis* (Yoon et al., 2012; Li et al., 2014; Lee et al., 2019). Based on this evidence, it is likely that ABA is capable of promoting the conversion of PLs to NLs in AM fungi in parallel with the collapse of arbuscules under abiotic stress.

CONCLUSIONS AND PERSPECTIVES

To date, studies on lipid metabolism in AM fungi in response to abiotic stress are quite rare, given that lipids are an essential component of AM fungi. In this review, we analyze the existing literature, especially studies recently published in this respect, and we speculate that the synthesis and metabolism of lipids may play a key role in AM fungi to acclimate to abiotic stress. In general, under abiotic stress, the expression of AM fungus-specific induced genes responsible for lipid biosynthesis (*RAM2*) and transfer (*STR/STR2*) from the roots to AM fungi dramatically decreases in parallel with a severe suppression of mycorrhizal colonization and especially a more severe suppression of arbuscule abundance. This may be attributed to the enormous demand for PLs during arbuscule formation. Concomitantly, vesicle formation is suppressed because NLs are required in large amounts for this process. Under abiotic stress conditions, arbuscules become senescent and collapse at a relatively fast rate, which is accompanied by the hydrolysis and conversion of PLs to NLs. Neutral lipids are exported outside through EH to sustain the formation of new spores, which more easily occurs under stress conditions. ABA may act as a signaling molecule during this process, which promotes sporulation in AM fungi.

Most, if not all, relevant studies have concentrated on the plant side with respect to the symbiosis of AM; few studies have paid close attention to the fungal side. This may be due to the impedance of pure cultures of AM fungi.

Since some interesting work has recently highlighted pure cultures of AM fungi, especially those concerning the uptake of environmental lipids by fungal hyphae (Sugiura et al., 2019) and the promotive effects of ABA on sporulation (Liu et al., 2019), now is the right time to devote increased effort to strengthen these efforts. For instance, it is essential to clarify whether the enzymes hydrolyzing PLs in senescent arbuscules originate from plants or AM fungi. If they are of plant origin, the elaborate cooperation between two symbiotic partners and signaling is attractive. Second, it is of the utmost importance to explore where ABA in AM fungal structures originates from and what results in the different distribution of ABA in various AM fungal structures, as revealed by Esch et al. (1994). More importantly, whether the process by which NLs produced from the hydrolysis of PLs in collapsed arbuscules are transported to the extraradical spores instead of the intraradical vesicles is related to the different distribution of ABA in intraradical and extraradical fungal structures merits further study. Overall, more studies,

especially those on the side of AM fungi, are needed to provide insights into the lipid biology of AM fungi.

AUTHOR CONTRIBUTIONS

ZF organized and drafted the manuscript. XL helped in organizing this manuscript. HZ and QY proposed the concept and conceived the structure of this manuscript. QY contributed to the editing of the manuscript. All authors contributed to the article and approved the submitted version.

FUNDING

This work was financially supported by the Natural Science Foundation of China (No. 42077040) and Guangdong Technological Innovation Strategy of Special Funds (Key Areas of Research and Development Program, grant no. 2018B020205003).

REFERENCES

- Aguilera, P., Larsen, J., Borie, F., Berríos, D., Tapia, C., and Cornejo, P. (2018). New evidences on the contribution of arbuscular mycorrhizal fungi inducing Al tolerance in wheat. *Rhizosphere* 5, 43–50. doi: 10.1016/j.rhishp.2017.11.002
- Alexander, T., Toth, R., Meier, R., and Weber, H. C. (1989). Dynamics of arbuscule development and degeneration in onion, bean, and tomato with reference to vesicular-arbuscular mycorrhizae in grasses. *Can. J. Bot.* 67, 2505–2513. doi: 10.1139/b89-320
- Apel, K., and Hirt, H. (2004). Reactive oxygen species: metabolism, oxidative stress, and signal transduction. *Annu. Rev. Plant Biol.* 55, 373–399. doi: 10.1146/annurev.arplant.55.031903.141701
- Arabolaza, A., Rodriguez, E., Altabe, S., Alvarez, H., and Gramajo, H. (2008). Multiple pathways for triacylglycerol biosynthesis in *Streptomyces coelicolor*. *Appl. Environ. Microbiol.* 74, 2573–2582. doi: 10.1128/AEM.02638-07
- Aranda, E., Scervino, J. M., Godoy, P., Reina, R., Ocampo, J. A., Wittich, R. M., et al. (2013). Role of arbuscular mycorrhizal fungus *Rhizophagus custos* in the dissipation of PAHs under root-organ culture conditions. *Environ. Pollut.* 181, 182–189. doi: 10.1016/j.envpol.2013.06.034
- Asselbergh, B., De Vleeschauwer, D., and Höfte, M. (2008). Global switches and fine-tuning—ABA modulates plant pathogen defense. *Mol. Plant Microbe Interact.* 21, 709–719. doi: 10.1094/MPMI-21-6-0709
- Augé, R. M., Toler, H. D., and Saxton, A. M. (2014). Arbuscular mycorrhizal symbiosis and osmotic adjustment in response to NaCl stress: a meta-analysis. *Front. Plant Sci.* 5:562. doi: 10.3389/fpls.2014.00562
- Bago, B., Pfeffer, P. E., Abubaker, J., Jun, J., Allen, J. W., Brouillette, J., et al. (2003). Carbon export from arbuscular mycorrhizal roots involves the translocation of carbohydrate as well as lipid. *Plant Physiol.* 131, 1496–1507. doi: 10.1104/pp.102.007765
- Bago, B., Pfeffer, P. E., Douds, D. D., Brouillette, J., Bécard, G., and Shachar-Hill, Y. (1999). Carbon metabolism in spores of the arbuscular mycorrhizal fungus *Glomus intraradices* as revealed by nuclear magnetic resonance spectroscopy. *Plant Physiol.* 121, 263–272. doi: 10.1104/pp.121.1.263
- Bago, B., Pfeffer, P. E., and Shachar-Hill, Y. (2000). Carbon metabolism and transport in arbuscular mycorrhizas. *Plant Physiol.* 124, 949–958. doi: 10.1104/pp.124.3.949
- Bago, B., Zipfel, W., Williams, R. M., Jun, J., Arreola, R., Lammers, P. J., et al. (2002). Translocation and utilization of fungal storage lipid in the arbuscular mycorrhizal symbiosis. *Plant Physiol.* 128, 108–124. doi: 10.1104/pp.010466
- Banaś, W., García, A. S., Banaś, A., and Szymne, S. (2013). Activities of acyl-CoA:diacylglycerol acyltransferase (DGAT) and phospholipid:diacylglycerol acyltransferase (PDAT) in microsomal preparations of developing sunflower and safflower seeds. *Planta* 237, 1627–1636. doi: 10.1007/s00425-013-1870-8
- Bates, P. D., and Browse, J. (2012). The significance of different diacylglycerol synthesis pathways on plant oil composition and bioengineering. *Front. Plant Sci.* 3:147. doi: 10.3389/fpls.2012.00147
- Begum, N., Qin, C., Ahanger, M. A., Raza, S., Khan, M. I., Ashraf, M., et al. (2019). Role of arbuscular mycorrhizal fungi in plant growth regulation: implications in abiotic stress tolerance. *Front. Plant Sci.* 10:1068. doi: 10.3389/fpls.2019.01068
- Benabdellah, K., Merlos, M., Azconaguilar, C., and Ferrol, N. (2009). GintGRX1, the first characterized glomeromycotan glutaredoxin, is a multifunctional enzyme that responds to oxidative stress. *Fungal Genet. Biol.* 46, 94–103. doi: 10.1016/j.fgb.2008.09.013
- Bitterlich, M., Roupael, Y., Graefe, J., and Franken, P. (2018). Arbuscular mycorrhizas: a promising component of plant production systems provided favorable conditions for their growth. *Front. Plant Sci.* 9:1329. doi: 10.3389/fpls.2018.01329
- Bouvier-Navé, P., Berna, A., Noiriel, A., Compagnon, V., Carlsson, A. S., Banas, A., et al. (2010). Involvement of the phospholipid sterol acyltransferase 1 in plant sterol homeostasis and leaf senescence. *Plant Physiol.* 152, 107–119. doi: 10.1104/pp.109.145672
- Bravo, A., Brands, M., Wewer, V., Dörmann, P., and Harrison, M. J. (2017). Arbuscular mycorrhiza-specific enzymes FatM and RAM2 fine-tune lipid biosynthesis to promote development of arbuscular mycorrhiza. *New Phytol.* 214, 1631–1645. doi: 10.1111/nph.14533
- Cabral, C., Sabine, R., Ivanka, T., and Bernd, W. (2016). Arbuscular mycorrhizal fungi modify nutrient allocation and composition in wheat (*Triticum aestivum* L.) subjected to heat-stress. *Plant and Soil* 408, 385–399. doi: 10.1007/s11104-016-2942-x
- Calonne, M., Fontaine, J., Debiane, D., Laruelle, F., Grandmougin-Ferjani, A., and Sahraoui, A. L. H. (2014). The arbuscular mycorrhizal *Rhizophagus irregularis* activates storage lipid biosynthesis to cope with the benzo[a]pyrene oxidative stress. *Phytochemistry* 97, 30–37. doi: 10.1016/j.phytochem.2013.10.014
- Chandrasekaran, M., Chanratana, M., Kim, K., Seshadri, S., and Sa, T. (2019). Impact of arbuscular mycorrhizal fungi on photosynthesis, water status, and gas exchange of plants under salt stress—a meta-analysis. *Front. Plant Sci.* 10:457. doi: 10.3389/fpls.2019.00457
- Chen, S., Jin, W., Liu, A., Zhang, S., Liu, D., Wang, F., et al. (2013). Arbuscular mycorrhizal fungi (AMF) increase growth and secondary metabolism in cucumber subjected to low temperature stress. *Sci. Hortic.* 160, 222–229. doi: 10.1016/j.scienta.2013.05.039
- Chu, Q., Zhang, L., Zhou, J., Yuan, L., Chen, F., Zhang, F., et al. (2020). Soil plant-available phosphorus levels and maize genotypes determine the phosphorus acquisition efficiency and contribution of mycorrhizal pathway. *Plant and Soil* 449, 357–371. doi: 10.1007/s11104-020-04494-4

- Dahlqvist, A., Stahl, U., Lenman, M., Banas, A., Lee, M., Sandager, L., et al. (2000). Phospholipid:diacylglycerol acyltransferase: an enzyme that catalyzes the acyl-CoA-independent formation of triacylglycerol in yeast and plants. *Proc. Natl. Acad. Sci. U. S. A.* 97, 6487–6492. doi: 10.1073/pnas.120067297
- Debiane, D., Calonne, M., Fontaine, J., Laruelle, F., Grandmougin-Ferjani, A., and Sahraoui, A. L. H. (2011). Lipid content disturbance in the arbuscular mycorrhizal, *glomus irregulare* grown in monoxenic conditions under PAHs pollution. *Fungal Biol.* 115, 782–792. doi: 10.1016/j.funbio.2011.06.003
- Dijksterhuis, J. (2018). Fungal spores: highly variable and stress-resistant vehicles for distribution and spoilage. *Food Microbiol.* 81, 2–11. doi: 10.1016/j.fm.2018.11.006
- Drissner, D., Kunze, G., Callewaert, N., Gehrig, P., Tamasloukht, M. B., Boller, T., et al. (2007). Lyso-phosphatidylcholine is a signal in the arbuscular mycorrhizal symbiosis. *Science* 318, 265–268. doi: 10.1126/science.1146487
- Esch, H., Hundeshagen, B., Schneider-Poetsch, H., and Bothe, H. (1994). Demonstration of abscisic acid in spores and hyphae of the arbuscular-mycorrhizal fungus *Glomus* and in the N_2 -fixing cyanobacterium *Anabaena variabilis*. *Plant Sci.* 99, 9–16. doi: 10.1016/0168-9452(94)90115-5
- Evelin, H., Devi, T. S., Gupta, S., and Kapoor, R. (2019). Mitigation of salinity stress in plants by arbuscular mycorrhizal symbiosis: current understanding and new challenges. *Front. Plant Sci.* 10:470. doi: 10.3389/fpls.2019.00470
- Feng, Z., Liu, X., Feng, G., Zhu, H., and Yao, Q. (2020). Linking lipid transfer with reduced arbuscule formation in tomato roots colonized by arbuscular mycorrhizal fungus under low pH stress. *Environ. Microbiol.* 22, 1036–1051. doi: 10.1111/1462-2920.14810
- Fester, T., and Hause, G. (2005). Accumulation of reactive oxygen species in arbuscular mycorrhizal roots. *Mycorrhiza* 15, 373–379. doi: 10.1007/s00572-005-0363-4
- Fujii, H., Chinnusamy, V., Rodrigues, A., Rubio, S., Antoni, R., Park, S. Y., et al. (2009). In vitro reconstitution of an abscisic acid signalling pathway. *Nature* 462, 660–664. doi: 10.1038/nature08599
- Garcia, K., and Zimmermann, S. D. (2014). The role of mycorrhizal associations in plant potassium nutrition. *Front. Plant Sci.* 5:337. doi: 10.3389/fpls.2014.00337
- Gaschler, M. M., and Stockwell, B. R. (2017). Lipid peroxidation in cell death. *Biochem. Biophys. Res. Commun.* 482, 419–425. doi: 10.1016/j.bbrc.2016.10.086
- Gaspar, M. L., Pollero, R. J., and Cabello, M. N. (1994). Triacylglycerol consumption during spore germination of vesicular-arbuscular mycorrhizal fungi. *J. Am. Oil Chem. Soc.* 71, 449–452. doi: 10.1007/BF02540529
- Gaspar, L., Pollero, R., and Cabello, M. (1997). Variations in the lipid composition of alfalfa roots during colonization with the arbuscular mycorrhizal fungus *Glomus versiforme*. *Mycologia* 89, 37–42. doi: 10.1080/00275514.1997.12026752
- Gong, Y., Guo, X., Wan, X., Liang, Z., and Jiang, M. (2013). Triacylglycerol accumulation and change in fatty acid content of four marine oleaginous microalgae under nutrient limitation and at different culture ages. *J. Basic Microbiol.* 53, 29–36. doi: 10.1002/jobm.201100487
- González-Guerrero, M., Benabdellah, K., Valderas, A., Azcón-Aguilar, C., and Ferrol, N. (2010). *GintABC1* encodes a putative ABC transporter of the MRP subfamily induced by Cu, Cd, and oxidative stress in *Glomus irregulare*. *Mycorrhiza* 20, 137–146. doi: 10.1007/s00572-009-0273-y
- González-Guerrero, M., Cano, C., Azcón-Aguilar, C., and Ferrol, N. (2007). *GintMT1* encodes a functional metallothionein in *Glomus irregulare* that responds to oxidative stress. *Mycorrhiza* 17, 327–335. doi: 10.1007/s00572-007-0108-7
- Göransson, P., Olsson, P. A., Postma, J., and Falkengren-Grerup, U. (2008). Colonisation by arbuscular mycorrhizal and fine endophytic fungi in four woodland grasses-variation in relation to pH and aluminium. *Soil Biol. Biochem.* 40, 2260–2265. doi: 10.1016/j.soilbio.2008.05.002
- Grümborg, B. C., María, U. C., Shroeder, A., Vargas-Gil, S., and Luna, C. M. (2015). The role of inoculum identity in drought stress mitigation by arbuscular mycorrhizal fungi in soybean. *Biol. Fertil. Soils* 51, 1–10. doi: 10.1007/s00374-014-0942-7
- Gutjahr, C., Radovanovic, D., Geoffroy, J., Zhang, Q., Siegler, H., Chiappello, M., et al. (2012). The half-size ABC transporters STR1 and STR2 are indispensable for mycorrhizal arbuscule formation in rice. *Plant J.* 69, 906–920. doi: 10.1111/j.1365-3113X.2011.04842.x
- Harrison, M. J. (2012). Cellular programs for arbuscular mycorrhizal symbiosis. *Curr. Opin. Plant Biol.* 15, 691–698. doi: 10.1016/j.pbi.2012.08.010
- Härtel, H., Dörmann, P., and Benning, C. (2000). DGD1-independent biosynthesis of extraplastidic galactolipids after phosphate deprivation in *Arabidopsis*. *Proc. Natl. Acad. Sci. U. S. A.* 97, 10649–10654. doi: 10.1073/pnas.180320497
- He, F., Sheng, M., and Tang, M. (2017). Effects of *Rhizophagus irregularis* on photosynthesis and antioxidative enzymatic system in *Robinia pseudoacacia* L. under drought stress. *Front. Plant Sci.* 8:183. doi: 10.3389/fpls.2017.00183
- Helber, N., Wippel, K., Sauer, N., Schaarschmidt, S., Hause, B., and Requena, N. (2011). A versatile monosaccharide transporter that operates in the arbuscular mycorrhizal fungus *Glomus* sp is crucial for the symbiotic relationship with plants. *Plant Cell* 23, 3812–3823. doi: 10.1105/tpc.111.089813
- Huang, L., He, Y., and Guo, Y. (2017a). The efficiency of arbuscular mycorrhizal fungi in promoting alfalfa growth in acid soils. *J. Agric. Sci.* 9, 186–204. doi: 10.5539/jas.v9n4p186
- Huang, X., Shi, H., Hu, Z., Liu, A., Amombo, E., Chen, L., et al. (2017b). ABA is involved in regulation of cold stress response in bermudagrass. *Front. Plant Sci.* 8:1613. doi: 10.3389/fpls.2017.01613
- Iwai, M., Ikeda, K., Shimojima, M., and Ohta, H. (2014). Enhancement of extraplastidic oil synthesis in *Chlamydomonas reinhardtii* using a type-2 diacylglycerol acyltransferase with a phosphorus starvation-inducible promoter. *Plant Biotechnol. J.* 12, 808–819. doi: 10.1111/pbi.12210
- Jabaji-Hare, S. (1988). Lipid and fatty acid profiles of some vesicular-arbuscular mycorrhizal fungi: contribution to taxonomy. *Mycologia* 80, 622–629. doi: 10.1080/00275514.1988.12025592
- Jabaji-Hare, S., Deschene, A., and Kendrick, B. (1984). Lipid content and composition of vesicles of a vesicular-arbuscular mycorrhizal fungus. *Mycologia* 76, 1024–1030. doi: 10.1080/00275514.1984.12023946
- Jiang, Y., Wang, W., Xie, Q., Liu, N., Liu, L., Wang, D., et al. (2017). Plants transfer lipids to sustain colonization by mutualistic mycorrhizal and parasitic fungi. *Science* 356, 1172–1175. doi: 10.1126/science.aam9970
- Jiang, M., and Zhang, J. (2002). Water stress-induced abscisic acid accumulation triggers the increased generation of reactive oxygen species and up-regulates the activities of antioxidant enzymes in maize leaves. *J. Exp. Bot.* 53, 2401–2410. doi: 10.1093/jxb/erf090
- Kameoka, H., Tsutsui, I., Saito, K., Kikuchi, Y., Handa, Y., Ezawa, T., et al. (2019). Stimulation of symbiotic sporulation in arbuscular mycorrhizal fungi by fatty acids. *Nat. Microbiol.* 4, 1654–1660. doi: 10.1038/s41564-019-0485-7
- Kim, Y. H., Choi, K. I., Khan, A. L., Waqas, M., and Lee, I. J. (2016). Exogenous application of abscisic acid regulates endogenous gibberellins homeostasis and enhances resistance of oriental melon (*Cucumis melo* var. L.) against low temperature. *Sci. Hortic.* 207, 41–47. doi: 10.1016/j.scienta.2016.05.009
- Kobae, Y., Gutjahr, C., Paszkowski, U., Kojima, T., Fujiwara, T., and Hata, S. (2014). Lipid droplets of arbuscular mycorrhizal fungi emerge in concert with arbuscule collapse. *Plant Cell Physiol.* 55, 1945–1953. doi: 10.1093/pcp/pcu123
- Kobae, Y., and Hata, S. (2010). Dynamics of periarbuscular membranes visualized with a fluorescent phosphate transporter in arbuscular mycorrhizal roots of rice. *Plant Cell Physiol.* 51, 341–353. doi: 10.1093/pcp/pcq013
- Koegel, S., Brulé, D., Wiemken, A., Boller, T., and Courty, P. (2015). The effect of different nitrogen sources on the symbiotic interaction between *Sorghum bicolor* and *Glomus intraradices*: expression of plant and fungal genes involved in nitrogen assimilation. *Soil Biol. Biochem.* 86, 159–163. doi: 10.1016/j.soilbio.2015.03.003
- Kong, Y., Chen, S., Yang, Y., and An, C. (2013). ABA-insensitive (ABI) 4 and ABI5 synergistically regulate *DGAT1* expression in *Arabidopsis* seedlings under stress. *FEBS Lett.* 587, 3076–3082. doi: 10.1016/j.febslet.2013.07.045
- Krishnamoorthy, R., Kim, K., Kim, C., and Sa, T. (2014). Changes of arbuscular mycorrhizal traits and community structure with respect to soil salinity in a coastal reclamation land. *Soil Biol. Biochem.* 72, 1–10. doi: 10.1016/j.soilbio.2014.01.017
- Lee, H. G., Park, M., Park, B. Y., Kim, H. U., and Seo, P. J. (2019). The *Arabidopsis* MYB96 transcription factor mediates ABA-dependent triacylglycerol accumulation in vegetative tissues under drought stress conditions. *Plant Theory* 8:296. doi: 10.3390/plants8090296
- Li, H., Chen, X. W., and Wong, M. H. (2016). Arbuscular mycorrhizal fungi reduced the ratios of inorganic/organic arsenic in rice grains. *Chemosphere* 145, 224–230. doi: 10.1016/j.chemosphere.2015.10.067
- Li, J., Han, D., Wang, D., Ning, K., Jia, J., Wei, L., et al. (2014). Choreography of transcriptomes and lipidomes of Nannochloropsis reveals the mechanisms of oil synthesis in microalgae. *Plant Cell* 26, 1645–1665. doi: 10.1105/tpc.113.121418
- Li, J., Meng, B., Chai, H., Yang, X., Song, W., Li, S., et al. (2019b). Arbuscular mycorrhizal fungi alleviate drought stress in C3 (*Leymus chinensis*) and C4

- (*Hemarthria altissima*) grasses via altering antioxidant enzyme activities and photosynthesis. *Front. Plant Sci.* 10:499. doi: 10.3389/fpls.2019.00499
- Li, C., Zhou, J., Wang, X., and Liao, H. (2019a). A purple acid phosphatase, GmPAP33, participates in arbuscule degeneration during AM symbiosis in soybean. *Plant Cell Environ.* 42, 2015–2027. doi: 10.1111/pce.13530
- Liu, X., Feng, Z., Zhao, Z., Zhu, H., and Yao, Q. (2020). Acidic soil inhibits the functionality of arbuscular mycorrhizal fungi by reducing arbuscule formation in tomato roots. *Soil Sci. Plant Nutr.* 66, 275–284. doi: 10.1080/00380768.2020.1721320
- Liu, X., Feng, Z., Zhu, H., and Yao, Q. (2019). Exogenous abscisic acid and root volatiles increase sporulation of *Rhizophagus irregularis* DAOM 197198 in asymbiotic and pre-symbiotic status. *Mycorrhiza* 29, 581–589. doi: 10.1007/s00572-019-00916-z
- Luginbuehl, L. H., Menard, G. N., Kurup, S., Van Erp, H., Radhakrishnan, G. V., Breakspear, A., et al. (2017). Fatty acids in arbuscular mycorrhizal fungi are synthesized by the host plant. *Science* 356, 1175–1178. doi: 10.1126/science.aan0081
- Luginbuehl, L. H., and Oldroyd, G. (2017). Understanding the arbuscule at the heart of endomycorrhizal symbioses in plants. *Curr. Biol.* 27, 952–963. doi: 10.1016/j.cub.2017.06.042
- Mathur, S., Sharma, M. P., and Jajoo, A. (2018). Improved photosynthetic efficacy of maize (*Zea mays*) plants with arbuscular mycorrhizal fungi (AMF) under high temperature stress. *J. Photochem. Photobiol. B* 180, 149–154. doi: 10.1016/j.jphotobiol.2018.02.002
- Millar, A. A., Smith, M. A., and Kunst, L. (2000). All fatty acids are not equal: discrimination in plant membrane lipids. *Trends Plant Sci.* 5, 95–101. doi: 10.1016/S1360-1385(00)01566-1
- Miller, G., Suzuki, N., Ciftci-Yilmaz, S., and Mittler, R. (2010). Reactive oxygen species homeostasis and signalling during drought and salinity stresses. *Plant Cell Environ.* 33, 453–467. doi: 10.1111/j.1365-3040.2009.02041.x
- Mo, Y., Wang, Y., Yang, R., Zheng, J., Liu, C., Li, H., et al. (2016). Regulation of plant growth, photosynthesis, antioxidant and osmosis by an arbuscular mycorrhizal fungus in watermelon seedlings under well-watered and drought conditions. *Front. Plant Sci.* 7:644. doi: 10.3389/fpls.2016.00644
- Mus, F., Toussaint, J. P., Cooksey, K. E., Fields, M. W., Gerlach, R., Peyton, B. M., et al. (2013). Physiological and molecular analysis of carbon source supplementation and pH stress-induced lipid accumulation in the marine diatom *Phaeodactylum tricornutum*. *Appl. Microbiol. Biotechnol.* 97, 3625–3642. doi: 10.1007/s00253-013-4747-7
- Nakatani, A. S., Mescolotti, D. L. C., Nogueira, M. A., Martinez, A. M., Miyachi, M. Y. H., Stürmer, S. L., et al. (2011). Dosage-dependent shift in the spore community of arbuscular mycorrhizal fungi following application of tannery sludge. *Mycorrhiza* 21, 515–522. doi: 10.1007/s00572-010-0359-6
- Oelkers, P., Tinkenberg, A., Erdeniz, N., Cromley, D., Billheimer, J. T., and Sturley, S. L. (2000). A lecithin cholesterol acyltransferase-like gene mediates diacylglycerol esterification in yeast. *J. Biol. Chem.* 275, 15609–15612. doi: 10.1074/jbc.C000144200
- Olsson, P. A., and Johansen, A. (2000). Lipid and fatty acid composition of hyphae and spores of arbuscular mycorrhizal fungi at different growth stages. *Mycol. Res.* 104, 429–434. doi: 10.1017/s0953756299001410
- Pant, B. D., Burgos, A., Pant, P., Cuadros-Inostroza, A., Willmitzer, L., and Scheible, W. R. (2015). The transcription factor PHR1 regulates lipid remodeling and triacylglycerol accumulation in *Arabidopsis thaliana* during phosphorus starvation. *J. Exp. Bot.* 66, 1907–1918. doi: 10.1093/jxb/eru535
- Pfeffer, P. E., Douds, J. D., Becard, G., and Shachar-Hill, Y. (1999). Carbon uptake and the metabolism and transport of lipids in an arbuscular mycorrhiza. *Plant Physiol.* 120, 587–598. doi: 10.1104/pp.120.2.587
- Pons, S., Fournier, S., Chervin, C., Becard, G., Rochange, S., and Puech-pages, V. (2020). Phytohormone production by the arbuscular mycorrhizal fungus *Rhizophagus irregularis*. *PLoS One* 15:e0240886. doi: 10.1101/2020.06.11.146126
- Rask, K. A., Johansen, J. L., Kjoller, R., and Ekelund, F. (2019). Differences in arbuscular mycorrhizal colonisation influence cadmium uptake in plants. *Environ. Exp. Bot.* 162, 223–229. doi: 10.1016/j.envexpbot.2019.02.022
- Remy, W., Taylor, T. N., Hass, H., and Kerp, H. (1994). Four hundred-million-year-old vesicular arbuscular mycorrhizae. *Proc. Natl. Acad. Sci. U. S. A.* 91, 11841–11843. doi: 10.1073/pnas.91.25.11841
- Roth, R., and Paszkowski, U. (2017). Plant carbon nourishment of arbuscular mycorrhizal fungi. *Curr. Opin. Plant Biol.* 39, 50–56. doi: 10.1016/j.pbi.2017.05.008
- Seguela, A., Cummingb, J., Cornejoa, P., and Borie, F. (2016). Aluminum tolerance of wheat cultivars and relation to arbuscular mycorrhizal colonization in a non-limed and limed andisol. *Appl. Soil Ecol.* 108, 228–237. doi: 10.1016/j.apsoil.2016.08.014
- Selvakumar, G., Krishnamoorthy, R., Kim, K., and Sa, T. (2016). Propagation technique of arbuscular mycorrhizal fungi isolated from coastal reclamation land. *Eur. J. Soil Biol.* 74, 39–44. doi: 10.1016/j.ejsobi.2016.03.005
- Selvakumar, G., Shagol, C. C., Kang, Y., Chung, B. N., Han, S. G., and Sa, T. M. (2018). Arbuscular mycorrhizal fungi spore propagation using single spore as starter inoculum and a plant host. *J. Appl. Microbiol.* 124, 1556–1565. doi: 10.1111/jam.13714
- Smith, S. E., and Read, D. J. (2008). *Mycorrhizal symbiosis*. San Diego, CA: Academic Press.
- Spatafora, J. W., Chang, Y., Benny, G. L., Lazarus, K., Smith, M. E., Berbee, M. L., et al. (2016). A phylum-level phylogenetic classification of zygomycete fungi based on genome-scale data. *Mycologia* 108, 1028–1046. doi: 10.3852/16-042
- Spraker, J. E., Sanchez, L. M., Lowe, T. M., Dorrestein, P. C., and Keller, N. P. (2016). *Ralstonia solanacearum* lipopeptide induces chlamydospore development in fungi and facilitates bacterial entry into fungal tissues. *ISME J.* 10, 2317–2330. doi: 10.1038/ismej.2016.32
- Sugiura, Y., Akiyama, R., Tanaka, S., Yano, K., Kameoka, H., Kawaguchi, M., et al. (2019). Myristate as a carbon and energy source for the asymbiotic growth of the arbuscular mycorrhizal fungus *Rhizophagus irregularis*. *Proc. Natl. Acad. Sci. U. S. A.* 117, 25779–25788. doi: 10.1101/731489
- Tan, W. J., Yang, Y. C., Zhou, Y., Huang, L. P., Xu, L., Chen, Q. F., et al. (2018). DIACYLGLYCEROL ACYLTRANSFERASE and DIACYLGLYCEROL KINASE modulate triacylglycerol and phosphatidic acid production in the plant response to freezing stress. *Plant Physiol.* 177, 1303–1318. doi: 10.1104/pp.18.00402
- Tanaka, Y., and Yano, K. (2005). Nitrogen delivery to maize via mycorrhizal hyphae depends on the form of N supplied. *Plant Cell Environ.* 28, 1247–1254. doi: 10.1111/j.1365-3040.2005.01360.x
- Tisserant, E., Malbreil, M., Kuo, A., Kohler, A., Symeonidi, A., Balestrini, R., et al. (2013). Genome of an arbuscular mycorrhizal fungus provides insight into the oldest plant symbiosis. *Proc. Natl. Acad. Sci. U. S. A.* 110, 20117–20122. doi: 10.1073/pnas.1313452110
- Tsikis, D. (2017). Assessment of lipid peroxidation by measuring malondialdehyde (MDA) and relatives in biological samples: analytical and biological challenges. *Anal. Biochem.* 524, 13–30. doi: 10.1016/j.ab.2016.10.021
- Van Aarle, I. M., and Olsson, P. A. (2003). Fungal lipid accumulation and development of mycelial structures by two arbuscular mycorrhizal fungi. *Appl. Environ. Microbiol.* 69, 6762–6767. doi: 10.1128/aem.69.11.6762-6767.2003
- Wang, N., Feng, Z., Zhou, Y., Zhu, H., and Yao, Q. (2017). External hyphae of *Rhizophagus irregularis* DAOM 197198 are less sensitive to low pH than roots in arbuscular mycorrhizae: evidence from axenic culture system. *Environ. Microbiol. Rep.* 9, 649–657. doi: 10.1111/1758-2229.12573
- Wewer, V., Brands, M., and Dörmann, P. (2014). Fatty acid synthesis and lipid metabolism in the obligate biotrophic fungus *Rhizophagus irregularis* during mycorrhization of *Lotus japonicus*. *Plant J.* 79, 398–412. doi: 10.1111/tj.12566
- Wewer, V., Dombink, I., vom Dorp, K., and Dörmann, P. (2011). Quantification of sterol lipids in plants by quadrupole time-of-flight mass spectrometry. *J. Lipid Res.* 52, 1039–1054. doi: 10.1194/jlr.D013987
- Wu, S., Hu, Y., Zhang, X., Sun, Y., Wu, Z., Li, T., et al. (2018). Chromium detoxification in arbuscular mycorrhizal symbiosis mediated by sulfur uptake and metabolism. *Environ. Exp. Bot.* 147, 43–52. doi: 10.1016/j.envexpbot.2017.11.010
- Yang, Y., Song, Y., Scheller, H. V., Ghosh, A., Ban, Y., Chen, H., et al. (2015). Community structure of arbuscular mycorrhizal fungi associated with *Robinia pseudoacacia* in uncontaminated and heavy metal contaminated soils. *Soil Biol. Biochem.* 86, 146–158. doi: 10.1016/j.soilbio.2015.03.018
- Yang, Y., Yu, X., Song, L., and An, C. (2011). ABI4 activates *DGAT1* expression in *Arabidopsis* seedlings during nitrogen deficiency. *Plant Physiol.* 156, 873–883. doi: 10.1104/pp.111.175950
- Ye, L., Zhao, X., Bao, E., Cao, K., and Zou, Z. (2019). Effects of arbuscular mycorrhizal fungi on watermelon growth, elemental uptake, antioxidant, and photosystem II activities and stress-response gene expressions under salinity-alkalinity stresses. *Front. Plant Sci.* 10:863. doi: 10.3389/fpls.2019.00863
- Yoon, K., Han, D., Li, Y., Sommerfeld, M., and Hu, Q. (2012). Phospholipid:diacylglycerol acyltransferase is a multifunctional enzyme involved

- in membrane lipid turnover and degradation while synthesizing triacylglycerol in the unicellular green microalga *Chlamydomonas reinhardtii*. *Plant Cell* 24, 3708–3724. doi: 10.1105/tpc.112.100701
- You, J., and Chan, Z. (2015). ROS regulation during abiotic stress responses in crop plants. *Front. Plant Sci.* 6:1092. doi: 10.3389/fpls.2015.01092
- Yuan, L., Mao, X., Zhao, K., Ji, X., Ji, C., Xue, J., et al. (2017). Characterisation of phospholipid:diacylglycerol acyltransferases (PDATs) from *Camelina sativa* and their roles in stress responses. *Biol. Open* 6, 1024–1034. doi: 10.1242/bio.026534
- Zhang, Q., Blaylock, L. A., and Harrison, M. J. (2010). Two *Medicago truncatula* half-ABC transporters are essential for arbuscule development in arbuscular mycorrhizal symbiosis. *Plant Cell* 22, 1483–1497. doi: 10.1105/tpc.110.074955
- Zhang, Y., Hu, J., Bai, J., Wang, J., Yin, R., Wang, J., et al. (2018). Arbuscular mycorrhizal fungi alleviate the heavy metal toxicity on sunflower (*Helianthus annuus* L.) plants cultivated on a heavily contaminated field soil at a WEEE-recycling site. *Sci. Total Environ.* 628, 282–290. doi: 10.1016/j.scitotenv.2018.01.331
- Zhang, T., Yang, X., Guo, R., and Guo, J. (2016). Response of AM fungi spore population to elevated temperature and nitrogen addition and their influence on the plant community composition and productivity. *Sci. Rep.* 6:24749. doi: 10.1038/srep24749
- Zhang, X., Zhang, H., Lou, X., and Tang, M. (2019). Mycorrhizal and non-mycorrhizal *Medicago truncatula* roots exhibit differentially regulated NADPH oxidase and antioxidant response under Pb stress. *Environ. Exp. Bot.* 164, 10–19. doi: 10.1016/j.envexpbot.2019.04.015
- Zhang, R., Zhu, H., Zhao, H., and Yao, Q. (2013). Arbuscular mycorrhizal fungal inoculation increases phenolic synthesis in clover roots via hydrogen peroxide, salicylic acid and nitric oxide signaling pathways. *J. Plant Physiol.* 170, 74–79. doi: 10.1016/j.jscinta.2014.12.015
- Zhu, X. C., Song, F. B., Liu, S. Q., and Liu, T. D. (2011). Effects of arbuscular mycorrhizal fungus on photosynthesis and water status of maize under high temperature stress. *Plant and Soil* 346, 189–199. doi: 10.1007/s11104-011-0809-8
- Zhu, H., Yao, Q., Sun, X., and Hu, Y. (2007). Colonization, ALP activity and plant growth promotion of native and exotic arbuscular mycorrhizal fungi at low pH. *Soil Biol. Biochem.* 39, 942–950. doi: 10.1016/j.soilbio.2006.11.006
- Zhu, H., Zhang, R., Chen, W., Gu, Z., Xie, X., Zhao, H., et al. (2015). The possible involvement of salicylic acid and hydrogen peroxide in the systemic promotion of phenolic biosynthesis in clover roots colonized by arbuscular mycorrhizal fungus. *J. Plant Physiol.* 178, 27–34. doi: 10.1016/j.jplph.2015.01.016
- Zienkiewicz, K., Du, Z., Ma, W., Vollheyde, K., and Benning, C. (2016). Stress-induced neutral lipid biosynthesis in microalgae—molecular, cellular and physiological insights. *BBA-Mol. Cell Biol. L.* 1861, 1269–1281. doi: 10.1016/j.bbalip.2016.02.008

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.

Copyright © 2020 Feng, Liu, Zhu and Yao. This is an open-access article distributed under the terms of the Creative Commons Attribution License (CC BY). The use, distribution or reproduction in other forums is permitted, provided the original author(s) and the copyright owner(s) are credited and that the original publication in this journal is cited, in accordance with accepted academic practice. No use, distribution or reproduction is permitted which does not comply with these terms.



Plant Salinity Tolerance Conferred by Arbuscular Mycorrhizal Fungi and Associated Mechanisms: A Meta-Analysis

Khondoker M. G. Dastogeer^{1,2*}, Mst Ishrat Zahan³, Md. Tahjib-Ul-Arif⁴, Mst Arjina Akter² and Shin Okazaki^{1*}

¹ Graduate School of Agriculture, Tokyo University of Agriculture and Technology, Fuchu, Japan, ² Department of Plant Pathology, Bangladesh Agricultural University, Mymensingh, Bangladesh, ³ Independent Researcher, Mymensingh, Bangladesh, ⁴ Department of Biochemistry and Molecular Biology, Bangladesh Agricultural University, Mymensingh, Bangladesh

OPEN ACCESS

Edited by:

Alvaro Sanz-Saez,
Auburn University, United States

Reviewed by:

Irakli Loladze,
Arizona State University, United States
Rebecca Bunn,
Western Washington University,
United States

*Correspondence:

Khondoker M. G. Dastogeer
dastogeer.ppath@bau.edu.bd
Shin Okazaki
sokazaki@cc.tuat.ac.jp

Specialty section:

This article was submitted to
Plant Abiotic Stress,
a section of the journal
Frontiers in Plant Science

Received: 29 July 2020

Accepted: 12 November 2020

Published: 09 December 2020

Citation:

Dastogeer KMG, Zahan MI,
Tahjib-Ul-Arif M, Akter MA and
Okazaki S (2020) Plant Salinity
Tolerance Conferred by Arbuscular
Mycorrhizal Fungi and Associated
Mechanisms: A Meta-Analysis.
Front. Plant Sci. 11:588550.
doi: 10.3389/fpls.2020.588550

Soil salinity often hinders plant productivity in both natural and agricultural settings. Arbuscular mycorrhizal fungal (AMF) symbionts can mediate plant stress responses by enhancing salinity tolerance, but less attention has been devoted to measuring these effects across plant-AMF studies. We performed a meta-analysis of published studies to determine how AMF symbionts influence plant responses under non-stressed vs. salt-stressed conditions. Compared to non-AMF plants, AMF plants had significantly higher shoot and root biomass ($p < 0.0001$) both under non-stressed conditions and in the presence of varying levels of NaCl salinity in soil, and the differences became more prominent as the salinity stress increased. Categorical analyses revealed that the accumulation of plant shoot and root biomass was influenced by various factors, such as the host life cycle and lifestyle, the fungal group, and the duration of the AMF and salinity treatments. More specifically, the effect of *Funneliformis* on plant shoot biomass was more prominent as the salinity level increased. Additionally, under stress, AMF increased shoot biomass more on plants that are dicots, plants that have nodulation capacity and plants that use the C3 plant photosynthetic pathway. When plants experienced short-term stress (<2 weeks), the effect of AMF was not apparent, but under longer-term stress (>4 weeks), AMF had a distinct effect on the plant response. For the first time, we observed significant phylogenetic signals in plants and mycorrhizal species in terms of their shoot biomass response to moderate levels of salinity stress, i.e., closely related plants had more similar responses, and closely related mycorrhizal species had similar effects than distantly related species. In contrast, the root biomass accumulation trait was related to fungal phylogeny only under non-stressed conditions and not under stressed conditions. Additionally, the influence of AMF on plant biomass was found to be unrelated to plant phylogeny. In line with the greater biomass accumulation in AMF plants, AMF improved the water status, photosynthetic efficiency and uptake of Ca and K in plants irrespective of salinity stress. The uptake of N and P was higher in AMF plants, and as the salinity increased, the trend showed a decline but had a clear upturn as the salinity stress increased to a high level. The activities of malondialdehyde (MDA), peroxidase (POD),

and superoxide dismutase (SOD) as well as the proline content changed due to AMF treatment under salinity stress. The accumulation of proline and catalase (CAT) was observed only when plants experienced moderate salinity stress, but peroxidase (POD) and superoxide dismutase (SOD) were significantly increased in AMF plants irrespective of salinity stress. Taken together, arbuscular mycorrhizal fungi influenced plant growth and physiology, and their effects were more notable when their host plants experienced salinity stress and were influenced by plant and fungal traits.

Keywords: AMF, antioxidant, standardized mean difference, plant physiology, photosynthesis, plant biomass, phylogenetic signal, effect size

INTRODUCTION

Salinity is a major environmental problem that limits agricultural productivity worldwide, especially in arid and semiarid regions (Munns and Gilliam, 2015). The deleterious effect of NaCl on plants is caused by both the reduced water availability as sodium accumulation reduces the soil water potential and the toxic effects of sodium and chlorine ions on plants. Reduced water and nutrient uptake lead to osmotic stress, ion toxicity, and nutrient imbalances, resulting in significant reductions in plant growth and crop production (Munns and Tester, 2008; Hanin et al., 2016). Soil salinity problems are projected to worsen in the coming years in many low-lying areas due to the changing climate (Zörb et al., 2019). Plants adapt physiologically and biochemically to mitigate the detrimental effects of salinity via ion homeostasis and compartmentalization, osmoprotectant and compatible solute biosynthesis, antioxidant enzyme activation, antioxidant compound synthesis, polyamine synthesis, nitric oxide (NO) generation, and hormone modulation (Gupta and Huang, 2014; Hernández, 2019; Van Zelm et al., 2020). Plant responses to stress have been studied extensively in the last few decades, and the role of plant-microbe interactions on plant stress responses has also been given attention in recent years. In particular, plant species are commonly associated with fungal symbionts such as mycorrhizal fungi and endophytes (within plants), which may influence their responses to environmental stimuli, including salinity stress.

The majority of terrestrial plants form mutualistic associations with arbuscular mycorrhizal fungi (AMF). Accumulating evidence suggests that AMF colonization in roots can help improve plant tolerance to salinity stress (Al-Karaki et al., 2001; Porcel et al., 2012; Begum et al., 2019; Evelin et al., 2019). AMF employ various mechanisms to mitigate plant salinity stress. For instance, AMF can augment nutrient uptake, increase water uptake, maintain osmotic balance, stimulate antioxidant activities to protect against damage by reactive oxygen species (ROS), increase the photosynthetic rate and regulate hormonal levels to abate the harmful effects of salts on plant growth and development (Evelin et al., 2009, 2019; Ruiz-Lozano et al., 2012; Augé et al., 2014; Khalloufi et al., 2017). Under salinity stress, AMF increase the uptake of plant nutrients such as P (phosphorus), N (nitrogen), K (potassium), Zn (zinc), and Cu (copper) and maintain ionic homeostasis (Marschner and Dell, 1994; Pang et al., 2007; Sheng et al., 2011). The accumulation of

proline is another mechanism associated with AMF-mediated plant salinity tolerance. However, the role of AMF in proline accumulation in plants is not consistent: several studies reported a higher proline content, whereas others reported a lower proline content in AMF-colonized plants under stress (Evelin et al., 2012; Hashem et al., 2015; Frosi et al., 2016). An efficient reactive oxygen species (ROS) scavenging system is paramount for alleviating salinity stress in plants. AMF colonization boosts the production of antioxidant molecules and enhances the activities of enzymes to provide an improved oxidation scavenging system (Serbinova and Packer, 1994; Evelin and Kapoor, 2014). Under salinity stress, increased activities of catalase (CAT), peroxidase (POD), superoxide dismutase (SOD), and ascorbate peroxidase (APX) have been reported in AMF-colonized plants compared to non-colonized plants in many studies (Li et al., 2012; Pandey and Garg, 2017; Hashem et al., 2018). Salinity stress affects stomatal conductance, disrupts photosynthetic machinery and decreases the activity of photosynthetic pigments, all of which impede photosynthesis in plants (Giri and Mukerji, 2004; Murkute et al., 2006; Sheng et al., 2008; Chaves et al., 2009). AMF help plants maintain water status, increase stomatal conductance and enhance photosynthetic pigments to combat the effects of salts and increase photosynthesis for growth and development (Hidri et al., 2016; Chen et al., 2017). In the last several years, considerable progress has been made to understand these mechanisms. Studies have examined various mechanisms considering different plant-AMF combinations. For a better understanding of the comprehensive biochemical and physiological mechanisms across host AMF settings, we need to systematically examine these studies to determine the relative importance of each mechanism. The magnitude of the effect of AMF on plant salinity tolerance differs greatly among various studies (Evelin et al., 2009; Porcel et al., 2012; Chandrasekaran et al., 2019). These differences can be attributed to various factors, such as the level of salinity, types of hosts and mycorrhizal partners, environmental conditions, and their complex interactions. Understanding the contribution of these factors to AMF-plant symbiosis is important to elucidate the mechanism of AMF-mediated plant salinity tolerance. It is a complex task to infer general findings from individual studies. Therefore, to determine a central tendency, identify different patterns of AMF influences on plants under stress and compare them with those under control conditions, it is paramount to integrate results from multiple studies to determine whether

general factors can be identified. To this end, we conducted a meta-analysis to measure the overall magnitude and direction of the summary effect size of AMF symbiosis on important plant characteristics associated with stress tolerance mechanisms.

A meta-analysis is a mathematical approach that combines data from different studies using weighted statistical methods to calculate a mean effect size for the treatment across a range of studies (Rosenberg et al., 2000). Meta-analysis helps us to understand the results of a study in the context of all other comparable studies to evaluate whether the effect of a particular treatment is coherent across studies, whether it is noticeably different across studies, and which element might be responsible for this disparity (Borenstein et al., 2009). Categorical variables or “moderators” are often included in meta-analyses to determine how various features modulate the treatment effect of interest. Meta-analyses have been used broadly in various disciplines and have become increasingly common in plant ecology and evolutionary biology (Lau et al., 2013; Koricheva and Gurevitch, 2014; Gerstner et al., 2017; Gurevitch et al., 2018). Several meta-analyses have been conducted to determine the impact of arbuscular mycorrhizae (AM) on the plant response to salinity stress. In a meta-analysis of 43 studies, Chandrasekaran et al. (2014) reported that AMF enhanced plant biomass and uptake of K and reduced uptake of Na and that the fungal species and the host functional groupings were important moderators of that effect. A recent meta-analysis by Pan et al. (2020) showed that AMF help halophytes increase the accumulation of inorganic ions (K and Ca) and decrease the accumulation of osmolytes (proline and soluble sugar) to augment biomass production under salinity stress. In contrast, in glycophytes, AMF increase salinity tolerance by their combined influence on increasing soluble sugar, nutrient acquisition, superoxide dismutase, and chlorophyll synthesis and decreasing sodium uptake. It was shown that AMF increased the performance of photosystem II in plants under salinity stress by improving the utilization of photons and electron transport and reducing photoinhibition. Under salinity stress, C4 species had better photosynthesis performance than C3 species when inoculated with AMF, and the annual, monocotyledon, and woody species showed better tolerance than plant types (Wang et al., 2019). Augé et al. (2014) carried out a meta-analysis to focus on osmotic and ionic adjustments in AMF plants under salinity. They reported that AMF increased root and shoot K^+ concentrations, K^+/Na^+ ratios, and soluble carbohydrates but had no consistent effect on glycine betaine, Cl^- concentrations, leaf Ψ_π , shoot proline or polyamine concentrations. However, in most studies, the results from the non-stressed condition have not been examined to compare the effects. More importantly, a systematic categorization of salinity levels was not used in these studies, which might result in inaccurate inferences due to the missing impact of the degree of salinity on the effect size. As salinity increases, mycorrhizal colonization and mycorrhizal dependence in plants decrease (Wang et al., 2018). We did not come across any paper that considered the categorization of salinity level and performed meta-analysis of AMF influence at various salinity levels. Additionally, since plant and fungal identity are important moderators (Chandrasekaran et al., 2014), we wanted to examine

whether there remained any phylogenetic relevance of fungi and plants in their interactions under varying levels of salinity stress, which has never been tested before.

The publication trend indicates an overall increase in published research on AMF-mediated plant salinity tolerance (Figure 1). Since 2007, there has been a sharp increase in the publication number, indicating an increasing interest in research in this area. However, there remains a paucity of information on the relative importance and magnitude of various factors and mechanisms of AMF-mediated plant salinity tolerance. In the present study, we accumulated data from 97 relevant papers as obtained by our literature search and measured the effects of AMF on 23 plant response parameters encompassing plant growth, photosynthesis, metabolites, and enzymatic activities that are subject to change under salinity stress conditions. We expected that an increased number of articles (97) in our studies as opposed to 43 studies in Chandrasekaran et al. (2014) and 60 studies in Chandrasekaran et al. (2016) would increase the statistical power and robustness in the analyses and accuracy of inferences. The aims of the current study were to answer the following questions:

- 1) What is the overall impact of AMF colonization on the growth, biomass, nutrient uptake, water relation, and photosynthesis of plants grown under normal conditions as well as those exposed to salinity stress?
- 2) Do the magnitude and duration of salinity imposition alter the impact of AMF on plants?
- 3) Is the outcome AMF-plant interaction influenced by various host factors (host species, family, photosynthetic type, lifecycle, lifestyle, nodulation) and symbiont factors (AMF genus), and is the outcome depends on the level of stress?
- 4) Does AMF effect on plant stress tolerance show any signaling on the phylogeny of AMF or plant at non-stress vs. stressed conditions?
- 5) What is the relative importance of different antioxidant enzymes (such as CAT, SOD, POD) and osmolytes (proline and sugars) in AMF-mediated plant stress tolerance, and does it vary with the magnitude of stress?

MATERIALS AND METHODS

Literature Search Study Selection

The general guidelines of Field and Gillett (2010) were followed in gathering the data for the meta-analysis. We performed a literature search in the Web of Science (Clarivate Analysis) and Scopus databases through May 2019. Our search terms were mycorrhiza* AND salt stress/or under salinity stress, arbuscular mycorrhiza* AND salinity stress/or under salt stress, AMF AND salinity stress/or under salt stress and mycorrhiza*/or arbuscular mycorrhiza* plant growth under salt stress. The Boolean truncation (“*”) was used to include variations of the word “mycorrhiza” such as mycorrhizae, mycorrhizas, and mycorrhizal. Out of all the search results obtained, 638 were considered likely to contain relevant information based on their title and/or abstract (Figure 2). To make the final selection of the articles for data collection, we used the following set of criteria:

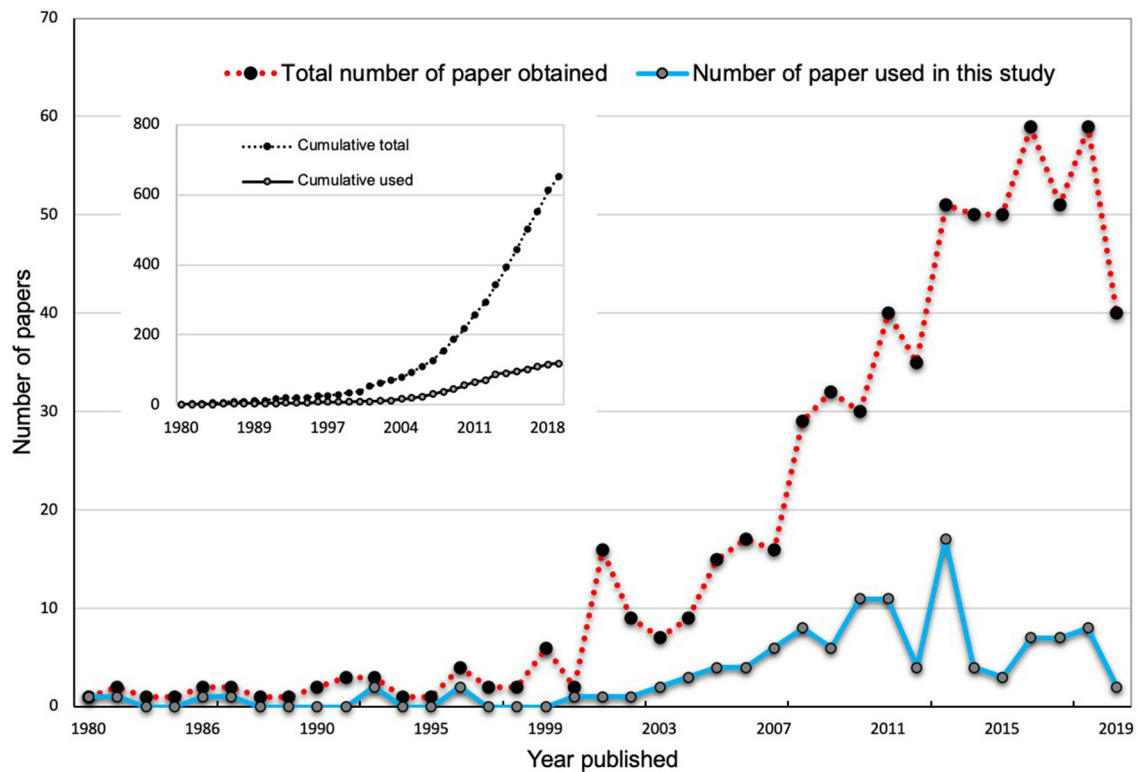


FIGURE 1 | The total number of publications about mycorrhizal effects on plant salinity stress physiology available in the “SCOPUS” and “Web of Science” databases from 1980 to 2019. The insets in the main plots show the total number of publications found in the search, while the main plots show the number of articles by year.

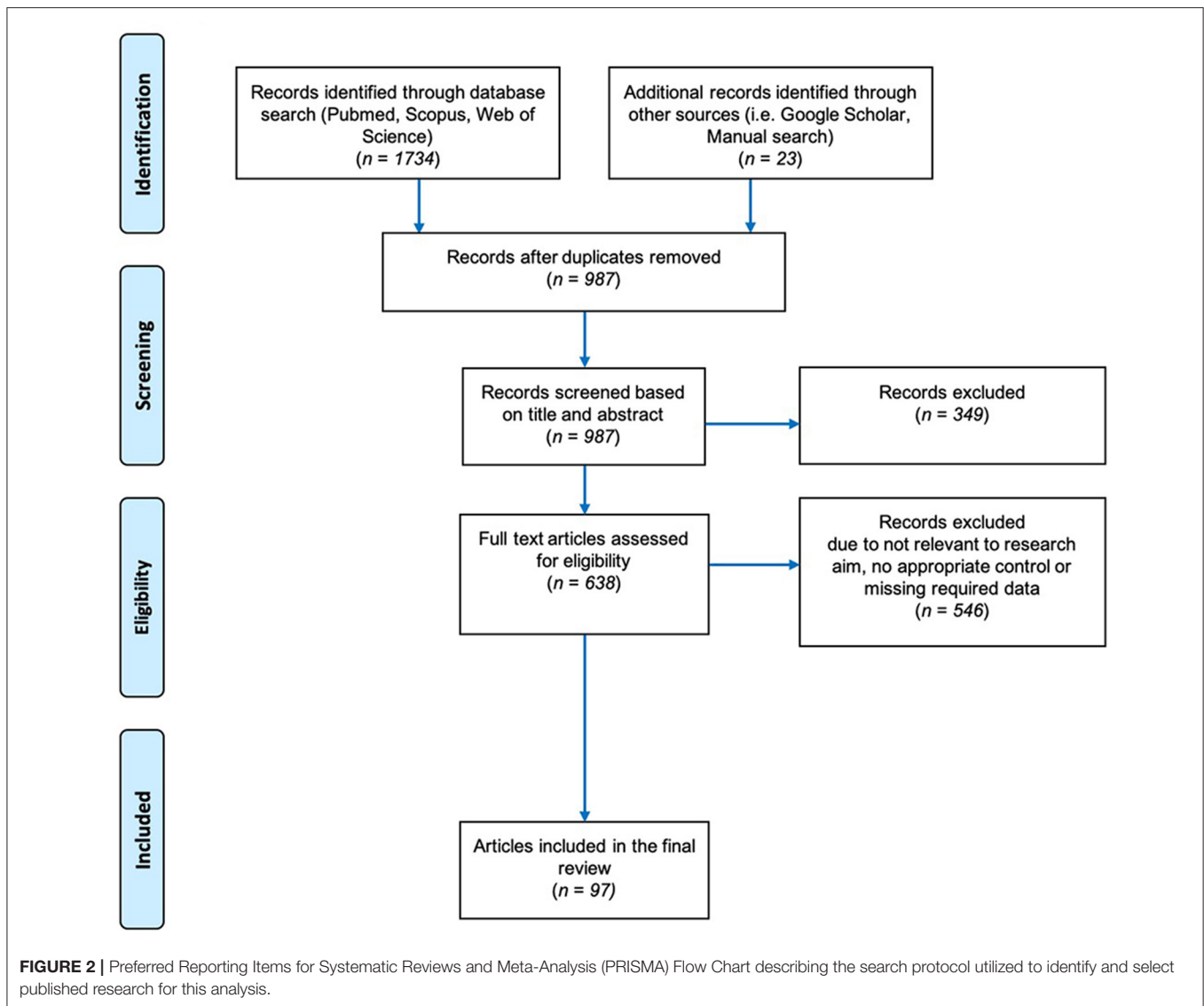
- i The experiment had to manipulate at least one AMF strain irrespective of inoculation method or colonization rate,
- ii The AMF inoculum was used singly, and we avoided mixed inoculation in this analysis,
- iii Both AMF-inoculated and non-inoculated plants were grown under salinity stress and non-stress conditions,
- iv Any physiological parameter, e.g., biomass, enzymes, metabolites, etc., was measured, and
- v The findings reported sample size, means, standard deviations/errors, and other relevant statistical information such that the outcome could be converted to a standardized measure of effect size.

Of all the studies found, most were rejected based on these criteria, and the list was refined to 97 articles (ST1). We allowed variation among the studies in our meta-analysis in terms of the levels of fertilizer applied, the growth conditions (greenhouse, growth chamber, or field), the duration of time before stress was applied, and the growth media. The papers spanned 39 years (1980–2019) and were in English.

Data Extraction

From these selected articles, we extracted information on plant biomass, AMF identity, photosynthetic parameters, enzyme parameters, and other relevant data (ST2). The means, sample sizes (replications), and standard deviations were recorded from each study. If standard errors (SEs) were presented, we converted

them to standard deviations with the equation $SD = SE \times \sqrt{(\text{sample size})}$. The 95% CIs (confidence intervals) reported were converted to SDs where necessary (Vohník et al., 2005). Often, the results were presented in a graph, and we used WebPlotDigitizer V4.2 (<https://automeris.io/WebPlotDigitizer/>) to digitize the values. Multiple treatments or host/AMF combinations from the same paper were regarded as separate studies and included as independent data units in the analysis. Extracting multiple studies from one experiment might increase the dependence on that study by assuming that the studies are independent (Gurevitch and Hedges, 1999). To examine the potential biases of publication due to non-independence from multiple observations, we calculated the mean effect size of the dataset considering only one random observation from each study and compared this with the effect size calculated with the whole dataset (He and Dijkstra, 2014). We compared effect sizes (full dataset vs. reduced dataset) using Welch's *t*-test to determine whether data reduction could significantly change the effect size. We did not observe any significant discrepancy due to data reduction, indicating that overrepresentation was less likely to occur in this study (ST3). We considered multiple observations as independent since this is thought to increase the statistical power of meta-analysis (Lajeunesse and Forbes, 2003). This approach has been used in various biological meta-analyses (Holmgren et al., 2012; Veresoglou et al., 2012; Mayerhofer et al., 2013; McGrath and Lobell, 2013; Eziz et al., 2017; Dastogeer, 2018).



Meta-Analysis

Meta-analyses were conducted using the “meta” package of Balduzzi et al. (2019) implemented in R version 4.14.0 (R Core Team, 2020). We calculated the standardized mean difference (SMD) using Hedge’s g statistic to measure the effect size for the difference between means, which is implemented in the “metacont” function by default. Hedge’s g expresses the difference of the means in units of the pooled standard deviation and is preferred in meta-analysis, as it has a lower Type I error rate than other measures, such as the log-response ratio (LRR) (Lajeunesse and Forbes, 2003; Van Kleunen et al., 2010; Xie et al., 2014). The SMD is suggested in meta-analyses that involve studies reporting continuous outcomes, which was the case in our study (Faraone, 2008). An SMD of zero means that the two treatments (AMF-treated or non-treated) have equivalent effects; SMDs >0 indicate the degree to which the AMF-inoculated samples outperformed the non-inoculated samples, and *vice*

versa. In general, SMD values of 0.3, 0.5, and 0.8 are interpreted to indicate small, medium, and large effect sizes, respectively (Cohen, 1988). A random-effects model was used to estimate the overall effect. A random-effects model was chosen because of the large number of diverse studies examined, and the studies were not anticipated to estimate a common effect size due to variable locations, conditions, experimental setups and methods used in the individual studies (Borenstein et al., 2011). We assumed that the differences among comparisons and among studies were not only due to sampling error but also due to true random variation, as is common for ecological data (Leimu et al., 2006). The effect size (SMD) was considered significant when the 95% CIs did not include zero. To estimate the random effects variance, the Sidik-Jonkman estimator (Sidik and Jonkman, 2005) was used with Hartung-Knapp adjustment (HKSJ) to make statistical inferences. This approach constructs the confidence interval based on the t -distribution and has been shown to improve

coverage probability compared to the DerSimonian and Laird (DL) method (Hartung and Knapp, 2001a,b; Int'Hout et al., 2014). HKSJ produces inflated error rates when the combined studies are of unequal size and show between-study heterogeneity, but it outperforms the widely used DL method (Sidik and Jonkman, 2007; Int'Hout et al., 2014). To quantify the heterogeneity and to test for statistical heterogeneity, Higgins's I^2 and Cochran's Q statistics were used, respectively. The I^2 statistic is defined as the ratio of true heterogeneity to total heterogeneity across the observed effect sizes, while Q represents the weighted deviations from the summary effect size that are due to heterogeneity rather than to sampling error (Higgins and Thompson, 2002; Higgins et al., 2003; Huedo-Medina et al., 2006). I^2 values range from 0 to 100%, and by convention, values of <25, 25–75, and >75% represent low, moderate, and high heterogeneity, respectively (Higgins et al., 2003). When the homogeneity statistic Q was found to be significant ($P < 0.05$ when tested against a chi-square distribution), the data were considered to be heterogeneous and further analyzed by single factor categorical analyses (Mayerhofer et al., 2013).

Publication Biases and Correction

We tested the publication bias for each dataset with different parameters. We visually inspected asymmetry in funnel plots, used “trim-and-fill” analysis and performed Begg and Mazumdar rank correlation tests based on Kendall's tau, the Egger regression test and p-curve analysis (Begg and Mazumdar, 1994; Egger et al., 1997; Simonsohn et al., 2014) to examine publication biases in the datasets. All these statistics suggested that there were substantial publication biases in some datasets (ST 3). If these tests indicated a bias, then we determined the effect sizes (SMD), CIs, and heterogeneity statistics after applying the trim-and-fill method to correct the biases. Thus, the trim-and-fill altered (decreased) the SMD values on average by 34% (12–48%) compared with the untrimmed SMD values (ST 3). After correcting for publication bias with trim-and-fill, we created subgroups from the studies based on the moderator subgroups and applied trim-and-fill to the subgroups if biases were identified in any of the subgroups by the tests mentioned above (Schmidt and Hunter, 2015). The “trim-and-fill” method is the most widely used method for assessing publication bias in meta-analyses (Duval and Tweedie, 2000a, b; Murad et al., 2018; Shi and Lin, 2019). This approach has so far been very seldom used in plant ecology meta-analyses. However, Nakagawa and Santos (2012) recommended the modification of funnel plots with the “trim-and-fill” method (Duval and Tweedie, 2000b), which allows one not only to test for but also to adjust for publication bias.

Subgroup Analyses

Subgroup analyses were performed on the data to determine the influence of factors such as plant or AMF identity, plant lifecycle, and salinity duration on the shoot and root dry biomass parameters because sufficient data were available. We ran a mixed-effects model that included the subgroups as the fixed-effects factor using the “*dmr*” package in R (Harrer et al., 2019). In this model, the overall effect size for each subgroup was

calculated using a random effects model where the variance of the summary effect for k studies is estimated as

$$V_M = \frac{s^2}{n} + \frac{T^2}{k} \text{ (Borenstein and Higgins, 2013)}$$

Then, we tested between subgroup differences using a fixed effects model where the variance of the summary effect for k studies is estimated as

$$V_M = \frac{s^2}{n} \text{ (Borenstein and Higgins, 2013).}$$

where n is the cumulative sample size across all studies, s is the standard deviation, T^2 is the estimated variance of effects across studies, and k is the number of studies.

This model is applicable when the subgroup levels under consideration can be assumed to be exhaustive for the characteristic and are not randomly chosen. Most of the subgroups in our study were fixed, such as the plant lifecycle (annual, perennial, or annual/perennial), photosynthesis (C3 or C4) or plant clade (monocot or dicot); therefore, we assumed a mixed effects model to be an appropriate choice. For a factor to be included in the analysis as a subgroup variable, it had to be reported in at least five studies across at least two different articles.

Mycorrhizal genera: Nine genera were included in the analyses, namely, *Claroideoglossum*, *Diversispora*, *Funneliformis*, *Gigaspora*, *Paraglossum*, *Rhizoglossum*, *Rhizophagus*, *Septoglossum*, and *Sieverdingia*. If enough data were not available for some of these genera, they were combined into “other genera” for inclusion in the subgroup analyses.

Plant family: There were 18 families included in the analysis. When enough data were not available for some of these families, they were combined as “other” for inclusion in the subgroup analyses. **Plant clade** comprised two levels: *eudicots* and *monocots*. **Plant life cycle:** Plants were categorized by life cycle as *annual*, *perennial* or *annual/perennial*. Plants that can live annually or perennially were included in the group *annual/perennial*. **Plant lifestyles:** This categorical variable classified plants into *herbaceous* and *woody* plants. **Plant life forms** were grouped into five groups: *forbs*, *grasses*, *shrubs*, *trees*, and *other*. Plants that can have variable life forms, such as shrubs/trees and forbs/shrubs, were included under “other.” We also conducted subgroup analyses based on the nitrogen-fixing ability of plants (legumes vs. non-legumes) as well as the photosynthetic pathway used by the plants (C3 vs. C4).

Duration of salinity: We considered the duration of salinity treatment to be short (<2 weeks), moderate (2–4 weeks), or long (>4 weeks). **Salinity level** was defined as three categories: *Low salinity* (<100 mM NaCl or <10 dS/m), *Moderate salinity* (100–200 mM NaCl or 10–20 dS/m), and *High salinity* (>200 mM NaCl or >20 dS/m). The categories of imposed soil salinity were established based on the reported salinity in the published studies.

Analysis of Phylogenetic Signal

To evaluate whether the effect of AMF symbionts on plant growth was based on the phylogeny of the fungi or plants, we calculated effect sizes for each plant species and fungal species and used each effect size as a trait value to identify phylogenetic signals. For plant phylogenetic signal analysis, we first created a phylogenetic tree by using the R package

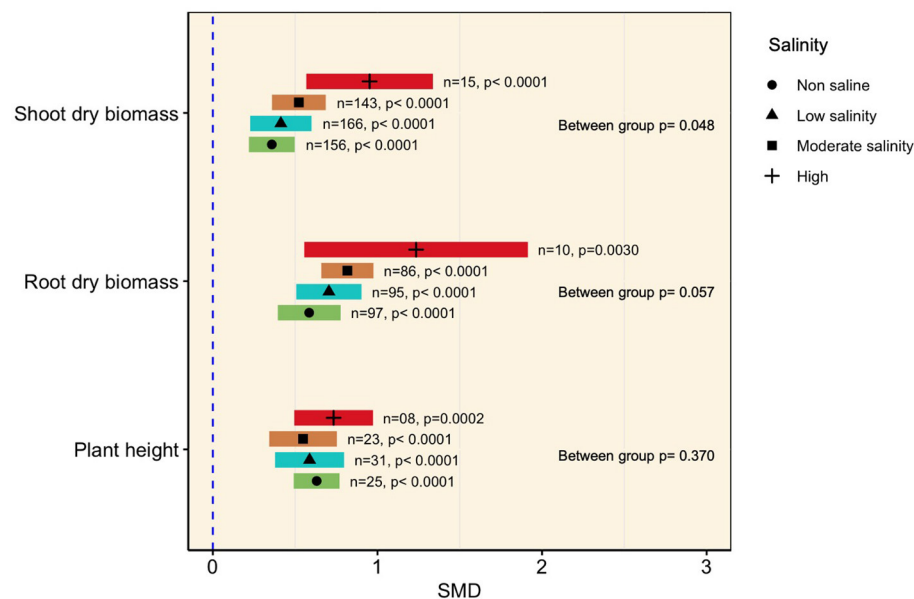


FIGURE 3 | Growth responses of AMF-inoculated plants compared with those of non-inoculated plants under non-stressed conditions and at various levels of salinity. Error bars are effect size (SMD) means $\pm$ 95% CIs. Where the CIs do not overlap the vertical dashed lines, the effect size for a parameter is significant, i.e., the growth responses of AMF plants were significantly different from those of non-AMF plants. *n*, number of studies included in the meta-analysis; *p*, significance level of SMD.

“V. PhyloMaker,” which is freely available at <https://github.com/jinyizju/V-PhyloMaker> (Jin and Qian, 2019). For fungal phylogenetic signal analysis, we created alignment using the 18S gene for mycorrhizal species and created maximum likelihood phylogenetic trees using MEGA X version 10.1.5 (Stecher et al., 2020). We based the phylogenies on the 18S region because it is sufficiently variable to distinguish among species, is the most commonly used molecular marker for studying AMF communities, and has a good balance between conserved and hypervariable regions (Öpik et al., 2013; Thiéry et al., 2016). We calculated phylogenetic signals using the R package “phylosignal” (Keck et al., 2016). We computed phylogenetic signal indices such as Abouheif’s C mean (Abouheif, 1999), Moran’s I (Moran, 1950), Pagel’s Lambda (Pagel, 1999), K and K.star (Blomberg et al., 2003) and their corresponding *p*-values using the R packages “adephylo” (Jombart and Dray, 2010), “ape” (Paradis and Schliep, 2019) and “phylobase” (Hackathon et al., 2020). The C mean is designed to detect phylogenetic autocorrelation in a quantitative trait (Abouheif, 1999). Moran’s I is a measure of spatial autocorrelation (Moran, 1950) that was adapted for use in phylogenetic analyses by Gittleman and Kot (1990). They refer to it as an autocorrelation coefficient that describes the relationship of cross-taxonomic trait variation to phylogeny.

RESULTS

We examined the influence of AMF on 23 plant response parameters at varying levels of salinity stress. The summary effect sizes for non-stressed plants in the studies were also considered for comparison. Plant hosts were represented by 51 species in 47

genera and 18 families across the 97 articles and a total of 2,555 experiments for 23 plant parameters. *Zea mays* (9%), *Solanum lycopersicum* (9%), *Cajanus cajan* (8%), and *Cicer arietinum* (7%) were the most commonly studied hosts (SF1A). Out of the 15 species of nine fungal genera recorded in all the studies, the most studied fungal species were *Funneliformis mosseae* (36%), *Rhizophagus intraradices* (23%), and *Rhizophagus irregularis* (11%) (SF1B). We used the latest fungal names as described in the Mycobank database (<http://www.mycobank.org/>).

Effects of AMF on Plant Growth Parameters Under Salinity Stress

Effects of AMF on Plant Shoot Biomass

Generally, mycorrhizal colonization significantly increased plant shoot biomass as the amount of saline increased ($p < 0.0001$, Figure 3). The subgroup analysis revealed that although the effect sizes under moderate (SMD = 0.523) and low salinity (SMD = 0.413) were relatively higher than those under normal conditions (SMD = 0.358), they were not significantly different from each other, as evident from the overlapping confidence interval values (Figure 3). At higher levels of salinity (>200 mM NaCl), however, the influence of mycorrhizae was more prominent (SMD = 0.952) in accumulating shoot biomass than under normal conditions, as apparent from non-overlapping CI values (Figure 3). A similar tendency was evident in the case of plant root biomass, which was substantially increased by AMF association regardless of the level of salinity imposed. It was also observed that the effect size increased with increasing salinity level (the SMDs at non-saline and low, moderate, and high salinity conditions were 0.585, 0.705, 0.817, and 1.25, respectively), but subgroup meta-analysis did not reveal any

TABLE 1 | Heterogeneity statistics for the three biomass summary effect sizes under non-stressed and salinity stress conditions.

Biomass	Salinity level	τ^2	τ	I^2	H	Q d.f. p-value
Shoot dry biomass	Non-saline	0.5311 [0.0802; 0.4572]	0.7288 [0.2831; 0.6761]	30.3% [14.8%; 43.1%]	1.20 [1.08; 1.33]	222.52 155 0.0003
	Low salinity	1.1841 [0.5051; 1.2423]	1.0882 [0.7107; 1.1146]	54.7% [46.0%; 62.0%]	0.49 [1.36; 1.62]	363.90 165 < 0.0001
	Moderate salinity	0.6901 [0.1700; 0.6693]	0.8307 [0.4123; 0.8181]	39.5% [25.8%; 50.6%]	1.29 [1.16; 1.42]	234.62 142 < 0.0001
	High salinity	0.3338 [0.0000; 0.9545]	0.5777 [0.0000; 0.9770]	33.3% [0.0%; 64.1%]	1.22 [1.00; 1.67]	20.99 14 0.1019
Root dry biomass	Non-saline	0.6366 [0.2014; 0.7278]	0.7979 [0.4488; 0.8531]	54.9% [43.2%; 64.1%]	1.49 [1.33; 1.67]	212.64 96 < 0.0001
	Low salinity	1.6824 [0.6516; 1.8343]	1.2971 [0.8072; 1.3544]	56.3% [46.7%; 64.1%]	1.51 [1.37; 1.67]	290.38 127 < 0.0001
	Moderate salinity	0.6160 [0.1298; 0.6368]	0.7849 [0.3603; 0.7980]	42.5% [27.8%; 54.2%]	1.32 [1.18; 1.48]	191.35 110 < 0.0001
	High salinity	0.7452 [0.0000; 4.2383]	0.8632 [0.0000; 2.0587]	44.0% [0.0%; 74.1%]	1.34 [1.00; 1.97]	14.28 8 0.0749
Plant height	Non-saline	11.4567 [3.5176; 21.1810]	3.3848 [1.8755; 4.6023]	63.7% [47.6%; 74.8%]	1.66 [1.38; 1.99]	90.85 33 < 0.0001
	Low salinity	96.2122 [40.4184; 186.5957]	9.8088 [6.3575; 13.6600]	81.7% [74.8%; 86.7%]	2.34 [1.99; 2.74]	164.00 30 < 0.0001
	Moderate salinity	4.8334 [0.7138; 8.7916]	2.1985 [0.8449; 2.9651]	49.8% [24.5%; 66.7%]	1.41 [1.15; 1.73]	61.80 31 0.0008
	High salinity	28.7921 [1.9750; 140.6920]	5.3658 [1.4053; 11.8614]	62.9% [23.8%; 82.0%]	1.64 [1.15; 2.36]	21.59 8 0.0057

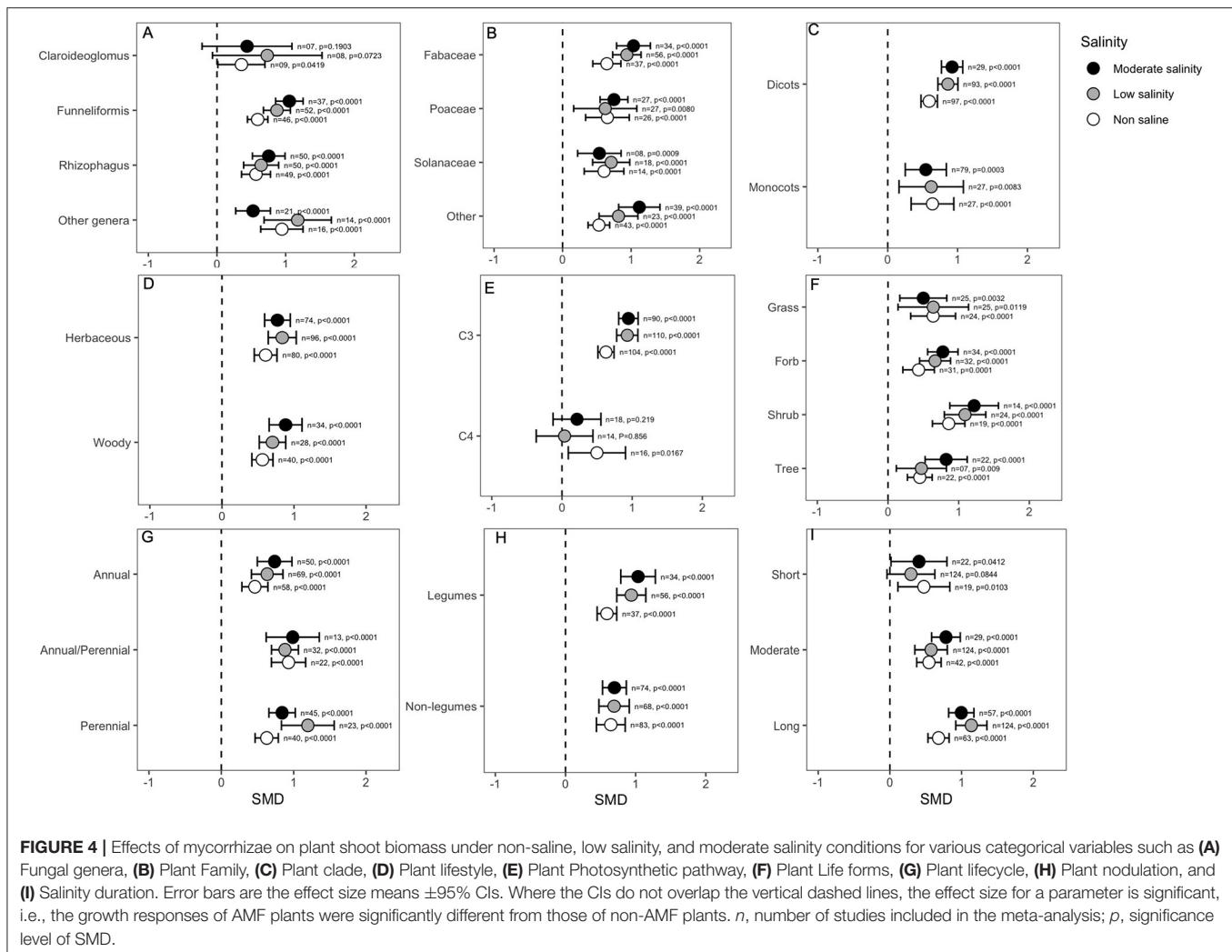
Q, total heterogeneity; p, significance of Q heterogeneity; I^2 , percentage of heterogeneity due to true variation in effect sizes.

differences among the effect sizes at various salinity levels ($p = 0.057$, **Figure 3**). The higher confidence interval values of the effect sizes at higher salinity levels indicated higher variability of mycorrhizal colonization on plant shoot and root biomass accumulation of AMF inoculation had a significant positive impact on plant height under non-stress as well as salt stress conditions. However, no apparent differences in plant height were noticed among the salinity levels (**Figure 3**, **Table 1**).

Categorical Analysis of the Effects of AMF on Plant Shoot Biomass

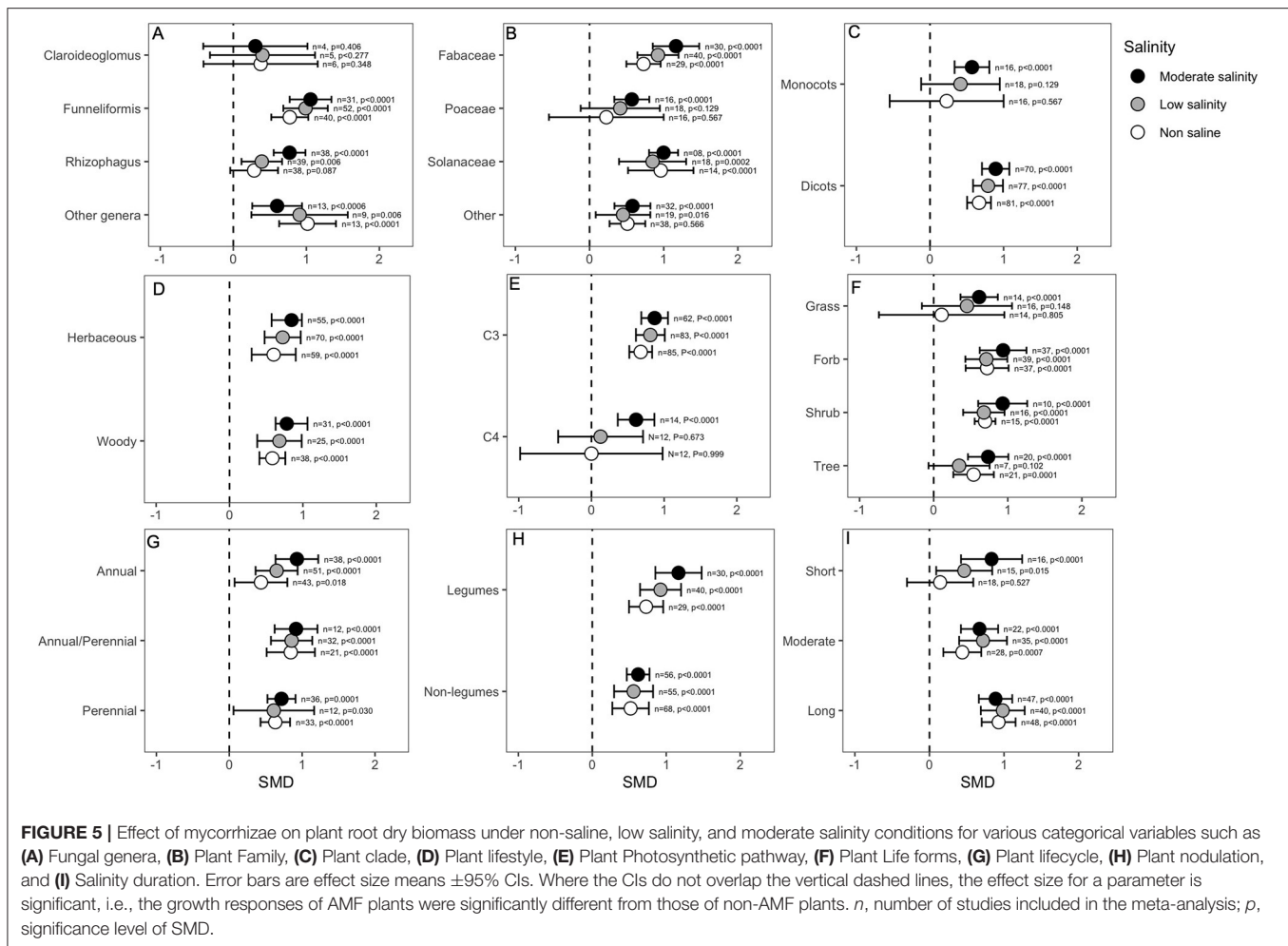
Categorical variables considered in the analysis indicated that the effect of mycorrhizal colonization on plant shoot biomass is influenced by several factors, such as host factors, fungus factors and salinity stress. For example, the effect of *Claroideoglomus* on plant shoot biomass was only marginally significant under non-saline conditions ($p = 0.04$), but it did not influence plant

shoots when hosts experienced salinity stress. In contrast, other AMF genera, including *Funneliformis* and *Rhizophagus*, showed highly significant effects on biomass in either the absence or presence of NaCl in soil (**Figure 4A**). Importantly, the effect of *Funneliformis* fungi was much greater at the moderate salinity level than at the normal salinity level (**Figure 4A**). In addition, relatively narrower CI values for *Funneliformis* implied that mycorrhizae that belong to this genus augmented plant shoot biomass, which is less influenced by other factors, such as host type or salinity level and duration, recorded in different studies. The plant family was not found to impact the AMF response, and the shoot biomass of all plants significantly increased under AMF colonization, although the effect sizes (SMDs) for the *Fabaceae* group were generally higher than those for the other plants (**Figure 4B**). Plants of the *Fabaceae* family and those included in the “other” group showed a significantly higher influence at moderate salinity than in the absence of salt stress. Both dicot



and monocot plants were significantly influenced by mycorrhizal colonization under normal and stressed conditions. Both dicots and monocots increased plant shoot biomass irrespective of salinity treatment. For dicots, the influence of AMF increased significantly as salinity was imposed on plants, which is evident from the non-overlapping CIs for the effect sizes under normal conditions and under salinity stress. Moreover, the higher CI values for monocot plants suggested a wide variability of results in the studies (Figure 4C) compared to the narrower range of CIs for dicots (Figure 4C). We did not observe any difference in shoot biomass effects due to the plant lifestyles; both herbaceous and woody plants accumulated significantly more ($p < 0.0001$) shoot biomass in response to AMF inoculation than non-AMF plants, irrespective of the salinity status (Figure 4D). The plant photosynthetic pathway was found to be very important, and C3 plants showed consistently increased shoot biomass as a result of mycorrhizal association. Importantly, under salinity stress, AMF had a significantly higher impact on the shoot biomass of C3 plants compared to their impact in the absence of stress. On the other hand, AMF inoculation in C4 plants only showed a

positive response for shoot biomass under non-saline conditions ($p = 0.016$), and in the presence of salinity stress, the effect was neutral or absent (Figure 4E). Moreover, the narrower CI range of the effect sizes for C3 plants than for C4 plants regardless of stress level indicated that the outcome of AMF on shoot biomass was less variable for C3 plants (Figure 4E). The plant life form was not very important in determining the impact of AMF on salinity tolerance. As is evident in the figure (Figure 4F), AMF effects on the forbs, shrubs and tree plants tended to increase as salinity increased, but the situation was reversed for grass plants, although the differences were not significant under normal conditions (Figure 4F). The plant life cycle was not an important factor, but the magnitude of AMF effects on plant shoot biomass was more striking for perennial than for annual plants, even though all plants showed significant positive effects regardless of the salinity treatment (Figure 4G). Plants that form nodules with bacteria, i.e., legumes, outperformed the non-legumes as a result of AMF treatment, as evident in their higher effect size (Figure 4H). Interestingly, the AMF effects on legume plants increased when plants were exposed to stress,



as evident from non-overlapping CIs of effect sizes at salinity stress with those under normal conditions (Figure 4H). When plants were exposed to salinity stress only for a short period (<2 weeks), the effect was only marginally significant under non-saline conditions. Under salinity, the AMF effect was not visible over a short period, but as the duration of salinity stress increased, the effect continued to increase. When plants were subjected to a long period (>4 weeks) of stress, the impact on AMF on plant shoot biomass was highly significant compared to their effects under normal conditions (Figure 4I).

Categorical Analysis of the Effects of AMF on Plant Root Biomass

Most categorical variables considered for analysis indicated that mycorrhizal colonization differentially influenced plant root biomass. For example, the fungal genus *Claroideoglomus* seemed to have no substantial effect on root biomass under any conditions. In contrast, *Funneliformis* and the genera classified into the “other” category also had positive effects on root mass under normal as well as salinity stress conditions. Interestingly, *Rhizophagus* increased root growth only in the presence of salt stress (Figure 5A). The root biomass of poaceous plants

was influenced positively only under moderate salinity stress conditions. Nevertheless, other plants, such as those belonging to Solanaceae and Fabaceae, had consistently higher root mass in the AMF-inoculated plants under both normal and stressed conditions (Figure 5B). The plant clade seemed to be a crucial determining factor for the AMF response. Dicots showed significantly higher ($p < 0.0001$) root growth in the AMF-inoculated plants irrespective of the salinity conditions, whereas monocot roots responded positively to AMF only at higher salinity levels (Figure 5C). Overall, the magnitude of effect sizes was not much different between woody vs. herbaceous plants, and the roots of both groups were influenced positively by AMF treatment (Figure 5D). The C3 plants had, in general, very high SMD values (~ 1.00), suggesting a positive correlation between AMF inoculation and root growth. In contrast, the root biomass of C4 plants was not influenced by AMF as the salinity increased to a moderate level ($p < 0.0001$), and the plants received benefits from the fungi and increased their root mass (Figure 5E). Grasses were less influenced by AMF treatment than forbs and shrubs, which showed significantly higher root mass in the colonized plants (Figure 5F). The life cycle of the plant did not strongly influence the response to AMF, and both annual

and perennial plants showed higher root mass in the presence of AMF regardless of the salinity stress level. Plants that are perennial but that are generally cultivated as annuals had higher SMD values than other plants (**Figure 5G**). Nodulating plants had overall higher effect sizes (~ 1.00) for root biomass than non-nodulating plants (~ 0.600), but in both groups, root mass significantly increased in AMF-treated plants compared to that in non-treated plants under all salinity conditions (**Figure 5H**). Notably, when the roots were harvested after a relatively longer period of AMF treatment (>4 weeks), the effect of AMF on increasing root biomass was highly significant regardless of the salinity condition. When the duration was <4 weeks but >2 weeks, AMF plants still had higher root mass than non-AMF plants, and salinity-stressed plants experienced a greater increase in root mass than non-stressed plants. If the treatment was very short (<2 weeks), the AMF effect on plant roots was neutral under normal conditions, but it became significant as the salinity increased (**Figure 5I**).

Phylogenetic Signal of AMF-Mediated Biomass Modulation Under Stress

We observed a significant plant phylogenetic signal, i.e., closely related plant species had more similar shoot biomass responses to AMF treatment than distantly associated species under moderate salinity stress but not under non-saline or low salinity conditions (**Figure 6A**). However, the response of root biomass to mycorrhizal association had no relationship with conserved plant phylogeny under any conditions (**Figure 6B**). Again, mycorrhizae that are more closely related phylogenetically had similar influences on plant shoot biomass under moderate salinity, but for the root mass trait, the phylogenetic signal could be identified only under normal conditions (**Figures 6C,D**).

Effects of AMF on Plant Photosynthetic Attributes and Water Status

Most of the photosynthetic parameters, such as the rate of photosynthesis (P_n), stomatal conductance (G_s) and chlorophyll a (Chla), and chlorophyll b (Chlb) content, were significantly influenced by AMF colonization under both stressed and non-stressed conditions (**Figure 7**). The subgroup analyses showed that the positive effects of AMF on certain plant photosynthetic parameters, especially Chla, G_s , and P_n , were higher when plants were exposed to salinity stress than those in non-stressed plants (**Figure 7**). For example, the quantum efficiency of PS II (F_v/F_m) was significantly influenced by AMF inoculation under moderate ($p = 0.002$) and high salinity ($p = 0.049$) conditions but not under low salinity or non-saline stress conditions (**Figure 7A**). The photosynthesis rate of AMF-treated plants was consistently higher than that of non-AMF plants regardless of salinity stress (**Figure 7B**). Stomatal conductance (G_s) was higher in AMF plants under salinity stress (SMD = 0.488), but it the difference at normal conditions (SMD = 0.382) were not very high in AMF plants than in non-inoculated plants (**Figure 7C**). Chlorophyll a increased more in AMF plants under salinity (SMD = 0.581 at moderate salinity, 0.644 at low salinity) than under normal (SMD = 0.277) conditions (between-group difference, $p = 0.012$) (**Figure 7D**). Chlorophyll b, on the other

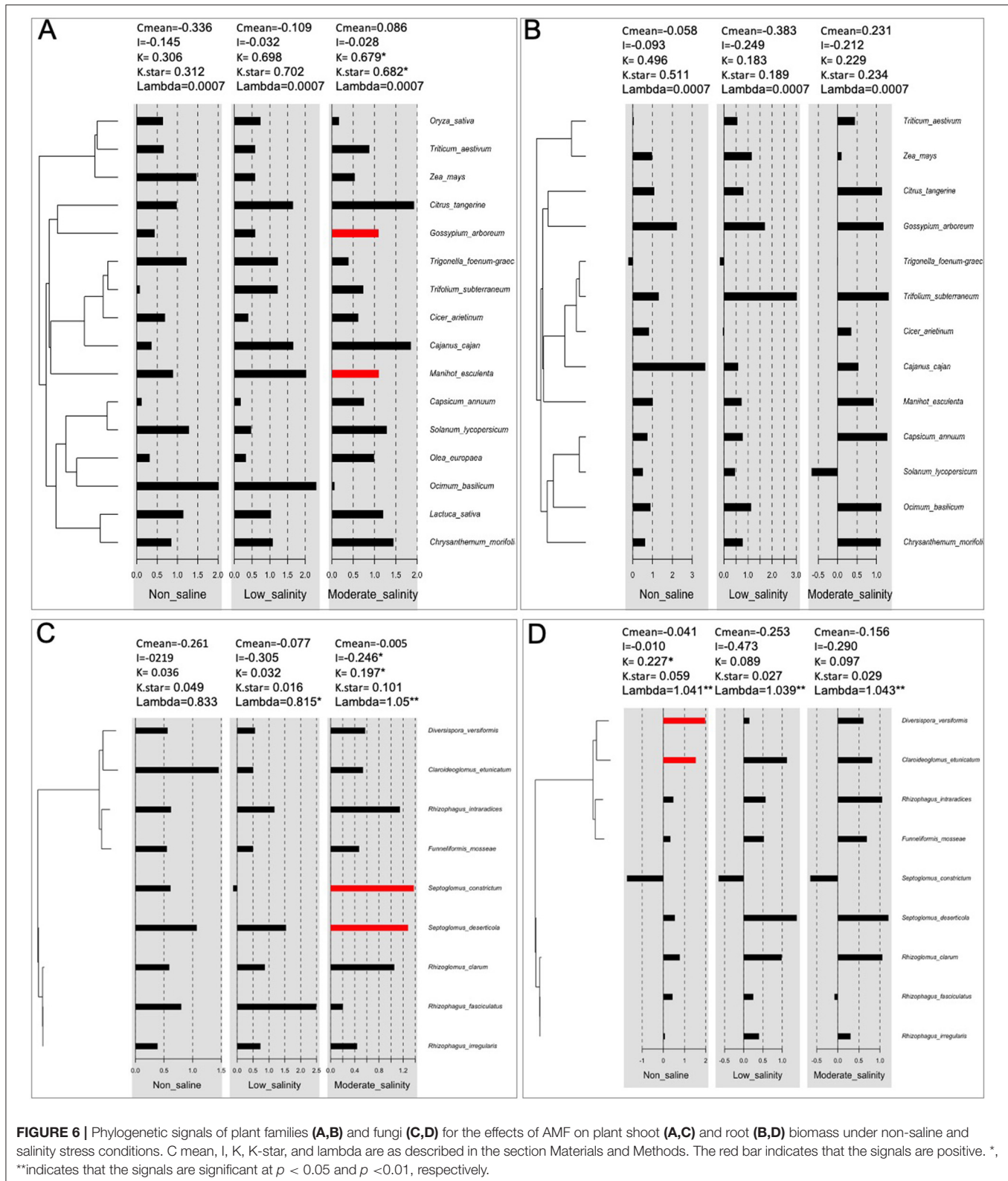
hand, increased with AMF but did not vary significantly due to salinity stress ($p = 0.956$). The leaf relative water content (RWC) in mycorrhiza-inoculated plants was consistently higher than that in non-inoculated plants regardless of the salinity stress level. Under low salinity conditions, the influence of AMF on RWC was significantly higher than under non-saline conditions (**Figure 7F**). All the parameters considered, however, tended to be more variable under salinity stress than under non-stressed conditions, as is evident from their larger confidence interval values (**Figure 7**).

AMF Effects on Plant Nutrient Homeostasis

The concentrations of nutrients such as N, P, K, Ca, and Na in the above-ground parts of plants were influenced significantly by AMF colonization under both stressed and non-stressed conditions (**Figure 8**), indicating the role of AMF in plant nutrient uptake. Plant P uptake showed an increase by AMF regardless of salinity although there was a decreasing trend as the salinity stress increases up to moderate level (**Figure 8A**). Interestingly, as the salinity stress increased from moderate to high level significantly higher-level P concentrations was measured in AMF plants compared to non-AMF plants (SMD = 0.679 at moderate salinity, 1.50 at high salinity). Like P, we observed a similar trend in N concentration in plant shoots (**Figure 8B**). Plants inoculated with AMF had consistently higher K levels in the shoot ($p < 0.0001$), and salinity had a slight impact on AMF-induced K uptake ($p = 0.544$) (**Figure 8C**). The concentration of Ca in the shoot was affected by AMF and salinity. Under normal conditions, AMF inoculation seemed to have an no effect ($p = 0.143$), but as the plants were exposed to a low (<100 mM NaCl) level of salinity, the Ca content in AMF-inoculated plants increased ($p = 0.001$). However, increasing the salinity beyond this level reduced this advantage although still significant ($p = 0.019$) than that in non-inoculated plants (**Figure 8D**). Plant Na uptake was decreased by mycorrhizal fungal inoculation. The magnitude of the decrease was higher at higher salinity levels ($p = 0.003$), e.g., Na uptake was significantly lower in AMF plants than in non-AMF plants at a high level of salinity compared to normal conditions (**Figure 8**).

AMF Effects on Plant Antioxidant and Enzyme Activities

The generation of malondialdehyde (MDA) under stress is related to the production of reactive oxygen species, including hydrogen peroxide (H_2O_2), in plant tissue. Under non-stressed conditions, there was no difference between AMF- and non-inoculated plants in terms of H_2O_2 and MDA (**Figures 9A,C**) concentrations. However, AMF-inoculated plants had significantly lower MDA levels than non-inoculated plants as the salinity stress increased. The effect of AMF on H_2O_2 production in plants was only substantial under moderate salinity ($p = 0.019$, **Figure 9**). Decreased electric leakage (EL) was consistently measured in AMF-inoculated plants compared with that in non-inoculated plants, but the AMF effects became more apparent ($p = 0.0003$) as the salinity increased from no salinity to a moderate level of salinity. Proline accumulation was not affected by AMF, but



moderate salinity caused a higher level of proline accumulation in AMF-treated plants that was marginally higher ($p = 0.034$) than that in non-AMF-treated plants (Figure 9D). Both

carotenoid and soluble sugar contents increased in AMF-treated plants compared with those in non-inoculated plants. No influence of salinity on AMF activity was noted in terms

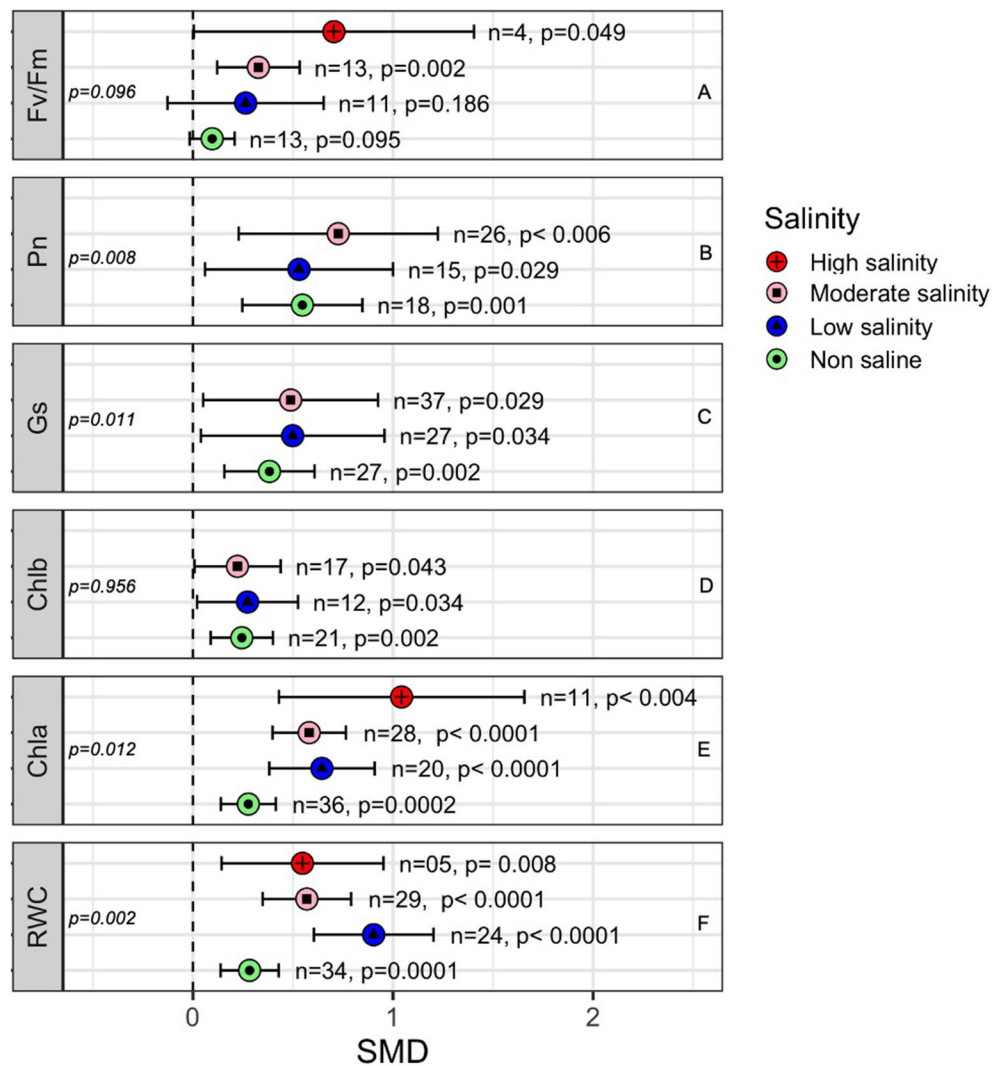


FIGURE 7 | Effects of mycorrhizae on plant photosynthetic parameters under different levels of salinity stress. Error bars are the effect size means $\pm$ 95% CIs. Where the CIs do not overlap the vertical dashed lines, the effect size for a parameter is significant. *n*, number of studies included in the meta-analysis. Chla, Chlorophyll a; Chlb, Chlorophyll b; Fv/Fm, maximal photochemical efficiency; Gs, Stomatal conductance; Pn, rate of photosynthesis; and RWC, Relative water content; *n*, number of studies included in the meta-analysis; *p*, significance level of SMD.

of carotenoid accumulation, but as salinity level increased, the effect size of AMF on the soluble sugar content increased (Figures 9E,F). The activity of catalase (CAT) did not change in plants due to AMF colonization under non-stressed or low salinity conditions, but moderate salinity caused the CAT activity to significantly increase in AMF plants (SMD = 0.559, $p = 0.026$). On the other hand, the activity of POD (peroxidase) and SOD (superoxide dismutase) increased substantially in AM-inoculated plants compared to that in non-inoculated plants. Salinity imposition did not seem to impact AMF influence on POD but on SOD. At moderate salinity significantly lower accumulation of SOD was observed in AMF plants when compared to the level at normal condition than in non-inoculated plants in the absence of salt stress (Figures 9G,H).

DISCUSSION

Effects of AMF on Plant Biomass

This study indicated that mycorrhizal inoculation has a significant impact on some plant physiological and biochemical variables related to plant growth, photosynthesis, and defense against oxidative damage under salinity stress.

Salinity induces both osmotic and ion stress in plants. Within a short exposure time, salinity-driven osmotic stress causes water scarcity in the root zone and directly impairs the water status of plants. However, the plant recovers over several hours and reaches a slow, steady rate of growth. The second phase develops with time and is driven by the toxicity of excess Na^+ and Cl^- ions that accumulate in the cytoplasm. Moreover, under salinity stress, plants need additional energy to reduce

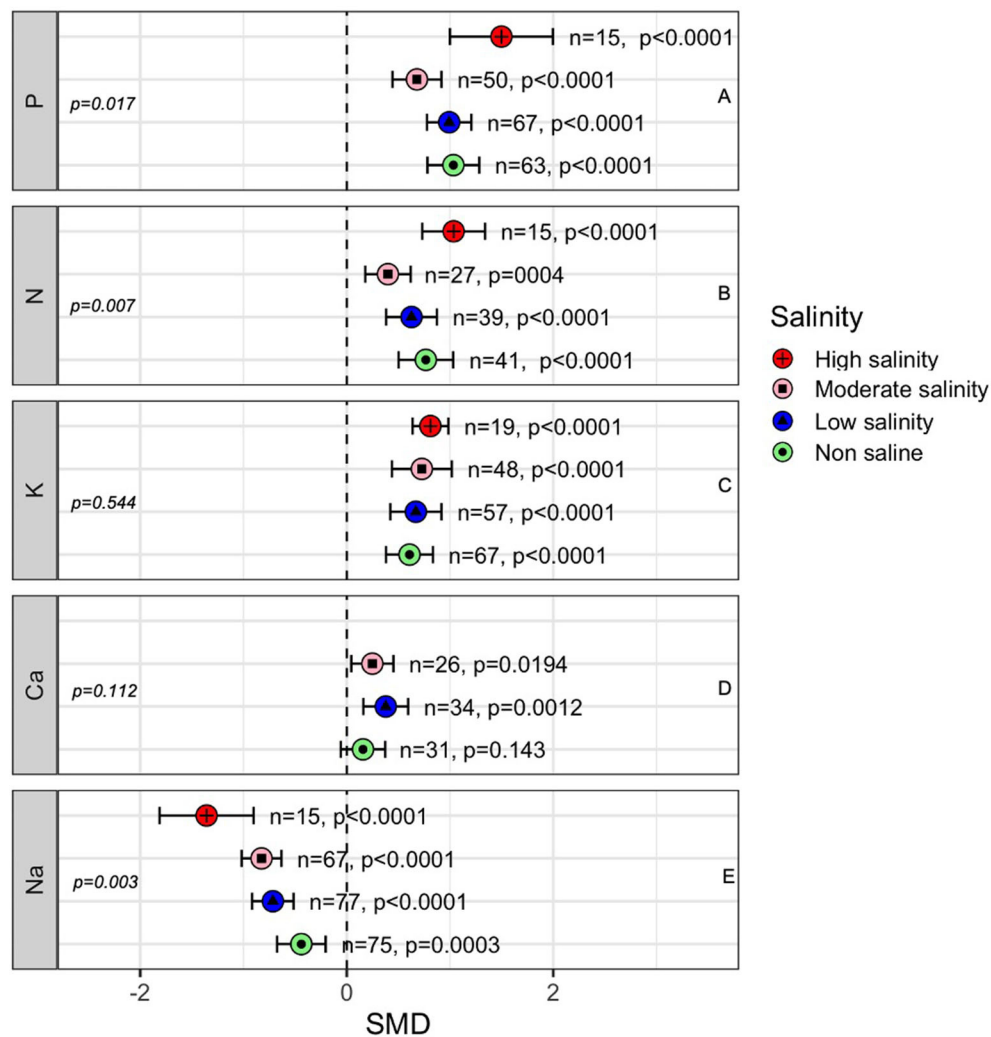


FIGURE 8 | Effects of mycorrhizae on plant nutrient uptake under non-stress, low salinity stress, and moderate salinity stress. Error bars are effect size means $\pm 95\%$ CIs. Where the CIs do not overlap the vertical dashed lines, the effect size for a parameter is significant. *n*, number of studies included in the meta-analysis. Ca, Calcium; K, potassium; Na, sodium; N, Nitrogen and P, phosphorus. *n*, number of studies included in the meta-analysis; *p*, significance level of SMD.

the toxic effects of Na^+ ions and also face nutrient deficiencies. All these processes negatively affect plant growth (Munns and Tester, 2008; Ilangumaran and Smith, 2017; Isayenkov and Maathuis, 2019). Mycorrhizal colonization has been shown to increase plant growth and photosynthetic efficiency under stress conditions (Chandrasekaran et al., 2014; Shamshiri and Fattahi, 2016; Elhindi et al., 2017). In the present meta-analysis, we confirmed that AMF inoculation increases the height, shoot biomass, and root biomass of host plants and that, interestingly, the effect of AMF on plant growth is more prominent under salinity stress than under normal conditions. AMF association in plants influences plant shoot biomass and root biomass more than plant height. Again, AMF influences on plant growth were context-dependent, with several factors playing important roles. For example, *Funneliformis* increased both the shoot and root biomass of plants under stress, whereas *Claroideoglomus*

increased only the shoot biomass, and *Rhizophagus* influenced only the root biomass. In most cases, the effects of fungi on plant growth parameters became more noticeable as the salinity level increased. This result indicates that the plant growth response to AMF colonization is context-dependent. The beneficial effects of fungal symbiosis increase under extreme environments (Redman et al., 2002; Bunn et al., 2009; Dastogeer, 2018). A meta-analysis with AM fungi and leaf endophytes and plant growth parameters reported similar results; the effects of the fungi increased as moisture stress increased (Worchel et al., 2013). Studying the effects of categorical variables on plant growth will help us to select some potential efficient plant-AMF associations in which AMF inoculation will more strongly modulate plant growth in the presence of salinity. Interestingly, under salinity stress conditions, AMF inoculation increased plant growth traits more efficiently in dicot plants than

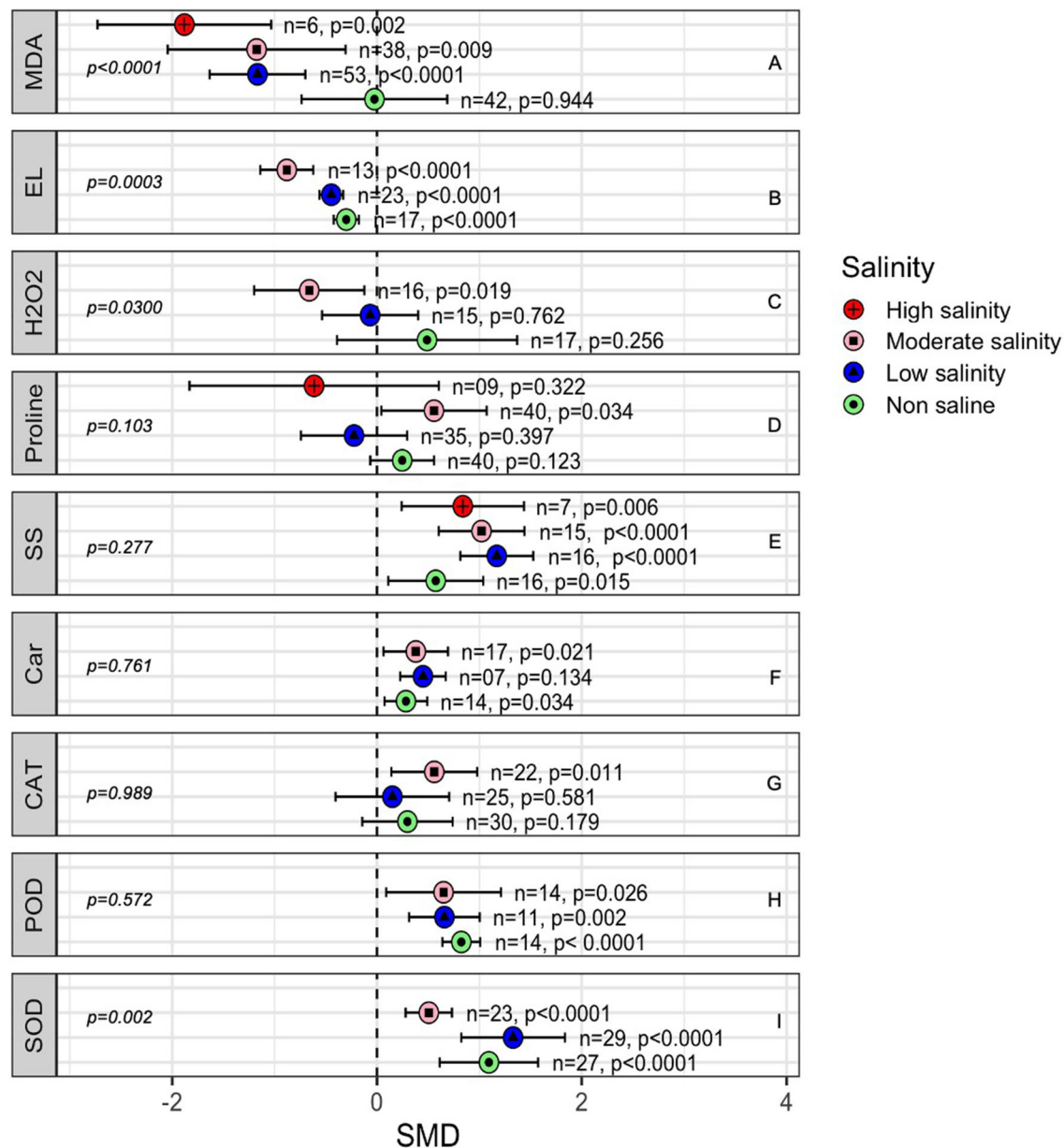


FIGURE 9 | Effects of mycorrhizae on plant enzymatic activity under non-stressed, low salinity, and moderate salinity stress. Error bars are means $\pm$ 95% CIs. Where the CIs do not overlap the vertical dashed lines, the effect size for a parameter is significant. *n*, number of studies included in the meta-analysis. CAT, catalase; Car, Carotenoids; EL, electrical leakage; H2O2, hydrogen peroxide; MDA, malondialdehyde; POD, peroxidase and SOD, superoxide dismutase and SS, soluble sugars. *n*, number of studies included in the meta-analysis, *p*, significance level of SMD.

in monocots. This result was supported by a previous finding that AMF colonization was higher in dicots than in monocots (Weishampel and Bedford, 2006). Similarly, the growth of C3 plants was influenced more than that of C4 plants by AMF inoculation under salinity stress, which corroborates the findings of another meta-analysis (Chandrasekaran et al., 2016). However, our analyses did not show any positive influence of AMF on shoot biomass of C4 plants which is somewhat contradictory to the findings of Chandrasekaran et al. (2016). Therefore, based on

the present meta-analysis, we can recommend the application of AMF to C3-dicot plants to effectively alleviate salinity-induced growth inhibition. Furthermore, in our meta-analysis, the effect of AMF treatment in plants was more pronounced at longer duration of salinity stress (>2 weeks).

Water Status and Photosynthesis

All photosynthetic parameters included in the meta-analysis were found to be present at a significantly higher level in AMF-treated

plants than in non-AMF plants, and this level increased with salinity stress (**Figure 7**). AMF can help plants mitigate or reduce the detrimental effects of salt stress on photosynthesis in various ways. As our meta-analysis showed, AMF improved the water status in plants and thus allowed them to maintain a larger leaf area and higher stomatal conductance, which improved the assimilation rate of CO₂ (Wu et al., 2015; Chen et al., 2017).

The higher water status in AMF-treated plants has been explained by several reports. For example, mycorrhizae can influence root morphology, and with far-reaching extramatrical mycelium, they can acquire macroelements beyond the depletion zone (Schnepp et al., 2011). Moreover, treatment with AMF increases the water use efficiency of plants by augmenting the concentration of compatible solutes to modulate the osmotic potential (Graham and Syvertsen, 1984). Several studies have suggested enhanced RuBisCO enzyme activity in AMF-treated plants, which reduces the intercellular CO₂ concentration (Ci) to provide better protection to the photosynthetic apparatus (Sheng et al., 2008; Chen et al., 2017). Mycorrhizae help plants reduce the degradation of D1 and D2 proteins under salt stress and thus maintain the function of photosystem II, which is important in adapting to stress conditions (Porcel et al., 2016; Chen et al., 2017; Hu et al., 2017). The presence of more polyamines and glycine betaine has also been reported in AMF plants, which is linked to safeguarding CO₂-fixing enzymes (Pang et al., 2007; Talaat and Shawky, 2014). We did not include Mg²⁺ data in our study, but several studies have suggested that a higher concentration of this cation is present in AMF-inoculated plants, which may be associated with higher chlorophyll in AMF plants (Evelin et al., 2012; Hashem et al., 2015). In addition, the enhanced photosynthesis in AMF-treated plants could be related to decreased non-photochemical quenching (NPQ) activity and increased Fv/Fm (Baker, 2008; Hu et al., 2017).

Nutrients

Na⁺ and K⁺ ions, which have similar physicochemical properties, compete at transport sites for entry into the symplast. Higher Na⁺ levels in the rhizosphere reduce K⁺ uptake in plants under saline conditions (Maathuis and Amtmann, 1999). Higher Na⁺ levels affect the integrity and selectivity of the root membrane (Grattan and Grieve, 1999). Plants must consistently maintain a low Na⁺ to K⁺ ratio to tolerate salinity stress (Evelin et al., 2012). AMF-treated plants showed higher K⁺ and lower Na⁺ than non-AMF-treated plants under salt stress conditions (**Figure 8**). AMF-inoculated plants can affect Na⁺ translocation to the upper plant parts and maintain the internal Na⁺ concentration. Mycorrhizae help plants remove Na⁺ from xylem and prevent its accumulation in photosynthetic tissues (Evelin et al., 2012; Maathuis, 2014). It was reported that salinity induces the accumulation of glomalin, a heat shock protein 60 (HSP60) homolog, in the AMF (Hammer and Rillig, 2011), which decreases the damage caused by Na⁺ in the cytosol (Maathuis and Amtmann, 1999). Several studies have detailed the molecular basis of the high K⁺: Na⁺ ratio in AMF-inoculated plants (Asins et al., 2013; Porcel et al., 2016; Chen et al., 2017). For example, in *Oryza sativa*, mycorrhizae compartmentalize Na⁺ into the vacuole by upregulating OsNHX3 (sodium/hydrogen exchanger) and facilitate the removal of cytosolic Na⁺ to the

apoplast through the increased expression of OsSOS1 (salt overly sensitive) and OsHKT2;1 (high-affinity potassium transporter) (Porcel et al., 2016). The higher nutrient uptake in AMF-treated plants is attributed to several factors, including the extramatrical hyphae of mycorrhizae (Marschner and Dell, 1994). AMF colonization influences organic acids and polyamines in plants, which play positive roles in decreasing soil EC, maintaining plant ion homeostasis and enhancing nutrient and water uptake under stress (Pang et al., 2007; Sheng et al., 2011; Evelin et al., 2013; Talaat and Shawky, 2013).

The higher P uptake in AMF-inoculated plants helps maintain membrane integrity by reducing ionic leakage, restricting toxic ions within vacuoles, and enforcing selective ion uptake (Rinaldelli and Mancuso, 1996; Evelin et al., 2012), which consequently reduce the adverse effects of salinity. The increased P uptake in colonized plants has several causes: (a) fungal hyphae secrete acid and alkaline phosphatases that release P and make it available to plants, (b) when P is available, high-affinity phosphate transporter genes (*GvPT*, *GiPT*, and *GmosPT*) are expressed, which can release P even at very low concentrations, and (c) mycorrhizal roots can obtain higher amounts of absorbed P than non-mycorrhizal roots, resulting in a consistent supply of P into the roots (Bolan et al., 1991; Marschner and Dell, 1994; Selvaraj and Chellappan, 2006; Abdel-Fattah and Asrar, 2012). Moreover, AMF colonization in roots helps improve nutrient uptake, specifically N uptake, with extensive underground extraradical mycelia ranging from the roots into the surrounding rhizosphere (Battini et al., 2017).

Oxidation and Antioxidants

Salinity stress induces oxidative stress by creating anomalies in the production and destruction of reactive oxygen species (ROS) (Gill and Tuteja, 2010). Salinity stress-induced lipid peroxidation results in uncontrolled membrane permeability and ion loss from the cells (Estrada et al., 2013; Fileccia et al., 2017). Plant malondialdehyde (MDA) levels are measured as a biomarker for lipid peroxidation to evaluate oxidative stress in plants. As salinity increases, MDA levels increase in plants, indicating the level of stress that the plants are experiencing (Asada and Takahashi, 1987; Gill and Tuteja, 2010; Sharma et al., 2012; Ozgur et al., 2013; Bose et al., 2014; Kumar et al., 2017). Our results suggest that AMF-colonized plants have significantly lower MDA levels, indicating less oxidative stress in these plants than in non-AMF plants. Plants use both enzymatic (SOD, POX, CAT, APX, and GR enzymes) and non-enzymatic antioxidative systems [ascorbate (AsA), glutathione (GSH), carotenoids, and α -tocopherol] to detoxify ROS (Evelin et al., 2009; Gill and Tuteja, 2010; Porcel et al., 2012). Our meta-analysis showed that AMF increased the enzymatic activity of plants in the presence of salts. Importantly, POD and SOD activity increased in mycorrhizal plants at low salinity levels. Higher SOD activity is correlated with higher plant tolerance to salinity (Benavides et al., 2000). SOD helps in the detoxification of excess O₂^{•−} to H₂O₂. H₂O₂ is then converted to H₂O by other enzymes, such as CAT, APX, and POX. Interestingly, some studies showed that mycorrhizal plants have higher CAT activity, but we found that the net effect was neutral when the salinity was low (**Figure 6**). This suggests that the role of CAT is not as important as that

of POD and SOD in AMF-mediated plant salinity tolerance at a lower level of stress, but as the salinity increases, CAT becomes activated, and a combination of these enzymes work toward antioxidation. Again, as noted elsewhere, the activities of these enzymes vary due to factors such as plant species, plant tissues, AMF species, level of salinity, and duration of stress (Evelin et al., 2019). While we did not estimate the magnitude of these factors, the relatively higher CI values of the effect sizes indicate the roles of these factors. One of the indicators of plant damage caused by salinity stress is the relative amount of electrolyte leakage. A higher level of relative electrolyte leakage implies more salinity injury to plant membrane systems (Verslues et al., 2006; Sánchez-Rodríguez et al., 2010; Alqarawi et al., 2014). We showed that electrical conductivity was significantly lower in AMF plants, which suggests lower damage to the cell membrane; electrical conductivity is also correlated with lower MDA levels (Figure 9) but is not correlated with the effect size for H_2O_2 production. The lower EL and MDA, as well as the higher POD and SOD, reflect better antioxidant responses that protect plants from oxidative injury in AMF-treated plants than in non-AMF-treated plants. POD is a group of enzymes that can detoxify H_2O_2 , organic hydroperoxide, and lipid peroxides and convert them to alcohol. They have a haem cofactor at their active sites. Haem is also related to iron homeostasis, which is involved in plant-microbe interactions (Briat et al., 2007). In addition, the redox-active cysteine residues in POD indicate the redox potential of cells or organelles. The plastid modulates the redox potential in leaves (Mühlenbock et al., 2008; Brautigam et al., 2009). Whether fungi interfere with the iron homeostasis and redox potential of the plant cell and increase plant stress tolerance *via* this mechanism has yet to be discovered.

Proline levels increased significantly under moderate salinity conditions in AMF-inoculated plants. Proline can play important roles as an osmoregulatory compound (Yoshida et al., 1997) as well as an ROS scavenger (Dickman and Chen, 2005). The accumulation of proline is correlated with both osmotic stress tolerance and responses to stress conditions involving dehydration (Aspinall and Paleg, 1981; Gzik, 1996; Verbruggen and Hermans, 2008). However, it is still unresolved whether its presence is an adaptive response that provides greater stress tolerance or if its increase is a symptom of stress injury (Ashraf and Foolad, 2007). Relatively high levels of proline in the presence of AMF could, therefore, indicate less damage to a moderately stressed plant in the presence of AMF.

Some Recommendations

This systematic review highlights the importance of AMF in mitigating plant salinity stress and the importance of AMF-mediated plant salinity tolerance. Our current analysis, as well as the recent review paper by Evelin et al. (2019), suggests several aspects that need more attention in order to develop a complete and robust understanding of AM-conferred plant salt stress tolerance. We discuss these aspects in the following bullet points:

- The presence of publication biases was apparent in our datasets. Publication bias is a serious issue in meta-analyses that can have profound effects on the validity and

generalization of the conclusions (Lin and Chu, 2018). Although publication bias has been discussed in medical and social sciences for several years, in ecology and plant sciences, this discussion has just started in the last few years (Alatalo et al., 1997; Gontard-Danek and Møller, 1999; Palmer, 2000; Møller and Jennions, 2001; Dieleman and Janssens, 2011; Koricheva and Gurevitch, 2014). It has been reported that the majority (61%) of meta-analyses in plant ecology did not show or mention publication bias or mention the term “publication bias” (Koricheva and Gurevitch, 2014). Therefore, it is uncertain how robust the conclusions of these meta-analyses are. Therefore, we, along with many other authors, urge researchers to report publication biases in meta-analysis reports. Additionally, factors that contribute to publication biases such as submission bias, editor bias, reviewer bias, etc., as discussed elsewhere (Møller and Jennions, 2001) should be avoided in the publication of scientific reports.

- Most research has focused on the effects of AMF on plant physiology by inoculating individual inocula or mixtures of only a few strains, and less attention has been paid to discerning the effects of complex inoculum mixtures. In nature, AMF colonize roots along with myriad other microbes, which are collectively called the “microbiome.” Many other microbial organisms, such as fungal endophytes and bacteria, can also confer stress tolerance in plants. The consideration of microbe-microbe interactions could be an exciting area for future research.
- The majority of these studies were conducted in controlled growth chamber or greenhouse conditions, while very few have been conducted in field conditions. We need more data regarding the variability or stability of AMF-conferred salinity tolerance before advocating for farm-level applications.
- From a mechanistic perspective, most papers measured the osmolyte concentration in AMF and non-AMF plants, but the underlying mechanisms of this response at the molecular level need further investigation.
- Very few studies have described the effects of sulfur, and they have made inconclusive findings (Evelin et al., 2019). We have yet to explore whether hormones, such as brassinosteroids, auxins, jasmonic acid, and salicylic acid, are involved in AMF-induced plant salinity tolerance, as some of these hormones have been found to improve plant tolerance to salinity (Pedranzani et al., 2003; Siddiqi and Husen, 2019).
- Under salinity stress, the plant lipid metabolism is altered, which is associated with alterations in membrane integrity, composition, and function (Parihar et al., 2015). Lipid peroxidation has been studied, but lipid metabolism in salt-stressed AMF-inoculated plants has received less attention.

Salinity stress inhibits plant growth and the accumulation of biomass by affecting photosynthesis, osmotic balance, enzymatic activities, and nutrient uptake. Mycorrhizal fungi consistently help plants to reduce the impact of salt stress by modulating their physiological processes. AMF-induced plant salinity stress tolerance has broad ecological and agricultural implications. In some regions, increased salinity tolerance can result in higher crop yields. Continued interest in AMF research to uncover the underlying mechanisms of plant-AMF interactions appears well-justified.

CONCLUSION

Our current meta-analysis of 97 published peer reviews related to the effect of AMF on plant responses under salinity stress revealed that compared to non-inoculated plants, AMF plants had significantly higher shoot and root biomass regardless of salinity stress and that the effect was more prominent as salinity stress increased. Consistent with the previous findings of Chandrasekaran et al. (2014, 2016, 2019), Wang et al. (2019), and Pan et al. (2020), we report that AMF-mediated plant salinity tolerance was influenced by fungal genera, plant clades and plant photosynthetic pathways. In addition, we observed for the first time that significant phylogenetic signals exist in AMF-mediated plant salinity tolerance, i.e., closely related plant species had more similar responses to moderate salinity stress when inoculated with closely related (phylogenetic) AMF species. Under salinity stress, the growth of C3 plants was more strongly influenced by AMF inoculation than that of C4 plants, which corroborates the findings of another meta-analysis (Chandrasekaran et al., 2016). However, our analyses did not show any positive influence of AMF on the shoot biomass of C4 plants, which is somewhat contradictory to the findings of Chandrasekaran et al. (2016). Furthermore, we showed that the effect of AMF treatment in plants was more pronounced at longer durations of salinity stress (>2 weeks). Therefore, choosing the appropriate host plant and AMF species is important for using plant-AMF symbionts to improve salt-affected soil in practical applications. The inoculation of AMF consistently increases shoot K and decreases shoot Na, as demonstrated by several investigators (Augé et al., 2014; Chandrasekaran et al., 2014; Pan et al., 2020) and the outcomes of this meta-analysis. The involvement of CAT, SOD, and POD has been reported in previous meta-analyses (Chandrasekaran et al., 2014; Pan et al., 2020), but we also found that AMF-mediated plant salinity tolerance at low salinity

was associated with higher SOD and POD activity, whereas at moderate salinity, more CAT and proline accumulation were also observed in addition to POD and SOD enzymes. AMF-mediated plant salinity tolerance has broad ecological and agricultural implications.

AUTHOR CONTRIBUTIONS

KD developed the concept, performed the database search, partially collected the data, analyzed and interpreted the results, and wrote the draft. MZ, MT, and MA extracted the data, produced the graphs, discussed the results, and revised the manuscript. SO revised the manuscript and supervised the project. All authors approved the final version of the manuscript.

FUNDING

The authors would like to thank Japan Society for the Promotion of Science (JSPS) for providing financial support through Grans-in-Aid for JSPS fellow (to the first author) and through the grant number 120199926.

SUPPLEMENTARY MATERIAL

The Supplementary Material for this article can be found online at: <https://www.frontiersin.org/articles/10.3389/fpls.2020.588550/full#supplementary-material>

Supplementary Figure 1 | Pie chart showing frequency (%) of occurrences of plant (A) and fungal (B) species in the whole metaanalysis dataset in current study.

Supplementary Table 1 | List of papers used in metanalysis.

Supplementary Table 2 | Metadata obtained and used for analyses in the current study.

Supplementary Table 3 | Evaluating bias caused by non-independence of multiple data points per study.

REFERENCES

- Abdel-Fattah, G. M., and Asrar, A.-W. A. (2012). Arbuscular mycorrhizal fungal application to improve growth and tolerance of wheat (*Triticum aestivum* L.) plants grown in saline soil. *Acta Physiol. Plant.* 34, 267–277. doi: 10.1007/s11738-011-0825-6
- Abouheif, E. (1999). A method for testing the assumption of phylogenetic independence in comparative data. *Evol. Ecol. Res.* 1, 895–909.
- Alatalo, R. V., Mappes, J., and Elgar, M. A. (1997). Heritabilities and paradigm shifts. *Nature* 385, 402–403. doi: 10.1038/385402a0
- Al-Karaki, G. N., Hammad, R., and Rusan, M. (2001). Response of two tomato cultivars differing in salt tolerance to inoculation with mycorrhizal fungi under salt stress. *Mycorrhiza* 11, 43–47. doi: 10.1007/s005720100098
- Alqarawi, A., Hashem, A., Abd Allah, E., Alshahrani, T., and Huqail, A. (2014). Effect of salinity on moisture content, pigment system, and lipid composition in *ephedra alata* decne. *Acta. Biol. Hung.* 65, 61–71. doi: 10.1556/ABiol.65.2014.1.6
- Asada, K., and Takahashi, M. (1987). "Production and scavenging of active oxygen in photosynthesis," in *Photoinhibition*. eds D. J. Kyle, C. B. Osmond and C. J. Arntzen (Amsterdam: Elsevier Science Publishers), 227–287.
- Ashraf, M., and Foolad, M. A. (2007). Improving plant abiotic-stress resistance by exogenous application of osmoprotectants glycine betaine and proline. *Environ. Exp. Bot.* 59, 206–216. doi: 10.1016/j.envexpbot.2005.12.006
- Asins, M. J., Villalta, I., Aly, M. M., Olias, R., Álvarez De Morales, P., Huertas, R., et al. (2013). Two closely linked tomato HKT coding genes are positional candidates for the major tomato QTL involved in Na⁺/K⁺ homeostasis. *Plant Cell Environ.* 36, 1171–1191. doi: 10.1111/pce.12051
- Aspinall, D., and Paleg, L. G. (1981). "Proline accumulation: physiological aspects," in *The Physiology and Biochemistry of Drought Resistance in Plants*, eds L. G. Paleg, and D. Aspinall (Sydney, NSW: Academic Press), 205–241.
- Augé, R. M., Toler, H. D., and Saxton, A. M. (2014). Arbuscular mycorrhizal symbiosis and osmotic adjustment in response to NaCl stress: a meta-analysis. *Front. Plant Sci.* 5:562. doi: 10.3389/fpls.2014.00562
- 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
- Balduzzi, S., Rücker, G., and Schwarzer, G. (2019). How to perform a meta-analysis with R: a practical tutorial. *Evid. Based Mental Health* 22, 153–160. doi: 10.1136/ebmental-2019-300117
- Battini, F., Grönlund, M., Agnolucci, M., Giovannetti, M., and Jakobsen, I. (2017). Facilitation of phosphorus uptake in maize plants by mycorrhizosphere bacteria. *Sci. Rep.* 7, 1–11. doi: 10.1038/s41598-017-04959-0
- Begg, C., and Mazumdar, M. (1994). Operating characteristics of a rank correlation test for publication bias. *Biometrics* 50, 1088–1101. doi: 10.2307/2533446
- Begum, N., Qin, C., Ahanger, M. A., Raza, S., Khan, M. I., Ahmed, N., et al. (2019). Role of arbuscular mycorrhizal fungi in plant growth regulation: implications in abiotic stress tolerance. *Front. Plant Sci.* 10:1068. doi: 10.3389/fpls.2019.01068

- Benavides, M. P., Marconi, P. L., Gallego, S. M., Comba, M. E., and Tomaro, M. L. (2000). Relationship between antioxidant defence systems and salt tolerance in *Solanum tuberosum*. *Funct. Plant Biol.* 27, 273–278. doi: 10.1071/PP99138
- Blomberg, S. P., Garland Jr., T., and Ives, A. R. (2003). Testing for phylogenetic signal in comparative data: behavioral traits are more labile. *Evolution* 57, 717–745. doi: 10.1111/j.0014-3820.2003.tb00285.x
- Bolan, N., Hedley, M., and White, R. (1991). Processes of soil acidification during nitrogen cycling with emphasis on legume based pastures. *Plant Soil* 134, 53–63. doi: 10.1007/BF00010717
- Borenstein, M., Hedges, L. V., Higgins, J. P., and Rothstein, H. R. (2011). *Introduction to Meta-analysis*. New York, NY: John Wiley and Sons.
- Borenstein, M., Hedges, L. V., Higgins, J. P. T., and Rothstein, H. R. (2009). *Introduction to Meta-analysis*. Chichester: Wiley. doi: 10.1002/9780470743386
- Borenstein, M., and Higgins, J. P. (2013). Meta-analysis and subgroups. *Prev. Sci.* 14, 134–143. doi: 10.1007/s11121-013-0377-7
- Bose, J., Rodrigo-Moreno, A., and Shabala, S. (2014). ROS homeostasis in halophytes in the context of salinity stress tolerance. *J. Exp. Bot.* 65, 1241–1257. doi: 10.1093/jxb/ert430
- Brautigam, K., Dietzel, L., Kleine, T., Stroher, E., Wormuth, D., and Dietz, K. J. (2009). Dynamic plastid redox signals integrate gene expression and metabolism to induce distinct metabolic states in photosynthetic acclimation in *Arabidopsis*. *Plant Cell* 21, 2715–2732. doi: 10.1105/tpc.108.062018
- Briat, J. F., Curie, C., and Gaymard, F. (2007). Iron utilization and metabolism in plants. *Curr. Opin. Plant Biol.* 10, 276–282. doi: 10.1016/j.pbi.2007.04.003
- Bunn, R., Lekberg, Y., and Zabinski, C. (2009). Arbuscular mycorrhizal fungi ameliorate temperature stress in thermophilic plants. *Ecology* 90, 1378–1388. doi: 10.1890/07-2080.1
- Chandrasekaran, M., Boughattas, S., Hu, S., Oh, S.-H., and Sa, T. (2014). A meta-analysis of arbuscular mycorrhizal effects on plants grown under salt stress. *Mycorrhiza* 24, 611–625. doi: 10.1007/s00572-014-0582-7
- Chandrasekaran, M., Chanratana, M., Kim, K., Seshadri, S., and Sa, T. (2019). Impact of arbuscular mycorrhizal fungi on photosynthesis, water status, and gas exchange of plants under salt stress—a meta-analysis. *Front. Plant Sci.* 10:457. doi: 10.3389/fpls.2019.00457
- Chandrasekaran, M., Kim, K., Krishnamoorthy, R., Walitang, D., Sundaram, S., Joe, M. M., et al. (2016). Mycorrhizal symbiotic efficiency on C3 and C4 plants under salinity stress—a meta-analysis. *Front. Microbiol.* 7:1246. doi: 10.3389/fmicb.2016.01246
- Chaves, M. M., Flexas, J., and Pinheiro, C. (2009). Photosynthesis under drought and salt stress: regulation mechanisms from whole plant to cell. *Ann. Bot.* 103, 551–560. doi: 10.1093/aob/mcn125
- Chen, J., Zhang, H., Zhang, X., and Tang, M. (2017). Arbuscular mycorrhizal symbiosis alleviates salt stress in black locust through improved photosynthesis, water status, and K⁺/Na⁺ homeostasis. *Front. Plant Sci.* 8:1739. doi: 10.3389/fpls.2017.01739
- Cohen, J. (1988). *Statistical Power Analysis for the Behavioral Sciences*. Erlbaum Associates: Hillsdale, NJ.
- Dastogeer, K. M. (2018). Influence of fungal endophytes on plant physiology is more pronounced under stress than well-watered conditions: a meta-analysis. *Planta* 248, 1403–1416. doi: 10.1007/s00425-018-2982-y
- Dickman, M. B., and Chen, C. B. (2005). Proline suppresses apoptosis in the fungal pathogen *Colletotrichum trifolii*. *Proc. Natl. Acad. Sci. U.S.A.* 102, 3459–3464. doi: 10.1073/pnas.0407960102
- Dieleman, W. I., and Janssens, I. A. (2011). Can publication bias affect ecological research? A case study on soil respiration under elevated CO₂. *New Phytol.* 190, 517–521. doi: 10.1111/j.1469-8137.2010.03499.x
- Duval, S., and Tweedie, R. (2000a). A nonparametric “trim and fill” method of accounting for publication bias in meta-analysis. *J. Am. Stat. Assoc.* 95, 89–98. doi: 10.1080/01621459.2000.10473905
- Duval, S., and Tweedie, R. (2000b). Trim and fill: a simple funnel-plot-based method of testing and adjusting for publication bias in meta-analysis. *Biometrics* 56, 455–463. doi: 10.1111/j.0006-341X.2000.00455.x
- Egger, M., Smith, G. D., Schneider, M., and Minder, C. (1997). Bias in meta-analysis detected by a simple, graphical test. Increase in studies of publication bias coincided with increasing use of meta-analysis. *Br. Med. J.* 316, 629–634. doi: 10.1136/bmj.315.7109.629
- Elhindi, K. M., El-Din, A. S., and Elgorban, A. M. (2017). The impact of arbuscular mycorrhizal fungi in mitigating salt-induced adverse effects in sweet basil (*Ocimum basilicum* L.). *Saudi J. Biol. Sci.* 24, 170–179. doi: 10.1016/j.sjbs.2016.02.010
- Estrada, B., Aroca, R., Maathuis, F. J. M., Barea, J. M., and Ruiz-Lozano, J. M. (2013). Arbuscular mycorrhizal fungi native from a Mediterranean saline area enhance maize tolerance to salinity through improved ion homeostasis. *Plant Cell Environ.* 36, 1771–1782. doi: 10.1111/pce.12082
- Evelin, H., Devi, T. S., Gupta, S., and Kapoor, R. (2019). Mitigation of salinity stress in plants by arbuscular mycorrhizal symbiosis: current understanding and new challenges. *Front. Plant Sci.* 10:470. doi: 10.3389/fpls.2019.00470
- Evelin, H., Giri, B., and Kapoor, R. (2012). Contribution of glomus intraradices inoculation to nutrient acquisition and mitigation of ionic imbalance in NaCl-stressed *Trigonella foenum-graecum*. *Mycorrhiza* 22, 203–217. doi: 10.1007/s00572-011-0392-0
- Evelin, H., Giri, B., and Kapoor, R. (2013). Ultrastructural evidence for AMF mediated salt stress mitigation in *Trigonella foenum-graecum*. *Mycorrhiza* 23, 71–86. doi: 10.1007/s00572-012-0449-8
- Evelin, H., and Kapoor, R. (2014). Arbuscular mycorrhizal symbiosis modulates antioxidant response in salt-stressed *Trigonella foenum-graecum* plants. *Mycorrhiza* 24, 197–208. doi: 10.1007/s00572-013-0529-4
- Evelin, H., Kapoor, R., and Giri, B. (2009). Arbuscular mycorrhizal fungi in alleviation of salt stress: a review. *Ann. Bot.* 104, 1263–1280. doi: 10.1093/aob/mcp251
- Eziz, A., Yan, Z., Tian, D., Han, W., Tang, Z., and Fang, J. (2017). Drought effect on plant biomass allocation: a meta-analysis. *Ecol. Evol.* 7, 11002–11010. doi: 10.1002/ece3.3630
- Faraone, S. V. (2008). Interpreting estimates of treatment effects: implications for managed care. *Pharm. Ther.* 33:700.
- Field, A. P., and Gillett, R. (2010). How to do a meta-analysis. *Br. J. Math. Stat. Psychol.* 63, 665–694. doi: 10.1348/000711010X502733
- Fileccia, V., Ruissi, P., Ingrassia, R., Giambalvo, D., Frenda, A. S., and Martinelli, F. (2017). Arbuscular mycorrhizal symbiosis mitigates the negative effects of salinity on durum wheat. *PLoS ONE* 12:e0184158. doi: 10.1371/journal.pone.0184158
- Frosi, G., Barros, V. A., Oliveira, M. T., Santos, M., Ramos, D. G., Maia, L. C., et al. (2016). Symbiosis with AMF and leaf Pi supply increases water deficit tolerance of woody species from seasonal dry tropical forest. *J. Plant Physiol.* 207, 84–93. doi: 10.1016/j.jplph.2016.11.002
- Gerstner, K., Moreno-Mateos, D., Gurevitch, J., Beckmann, M., Kambach, S., Jones, H. P., et al. (2017). Will your paper be used in a meta-analysis? Make the reach of your research broader and longer lasting. *Methods Ecol. Evol.* 8, 777–784. doi: 10.1111/2041-210X.12758
- Gill, S. S., and Tuteja, N. (2010). Reactive oxygen species and antioxidant machinery in abiotic stress tolerance in crop plants. *Plant Physiol. Biochem.* 48, 909–930. doi: 10.1016/j.plaphy.2010.08.016
- Giri, B., and Mukerji, K. G. (2004). Mycorrhizal inoculant alleviates salt stress in *Sesbania aegyptiaca* and *Sesbania grandiflora* under field conditions: evidence for reduced sodium and improved magnesium uptake. *Mycorrhiza* 14, 307–312. doi: 10.1007/s00572-003-0274-1
- Gittleman, J. L., and Kot, M. (1990). Adaptation: statistics and a null model for estimating phylogenetic effects. *Syst. Zool.* 39, 227–241. doi: 10.2307/2992183
- Gontard-Danek, M.-C., and Möller, A. (1999). The strength of sexual selection: a meta-analysis of bird studies. *Behav. Ecol.* 10, 476–486. doi: 10.1093/beheco/10.5.476
- Graham, J., and Syvertsen, J. (1984). Influence of vesicular-arbuscular mycorrhiza on the hydraulic conductivity of roots of two citrus rootstocks. *N. Phytol.* 97, 277–284. doi: 10.1111/j.1469-8137.1984.tb04132.x
- Grattan, S., and Grieve, C. (1999). Mineral nutrient acquisition and response by plants grown in saline environments. *Handb. Plant Crop Stress* 2, 203–229. doi: 10.1201/9780824746728.ch9
- Gupta, B., and Huang, B. (2014). Mechanism of salinity tolerance in plants: physiological, biochemical, and molecular characterization. *Int. J. Genomics* 2014:701596. doi: 10.1155/2014/701596
- Gurevitch, J., and Hedges, L. (1999). Statistical issues in ecological metaanalyses. *Ecology* 80, 1142–1149. doi: 10.1890/0012-9658(1999)080[1142:SIEMA]2.0.CO;2
- Gurevitch, J., Koricheva, J., Nakagawa, S., and Stewart, G. (2018). Meta-analysis and the science of research synthesis. *Nature* 555, 175–182. doi: 10.1038/nature25753

- Gzik, A. (1996). Accumulation of proline and pattern of α -amino acids in sugar beet plants in response to osmotic, water and salt stress. *Environ. Exp. Bot.* 36, 29–38. doi: 10.1016/0098-8472(95)00046-1
- Hackathon, R., Bolker, B., Butler, M., Cowan, P., Vienne, D., and Eddelbuettel, D. (2020). phylobase: base package for phylogenetic structures and comparative data. *R Package Version* 0.8.10.
- Hammer, E. C., and Rillig, M. C. (2011). The influence of different stresses on glomalin levels in an arbuscular mycorrhizal fungus—salinity increases glomalin content. *PLoS ONE* 6:e28426. doi: 10.1371/journal.pone.0028426
- Hanin, M., Ebel, C., Ngom, M., Laplace, L., and Masmoudi, K. (2016). New insights on plant salt tolerance mechanisms and their potential use for breeding. *Front. Plant Sci.* 7:1787. doi: 10.3389/fpls.2016.01787
- Harrer, M., Cuijpers, P., Furukawa, T., and Ebert, D. (2019). dmetar: companion R package for the guide doing meta-analysis in R. *R Package Version* 0.0.9000.
- Hartung, J., and Knapp, G. (2001a). On tests of the overall treatment effect in meta-analysis with normally distributed responses. *Stat. Med.* 20, 1771–1782. doi: 10.1002/sim.791
- Hartung, J., and Knapp, G. (2001b). A refined method for the meta-analysis of controlled clinical trials with binary outcome. *Stat. Med.* 20, 3875–3889. doi: 10.1002/sim.1009
- Hashem, A., Abd Allah, E. F., Alqarawi, A. A., Aldubise, A., and Egamberdieva, D. (2015). Arbuscular mycorrhizal fungi enhances salinity tolerance of panicum turgidum forssk by altering photosynthetic and antioxidant pathways. *J. Plant Interact.* 10, 230–242. doi: 10.1080/17429145.2015.1052025
- Hashem, A., Alqarawi, A. A., Radhakrishnan, R., Al-Arjani, A.-B. F., Aldehaish, H. A., Egamberdieva, D., et al. (2018). Arbuscular mycorrhizal fungi regulate the oxidative system, hormones and ionic equilibrium to trigger salt stress tolerance in *Cucumis sativus* L. *Saudi J. Biol. Sci.* 25, 1102–1114. doi: 10.1016/j.sjbs.2018.03.009
- He, M., and Dijkstra, F. A. (2014). Drought effect on plant nitrogen and phosphorus: a meta-analysis. *New Phytol.* 204, 924–931.
- Hernández, J. A. (2019). Salinity Tolerance in Plants: Trends and Perspectives. *Int. J. Mol. Sci.* 20:2408. doi: 10.3390/ijms20102408
- Hidri, R., Barea, J., Mahmoud, O. M.-B., Abdelly, C., and Azcón, R. (2016). Impact of microbial inoculation on biomass accumulation by *Sulla carnosa* provenances, and in regulating nutrition, physiological and antioxidant activities of this species under non-saline and saline conditions. *J. Plant Physiol.* 201, 28–41. doi: 10.1016/j.jplph.2016.06.013
- Higgins, J. P., Thompson, S. G., Deeks, J. J., and Altman, D. G. (2003). Measuring inconsistency in meta-analyses. *Br. Med. J.* 327, 557–560. doi: 10.1136/bmj.327.7414.557
- Higgins, J. P. T., and Thompson, S. G. (2002). Quantifying heterogeneity in a meta-analysis. *Stat. Med.* 21, 1539–1558. doi: 10.1002/sim.1186
- Holmgren, M., Gómez-Aparicio, L. Q., and Valladares, F. (2012). Non-linear effects of drought under shade-reconciling physiological and ecological models in plant communities. *Oecologia* 169, 293–305. doi: 10.1007/s00442-011-2196-5
- Hu, W., Zhang, H., Chen, H., and Tang, M. (2017). Arbuscular mycorrhizas influence *Lycium barbarum* tolerance of water stress in a hot environment. *Mycorrhiza* 27, 451–463. doi: 10.1007/s00572-017-0765-0
- Huedo-Medina, T. B., Sánchez-Meca, J., Marín-Martínez, F., and Botella, J. (2006). Assessing heterogeneity in meta-analysis: Q statistic or I² index? *Psychol. Methods* 11, 193–206. doi: 10.1037/1082-989X.11.2.193
- Ilangumaran, G., and Smith, D. L. (2017). Plant growth promoting rhizobacteria in amelioration of salinity stress: a systems biology perspective. *Front. Plant Sci.* 8:1768. doi: 10.3389/fpls.2017.01768
- Int'Hout, J., Ioannidis, J. P., and Borm, G. F. (2014). The Hartung-Knapp-Sidik-Jonkman method for random effects meta-analysis is straightforward and considerably outperforms the standard DerSimonian-Laird method. *BMC Med. Res. Methodol.* 14:25. doi: 10.1186/1471-2288-14-25
- Isayenkov, S. V., and Maathuis, F. J. (2019). Plant salinity stress: many unanswered questions remain. *Front. Plant Sci.* 10:80. doi: 10.3389/fpls.2019.00080
- Jin, Y., and Qian, H. (2019). VPhyloMaker: an R package that can generate very large phylogenies for vascular plants. *Ecography* 42, 1353–1359. doi: 10.1111/ecog.04434
- Jombart, T., and Dray, S. (2010). adephylo: exploratory analyses for the phylogenetic comparative method. *Bioinformatics* 26, 1–21. doi: 10.1093/bioinformatics/btq292
- Keck, F., Rimet, F., Bouchez, A., and Franc, A. (2016). phyloSignal: an R package to measure, test, and explore the phylogenetic signal. *Ecol. Evol.* 6, 2774–2780. doi: 10.1002/ece3.2051
- Khallof, M., Martínez-Andújar, C., Lachaâl, M., Karray-Bourauoui, N., Pérez-Alfocea, F., and Albacete, A. (2017). The interaction between foliar GA3 application and arbuscular mycorrhizal fungi inoculation improves growth in salinized tomato (*Solanum lycopersicum* L.) plants by modifying the hormonal balance. *J. Plant Physiol.* 214, 134–144. doi: 10.1016/j.jplph.2017.04.012
- Koricheva, J., and Gurevitch, J. (2014). Uses and misuses of meta-analysis in plant ecology. *J. Ecol.* 102, 828–844. doi: 10.1111/1365-2745.12224
- Kumar, D., Al Hassan, M., Naranjo, M. A., Agrawal, V., Boscaiu, M., and Vicente, O. (2017). Effects of salinity and drought on growth, ionic relations, compatible solutes and activation of antioxidant systems in oleander (*Nerium oleander* L.). *PLoS ONE* 12:e0185017. doi: 10.1371/journal.pone.0185017
- Lajeunesse, M. J., and Forbes, M. R. (2003). Variable reporting and quantitative reviews: a comparison of three meta-analytical techniques. *Ecol. Lett.* 6, 448–454. doi: 10.1046/j.1461-0248.2003.00448.x
- Lau, J., Rothstein, H. R., and Stewart, G. B. (2013). “History and progress of meta-analysis,” in *Handbook of Meta-analysis in Ecology and Evolution*, eds J. Koricheva, J. Gurevitch, and K. Mengersen (Princeton, NJ: Princeton University Press), 407–419. doi: 10.1515/9781400846184-027
- Leimu, R., Mutikainen, P., Koricheva, J., and Fischer, M. (2006). How general are positive relationships between plant population size, fitness and genetic variation? *J. Ecol.* 94, 942–952. doi: 10.1111/j.1365-2745.2006.01150.x
- Li, T., Liu, R.-J., He, X.-H., and Wang, B.-S. (2012). Enhancement of superoxide dismutase and catalase activities and salt tolerance of euhalophyte *Suaeda salsa* L. by mycorrhizal fungus *Glomus mosseae*. *Pedosphere* 22, 217–224. doi: 10.1016/S1002-0160(12)60008-3
- Lin, L., and Chu, H. (2018). Quantifying publication bias in meta-analysis. *Biometrics* 74, 785–794. doi: 10.1111/biom.12817
- Maathuis, F. J. (2014). Sodium in plants: perception, signalling, and regulation of sodium fluxes. *J. Exp. Bot.* 65, 849–858. doi: 10.1093/jxb/ert326
- Maathuis, F. J., and Amtmann, A. (1999). K⁺ nutrition and Na⁺ toxicity: the basis of cellular K⁺/Na⁺ ratios. *Ann. Bot.* 84, 123–133. doi: 10.1006/anbo.1999.0912
- Marschner, H., and Dell, B. (1994). “Nutrient uptake in mycorrhizal symbiosis,” in *Management of Mycorrhizas in Agriculture, Horticulture and Forestry*, eds A. D. Robson, L. K. Abbott, and N. Malajczuk (Dordrecht: Kluwer Academic Publishers), 89–102. doi: 10.1007/BF00000098
- Mayerhofer, M. S., Kernaghan, G., and Harper, K. A. (2013). The effects of fungal root endophytes on plant growth. *Mycorrhiza* 23, 199–128. doi: 10.1007/s00572-012-0456-9
- McGrath, J. M., and Lobell, D. B. (2013). Reduction of transpiration and altered nutrient allocation contribute to nutrient decline of crops grown in elevated CO₂ concentrations. *Plant Cell Environ.* 36, 697–705. doi: 10.1111/pce.12007
- Møller, A. P., and Jennions, M. D. (2001). Testing and adjusting for publication bias. *Trends Ecol. Evol.* 16, 580–586. doi: 10.1016/S0169-5347(01)02235-2
- Moran, P. A. (1950). Notes on continuous stochastic phenomena. *Biometrika* 37, 17–23. doi: 10.1093/biomet/37.1-2.17
- Mühlenbock, P., Szechyńska-Hebda, M., Plaszczyca, M., Baudo, M., Mateo, A., Mullineaux, P. M., et al. (2008). Chloroplast signaling and lesion simulating disease1 regulate crosstalk between light acclimation and immunity in *Arabidopsis*. *Plant Cell* 20, 2339–2356. doi: 10.1105/tpc.108.059618
- Munns, R., and Gilliam, M. (2015). Salinity tolerance of crops—what is the cost? *N. Phytol.* 208, 668–673. doi: 10.1111/nph.13519
- Munns, R., and Tester, M. (2008). Mechanisms of salinity tolerance. *Annu. Rev. Plant Biol.* 59, 651–681. doi: 10.1146/annurev.arplant.59.032607.092911
- Murad, M. H., Chu, H., Lin, L., and Wang, Z. (2018). The effect of publication bias magnitude and direction on the certainty in evidence. *BMJ Evid. Based Med.* 23, 84–86. doi: 10.1136/bmjebm-2018-110891
- Murkute, A., Sharma, S., and Singh, S. (2006). Studies on salt stress tolerance of citrus rootstock genotypes with arbuscular mycorrhizal fungi. *Hortic. Sci.* 33, 70–76. doi: 10.17221/3742-HORTSCI
- Nakagawa, S., and Santos, E. S. (2012). Methodological issues and advances in biological meta-analysis. *Evol. Ecol.* 26, 1253–1274. doi: 10.1007/s10682-012-9555-5

- Öpik, M., Zobel, M., Cantero, J. J., Davison, J., and Facelli, J. M., Hiiesalu, I., et al. (2013). Global sampling of plant roots expands the described molecular diversity of arbuscular mycorrhizal fungi. *Mycorrhiza* 23, 411–430. doi: 10.1007/s00572-013-0482-2
- Ozgur, R., Uzilday, B., Sekmen, A. H., and Turkan, I. (2013). Reactive oxygen species regulation and antioxidant defence in halophytes. *Funct. Plant Biol.* 40, 832–847. doi: 10.1071/FP12389
- Pagel, M. (1999). Inferring the historical patterns of biological evolution. *Nature* 401, 877–884. doi: 10.1038/44766
- Palmer, A. R. (2000). Quasi-replication and the contract of error: lessons from sex ratios, heritabilities and fluctuating asymmetry. *Ann. Rev. Ecol. Syst.* 31, 441–480. doi: 10.1146/annurev.ecolsys.31.1.441
- Pan, J., Peng, F., Tedeschi, A., Xue, X., Wang, T., Liao, J., et al. (2020). Do halophytes and glycophytes differ in their interactions with arbuscular mycorrhizal fungi under salt stress? A meta-analysis. *Bot. Stud.* 61, 1–13. doi: 10.1186/s40529-020-00290-6
- Pandey, R., and Garg, N. (2017). High effectiveness of *Rhizophagus irregularis* is linked to superior modulation of antioxidant defence mechanisms in *Cajanus cajan* (L.) Millsp. genotypes grown under salinity stress. *Mycorrhiza* 27, 669–682. doi: 10.1007/s00572-017-0778-8
- Pang, X.-M., Zhang, Z.-Y., Wen, X.-P., Ban, Y., and Moriguchi, T. (2007). Polyamines, all-purpose players in response to environment stresses in plants. *Plant Stress* 1, 173–188.
- Paradis, E., and Schliep, K. (2019). ape 5.0: an environment for modern phylogenetics and evolutionary analyses in R. *Bioinformatics* 35, 526–528. doi: 10.1093/bioinformatics/bty633
- Parihar, P., Singh, S., Singh, R., Singh, V. P., and Prasad, S. M. (2015). Effect of salinity stress on plants and its tolerance strategies: a review. *Environ. Sci. Pollut. Res.* 22, 4056–4075. doi: 10.1007/s11356-014-3739-1
- Pedranzani, H., Racagni, G., Alemano, S., Miersch, O., Ramírez, I., Peña-Cortés, H., et al. (2003). Salt tolerant tomato plants show increased levels of jasmonic acid. *Plant Growth Regul.* 41, 149–158. doi: 10.1023/A:1027311319940
- Porcel, R., Aroca, R., Azcon, R., and Ruiz-Lozano, J. M. (2016). Regulation of cation transporter genes by the arbuscular mycorrhizal symbiosis in rice plants subjected to salinity suggests improved salt tolerance due to reduced Na⁺ root-to-shoot distribution. *Mycorrhiza* 26, 673–684. doi: 10.1007/s00572-016-0704-5
- Porcel, R., Aroca, R., and Ruiz-Lozano, J. M. (2012). Salinity stress alleviation using arbuscular mycorrhizal fungi. A review. *Agron. Sust. Dev.* 32, 181–200. doi: 10.1007/s13593-011-0029-x
- R Core Team (2020). *R: A Language and Environment for Statistical Computing*. Vienna: R Foundation for Statistical Computing. Available online at: <https://www.R-project.org/>.
- Redman, R. S., Sheehan, K. B., Stout, R. G., Rodriguez, R. J., and Henson, J. M. (2002). Thermotolerance generated by plant/fungal symbiosis. *Science* 298, 1581–1581. doi: 10.1126/science.1072191
- Rinaldelli, E., and Mancuso, S. (1996). Response of young mycorrhizal and non-mycorrhizal plants of olive tree (*Olea europaea* L.) to saline conditions. I. Short-term electrophysiological and long-term vegetative salt effects. *Adv. Hortic. Sci.* 10, 126–134.
- Rosenberg, M. S., Adams, D. C., and Gurevitch, J. (2000). *Metawin: Statistical Software for Meta-analysis*. Version 2. Sunderland, MA: Sinauer Associates.
- Ruiz-Lozano, J. M., Porcel, R., Azcón, C., and Aroca, R. (2012). Regulation by arbuscular mycorrhizae of the integrated physiological response to salinity in plants: new challenges in physiological and molecular studies. *J. Exp. Bot.* 63, 4033–4044. doi: 10.1093/jxb/ers126
- Sánchez-Rodríguez, E., Rubio-Wilhelmi, M. M., Cervilla, L. M., Blasco, B., Rios, J. J., Rosales, M. A., et al. (2010). Genotypic differences in some physiological parameters symptomatic for oxidative stress under moderate drought in tomato plants. *Plant Sci.* 178, 30–40. doi: 10.1016/j.plantsci.2009.10.001
- Schmidt, F., and Hunter, J. (2015). *Methods of Meta-analysis*. London: SAGE Publications, Ltd. doi: 10.4135/9781483398105
- Schnepf, A., Leitner, D., Klepsch, S., Pellerin, S., and Mollier, A. (2011). “Modelling phosphorus dynamics in the soil-plant system,” in *Phosphorus in Action*, eds E. Bünemann, A. Oberson, and E. Frossard (Berlin: Springer), 113–133. doi: 10.1007/978-3-642-15271-9_5
- Selvaraj, T., and Chellappan, P. (2006). Arbuscular mycorrhizae: a diverse personality. *J. Cent. Eur. Agric.* 7, 349–358.
- Serbinova, E. A., and Packer, L. (1994). [34] antioxidant properties of α -tocopherol and α -tocotrienol. *Methods Enzymol.* 234, 354–366. doi: 10.1016/0076-6879(94)34105-2
- Shamshiri, M., and Fattahi, M. (2016). Effects of arbuscular mycorrhizal fungi on photosystem II activity of three pistachio rootstocks under salt stress as probed by the OJIP-test. *Russ. J. Plant Physiol.* 63, 101–110. doi: 10.1134/S1021443716010155
- Sharma, P., Jha, A. B., Dubey, R. S., and Pessarakli, M. (2012). Reactive oxygen species, oxidative damage, and antioxidative defense mechanism in plants under stressful conditions. *J. Bot.* 2012:217037. doi: 10.1155/2012/217037
- Sheng, M., Tang, M., Chen, H., Yang, B., Zhang, F., and Huang, Y. (2008). Influence of arbuscular mycorrhizae on photosynthesis and water status of maize plants under salt stress. *Mycorrhiza* 18, 287–296. doi: 10.1007/s00572-008-0180-7
- Sheng, M., Tang, M., Zhang, F., and Huang, Y. (2011). Influence of arbuscular mycorrhiza on organic solutes in maize leaves under salt stress. *Mycorrhiza* 21, 423–430. doi: 10.1007/s00572-010-0353-z
- Shi, L., and Lin, L. (2019). The trim-and-fill method for publication bias: practical guidelines and recommendations based on a large database of meta-analyses. *Medicine* 98:e15987. doi: 10.1097/MD.00000000000015987
- Siddiqi, K. S., and Husen, A. (2019). Plant response to jasmonates: current developments and their role in changing environment. *Bull. Natl. Res. Centre* 43, 1–11. doi: 10.1186/s42269-019-0195-6
- Sidik, K., and Jonkman, J. N. (2005). Simple heterogeneity variance estimation for meta-analysis. *J. R. Stat. Soc. [Ser. C]* 54, 367–384. doi: 10.1111/j.1467-9876.2005.00489.x
- Sidik, K., and Jonkman, J. N. (2007). A comparison of heterogeneity variance estimators in combining results of studies. *Stat. Med.* 26, 1964–1981. doi: 10.1002/sim.2688
- Simonsohn, U., Nelson, L. D., and Simmons, J. P. (2014). P-curve: a key to the file-drawer. *J. Exp. Psychol.* 143:534. doi: 10.1037/a0033242
- Stecher, G., Tamura, K., and Kumar, S. (2020). Molecular evolutionary genetics analysis (MEGA) for macOS. *Mol. Biol. Evol.* 37, 1237–1239. doi: 10.1093/molbev/msz312
- Talaat, N. B., and Shawky, B. T. (2013). Modulation of nutrient acquisition and polyamine pool in salt-stressed wheat (*Triticum aestivum* L.) plants inoculated with arbuscular mycorrhizal fungi. *Acta Physiol. Plant.* 35, 2601–2610. doi: 10.1007/s11738-013-1295-9
- Talaat, N. B., and Shawky, B. T. (2014). Protective effects of arbuscular mycorrhizal fungi on wheat (*Triticum aestivum* L.) plants exposed to salinity. *Environ. Exp. Bot.* 98, 20–31. doi: 10.1016/j.envexpbot.2013.10.005
- Thiery, O., Vasar, M., Jairus, T., Davison, J., Roux, C., Kivistik, P. A., et al. (2016). Sequence variation in nuclear ribosomal small subunit, internal transcribed spacer and large subunit regions of *Rhizophagus irregularis* and *Gigaspora margarita* is high and isolate-dependent. *Mol. Ecol.* 25, 2816–2832. doi: 10.1111/mec.13655
- 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
- Van Zelm, E., Zhang, Y., and Testerink, C. (2020). Salt tolerance mechanisms of plants. *Annu. Rev. Plant Biol.* 71, 403–433. doi: 10.1146/annurev-arplant-050718-100005
- Verbruggen, N., and Hermans, C. (2008). Proline accumulation in plants, a review. *Amino Acids* 35, 753–759. doi: 10.1007/s00726-008-0061-6
- Veresoglou, S. D., Meneses, G., and Rillig, M. C. (2012). Do arbuscular mycorrhizal fungi affect the allometric partition of host plant biomass to shoots and roots? A meta-analysis of studies from 1990 to 2010. *Mycorrhiza* 22, 227–235. doi: 10.1007/s00572-011-0398-7
- Verslues, P. E., Agarwal, M., Katiyar-Agarwal, S., Zhu, J., and Zhu, J. K. (2006). Methods and concepts in quantifying resistance to drought, salt and freezing, abiotic stresses that affect plant water status. *Plant J.* 45, 523–539. doi: 10.1111/j.1365-3113.2005.02593.x
- Vohník, M., Albrechtova, J., and Vosatka, M. (2005). The inoculation with *Oidiodendron maius* and *Phialocephala fortinii* alters phosphorus and nitrogen uptake, foliar C:N ratio and root biomass distribution in *Rhododendron* cv. *Azurro*. *Symbiosis* 40, 87–96.
- Wang, Y., Wang, J., Yan, X., Sun, S., and Lin, J. (2019). The effect of arbuscular mycorrhizal fungi on photosystem II of the host plant under salt stress: a meta-analysis. *Agronomy* 9:806. doi: 10.3390/agronomy9120806

- Wang, Y., Wang, M., Li, Y., Wu, A., and Huang, J. (2018). Effects of arbuscular mycorrhizal fungi on growth and nitrogen uptake of *Chrysanthemum morifolium* under salt stress. *PLoS ONE* 13:e0196408. doi: 10.1371/journal.pone.0196408
- Weishampel, P. A., and Bedford, B. L. (2006). Wetland dicots and monocots differ in colonization by arbuscular mycorrhizal fungi and dark septate endophytes. *Mycorrhiza* 16, 495–502. doi: 10.1007/s00572-006-0064-7
- Worchel, E. R., Giaque, H. E., and Kivlin, S. N. (2013). Fungal symbionts alter plant drought response. *Microb. Ecol.* 65, 671–678. doi: 10.1007/s00248-012-0151-6
- Wu, N., Li, Z., Liu, H., and Tang, M. (2015). Influence of arbuscular mycorrhiza on photosynthesis and water status of *populus cathayana* Rehder males and females under salt stress. *Acta Physiol. Plant.* 37:183. doi: 10.1007/s11738-015-1932-6
- Xie, X.-F., Song, Y.-B., Zhang, Y.-L., Pan, X., and Dong, M. (2014). Phylogenetic meta-analysis of the functional traits of clonal plants foraging in changing environments. *PLoS ONE* 9:e107114. doi: 10.1371/journal.pone.0107114
- Yoshida, Y., Kiyosue, T., Nakashima, K., Yamaguchi-Shinozaki, K., and Shinozaki, K. (1997). Regulation of levels of proline as an osmolyte in plants under water stress. *Plant Cell Physiol.* 38, 1095–1102.
- Zöhrb, C., Geilfus, C. M., and Dietz, K. J., (2019). Salinity and crop yield. *Plant Biol.* 21, 31–38. doi: 10.1111/plb.12884

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.

Copyright © 2020 Dastogeer, Zahan, Tahjib-Ul-Arif, Akter and Okazaki. This is an open-access article distributed under the terms of the Creative Commons Attribution License (CC BY). The use, distribution or reproduction in other forums is permitted, provided the original author(s) and the copyright owner(s) are credited and that the original publication in this journal is cited, in accordance with accepted academic practice. No use, distribution or reproduction is permitted which does not comply with these terms.



Arbuscular Mycorrhizal Fungi Regulate Polyamine Homeostasis in Roots of Trifoliolate Orange for Improved Adaptation to Soil Moisture Deficit Stress

Ying-Ning Zou¹, Fei Zhang¹, Anoop K. Srivastava², Qiang-Sheng Wu^{1,3*} and Kamil Kuča^{3*}

¹ College of Horticulture and Gardening, Yangtze University, Jingzhou, China, ² ICAR-Central Citrus Research Institute, Nagpur, India, ³ Department of Chemistry, Faculty of Science, University of Hradec Kralove, Hradec Kralove, Czechia

OPEN ACCESS

Edited by:

Alvaro Sanz-Saez,
Auburn University, United States

Reviewed by:

Yanbo Hu,
Northeast Forestry University, China
Christian Santander,
Centro de Recursos Hídricos para La
Agricultura y La Minería, Temuco,
Chile

*Correspondence:

Qiang-Sheng Wu
wuqiangsh@163.com
Kamil Kuča
kamil.kuca@uhk.cz

Specialty section:

This article was submitted to
Plant Abiotic Stress,
a section of the journal
Frontiers in Plant Science

Received: 31 August 2020

Accepted: 25 November 2020

Published: 12 January 2021

Citation:

Zou Y-N, Zhang F, Srivastava AK,
Wu Q-S and Kuča K (2021)
Arbuscular Mycorrhizal Fungi
Regulate Polyamine Homeostasis
in Roots of Trifoliolate Orange
for Improved Adaptation to Soil
Moisture Deficit Stress.
Front. Plant Sci. 11:600792.
doi: 10.3389/fpls.2020.600792

Soil arbuscular mycorrhizal fungi (AMF) enhance the tolerance of plants against soil moisture deficit stress (SMDS), but the underlying mechanisms are still not fully understood. Polyamines (PAs) as low-molecular-weight, aliphatic polycations have strong roles in abiotic stress tolerance of plants. We aimed to investigate the effect of AMF (*Funneliformis mosseae*) inoculation on PAs, PA precursors, activities of PA synthases and degrading enzymes, and concentration of reactive oxygen species in the roots of trifoliolate orange (*Poncirus trifoliata*) subjected to 15 days of SMDS. Leaf water potential and total chlorophyll levels were comparatively higher in AMF-inoculated than in non-AMF-treated plants exposed to SMDS. Mycorrhizal plants recorded a significantly higher concentration of precursors of PA synthesis such as L-ornithine, agmatine, and S-adenosyl methionine, besides higher putrescine and cadaverine and lower spermidine during the 15 days of SMDS. AMF colonization raised the PA synthase (arginine decarboxylase, ornithine decarboxylase, spermidine synthase, and spermine synthase) activities and PA-degrading enzymes (copper-containing diamine oxidase and FAD-containing polyamine oxidase) in response to SMDS. However, mycorrhizal plants showed a relatively lower degree of membrane lipid peroxidation, superoxide anion free radical, and hydrogen peroxide than non-mycorrhizal plants, whereas the difference between them increased linearly up to 15 days of SMDS. Our study concluded that AMF regulated PA homeostasis in roots of trifoliolate orange to tolerate SMDS.

Keywords: citrus, mycorrhiza, polyamine, *Poncirus trifoliata*, water deficit

INTRODUCTION

Polyamines (PAs) are low-molecular-weight, aliphatic polycationic compounds that widely occur in prokaryotic and eukaryotic cells (Takahashi, 2020). In plants, the abundant PAs include putrescine (Put), spermidine (Spd), and spermine (Spm) as well as the less abundant cadaverine (Cad) (Upadhyay et al., 2020). Plant Put originates from L-arginine (L-Arg) and L-ornithine (L-Orn)

via the catalytic action of arginine decarboxylase (ADC) and ornithine decarboxylase (ODC), respectively (Liu et al., 2015). Put serves as the substrate for the Spd biosynthesis under the combination with decarboxylated S-adenosyl methionine (SAM) and then Spm biosynthesis via the action of S-adenosyl methionine decarboxylase (SAMDC), spermidine synthases (SPDS), and spermine synthases (SPMS) (Salloum et al., 2018). PA catabolism in plants is operated through copper-containing diamine oxidase (CuAO) and FAD-containing polyamine oxidase (PAO) predominantly localized in the cell wall (Šebela et al., 2001). CuAO mainly catalyzes the oxidation of Put and Cad, and PAO catalyzes the oxidation of Spd and Spm. Hydrogen peroxide (H_2O_2), a kind of reactive oxygen species (ROS), is the byproduct of PA catabolism.

A large number of studies have shown that plant PAs are involved in a variety of responses against abiotic stress (Alcázar et al., 2020; Upadhyay et al., 2020; Zhang et al., 2020). The roles of PAs in response to plant stress include (1) acting as a compatible solute along with γ -aminobutyric acid, glycinebetaine, and proline; (2) stabilizing cellular organellar membranes and macromolecules; (3) acting as scavengers of ROS and triggering the antioxidant defense system; (4) considering as signal molecules in the ABA-regulated stress responses; (5) regulating the ion channels; and (6) participating in programmed cell death (Gupta et al., 2013; Minocha et al., 2014). These multiple roles of PAs suggest important cellular functions in plants while coping with abiotic stress.

Soil moisture deficit stress (SMDS) is one of the abiotic stresses, which seriously affects the growth and yield of crops (Muthusamy et al., 2014). It is well established that arbuscular mycorrhizal fungi (AMF) in soil colonize host roots, thereby establishing the arbuscular mycorrhizal (AM) symbionts. Such symbionts enhance the drought tolerance of host plants through a series of morphological, physiological, and molecular mechanisms (Baslam and Giocoechea, 2012; Wu et al., 2013, 2019; Millar and Bennett, 2016; Huang et al., 2017; Venice et al., 2017; He et al., 2019, 2020; Lokhandwala and Hoeksema, 2019; Zou et al., 2019, 2020; Cheng et al., 2020). In maize, AMF triggered Put catabolism into γ -aminobutyric acid along with an improved N-assimilation, which is an important metabolic process in mycorrhizal plants in response to drought stress (Hu and Chen, 2020). A higher Put and Cad and lower Spd and Spm level in mycorrhizal trifoliate orange (*Poncirus trifoliata* L. Raf.) compared with non-mycorrhizal plants is reported under drought stress (Zhang et al., 2020). Proteomics techniques further showed that when the mycelium of ericoid mycorrhizal fungi was exposed to excess of zinc and cadmium, agmatinase (an enzyme associated with PA biosynthesis) was accumulated in mycorrhizal plants in response to abiotic stress (Chiapello et al., 2015). However, Luo (2009) observed comparatively lower Put levels in mycorrhizal over non-mycorrhizal trifoliate orange seedlings exposed to drought stress. In alfalfa plants, AMF inoculation induced an increase in concentration of Spd and Spm under drought stress (Goicoechea et al., 1998). These inconsistent responses on PA changes indicate that changes of PA pool are one of the mechanisms responsible for improved adaptation under abiotic stress. The relationship between AMF and PAs of host

plants is rather complex, especially with regard to changes in the PA homeostasis and metabolic pathways involved.

Citrus is globally one of the most traded fruit crops (Srivastava and Singh, 2008). Likewise, trifoliate orange is the most extensively used rootstock for satsuma mandarin (*Citrus unshiu* Marc.) grown on Oxisols, Alfisols, and Ultisols, where exposure to SMDS is a common feature (Srivastava and Singh, 2009). Trifoliate orange is a drought-sensitive rootstock. In a plant, the roots act as first sensor to perceive the soil water deficit (Davies et al., 1994). Considering AMF as root colonizer, biochemical parameters in roots are expected to be more revealing than analysis of any other plant parts. In this study, we hypothesized that AMF inoculation could modulate root PA homeostasis of host plants to cope with drought stress. In this background, we studied the changes in four PA levels, four PA precursor levels, and PA synthase and catabolase activities, accompanied with ROS and degree of membrane lipid peroxidation in roots of trifoliate orange inoculated with AMF followed by exposure to SMDS under controlled-environment conditions.

MATERIALS AND METHODS

Preparation of Mycorrhizal Fungal Inoculum

An arbuscular mycorrhizal fungus, *Funneliformis mosseae* (Nicol. & Gerd.) C. Walker & A. Schüßler, was chosen based on its ability in enhancing drought tolerance of trifoliate orange on inoculation with this strain (He et al., 2020). The fungal strain was propagated with identified spores and white clover in pots for 3 months. At harvest, the aboveground part of white clover was removed, and both roots and growth substrates were collected as the mycorrhizal fungus inoculant, which contained soil hyphae, AMF-colonized root segments, and spores (16 spores g^{-1}).

Plant Culture

The seeds of trifoliate orange were surface sterilized with 75% of ethyl alcohol solutions for 10 min, rinsed with distilled water, and placed in autoclaved sand for germination under 26°C and 72% relative air humidity. A month later, the seedlings of four-leaf age and uniform in size were transplanted into 2.3-L plastic pots filled with 2.8 kg of autoclaved mixture of soil and sand (1:1, v/v). The soil belonged to the Ferralsol (FAO system) with pH 6.2, Brays-P 17.43 mg kg^{-1} , and soil organic carbon content 10.68 mg kg^{-1} . At the same time, 100 g of mycorrhizal inoculum was added into the rhizosphere of potted seedlings in each pot. An equal amount of sterilized mycorrhizal inoculant, plus 2 ml inoculum filtrate using $25\text{-}\mu\text{m}$ filters, was applied into the seedlings' rhizosphere as the non-AMF treatment. The non-AMF- and AMF-inoculated seedlings were kept at 75% of maximum field water capacity (24.11%), corresponding to well-watered (WW) status, in a growth chamber of Yangtze University (Jingzhou, China) maintained at $900 \mu\text{mol m}^{-2} \text{ s}^{-1}$ photon flux density, $28/20^\circ\text{C}$ day/night temperature, and 68% relative air humidity. During this experiment, the plants did not receive any nutrient solution.

Fifteen weeks later, soil moisture in all the treated pots was adjusted to soil WW status (18.08%), designed as 0 days, before SMDS. Subsequently, SMDS priming was executed by stopping the water supply. AMF- and non-AMF-inoculated plants were harvested at 0th, 5th, 10th, and 15th day of SMDS. During the 15 days of SMDS, the soil moisture content reduced from 18.08 to 4.18% in AM plants and from 18.08 to 4.24% in non-AM plants. At each harvest, 12 seedlings (three seedlings per pot) of four pots of each treatment were harvested. As a result, the experiment was laid out in a completely randomized blocked design with AMF and non-AMF inoculations under SMDS of 0, 5, 10, and 15 days. A total of 32 pots containing 16 AMF-inoculated pots and 16 non-AMF-inoculated pots were used. The experiment was conducted for 120 days.

Determination of Plant Growth and Root Mycorrhizal Colonization

Plant growth parameters such as plant height, stem diameter, and leaf number per plant were recorded before SMDS began. At 0 days of SMDS, AMF- and non-AMF-treated plants were harvested and divided into shoots and roots, followed by determination of their biomass. And then, at each harvest, the treated plants were divided into shoots and roots. Part root samples were frozen with liquid nitrogen and then stored at -80°C for the subsequent analysis of biochemical parameters. Six 1-cm-long root segments per plant were cleared in 10% KOH solution and stained by trypan blue in lactic acid (Phillips and Hayman, 1970). Root mycorrhizas were observed under an optical microscope, and the rate of root mycorrhizal colonization (%) was calculated as the percentage of AMF-colonized root lengths versus total observed root lengths (Zhang et al., 2018).

Determination of Leaf Water Potential and Total Chlorophyll Contents

Total chlorophyll ($a + b$) content in leaves was determined using the procedure as suggested by Lichtenthaler and Wellburn (1983) by extraction with 80% acetone. Leaf water potential (Ψ) was measured by PS Ψ PRO matched with a dew point microvoltmeter (HR-33T; Wescor, Logan, UT, United States) on a fully expanded second leaf at the top.

Determination of Root PA Contents

Root PAs (Spm, Spd, Put, and Cad) and precursors (L-Arg, L-Orn, agmatine, and SAM) to PA synthesis were extracted and quantified according to the protocol outlined by Ducros et al. (2009) with minor modification. A 50-mg fresh root sample was ground in an ice bath with 0.5 ml of pre-cooled solutions containing acetonitrile, methanol, and distilled water (2:2:1, v/v), followed by ultrasonic treatment for 5 min. The samples were placed at -20°C for 1 h and then centrifuged at $12,000 \times g$ for 15 min. The 0.1-ml supernatant was mixed with 50 μl sodium carbonate solutions and 50 μl 20 mg/ml dansulfonyl chloride solution at 40°C for 1 h in the dark. The mixture was incubated with 1% formic acid for 30 s and centrifuged at $12,000 \times g$ for 15 min. A total of 80 μl supernatants were used for analysis of free PAs and precursors by the ultra-high

performance liquid chromatograph (UHPLC)–MS. The Agilent 1290 Infinity II series (Agilent Technologies, Santa Clara, CA, United States) UHPLC was used, and the liquid chromatography column namely ACQUITY UPLC HSS T3 (100×2.1 mm, $1.8 \mu\text{m}$; Waters) was used for the separation. The A phase of the liquid chromatograph was 10 mM formic acid solution, while the B phase was acetonitrile solution using the column temperature of $35 \pm 4^{\circ}\text{C}$ as the sample tray temperature and 1 μl as the sample volume. Mass spectrometry data acquisition and quantitative analysis of target compounds were carried out through Agilent MassHunter Work Station Software (B.08.00; Agilent Technologies).

Determination of Root PA-Metabolized Enzyme Activities

Root samples were washed in precooled phosphate buffer (0.01 M, pH 7.4), followed by incubation of 0.1 g treated root samples with 0.9 ml 0.01 M (pH 7.4) phosphate buffer. The homogenate was placed in an ice bath for ultrasonic crushing (20 kHz) with a total of three crushings, every crushing lasting for 5 s intermittently involving of the interval of 3 s. Finally, the homogenate was centrifuged at $5,000 \times g$ for 10 min, and the supernatant was taken for the analysis of PA-metabolized enzyme activities, based on ELISA, sourced from Shanghai Enzyme-Linked Biotechnology Co. Ltd (Shanghai, China). All the determinations were carried out according to the recommended user's manuals. The following kits in the order of ml090024-S, ml090861-S, ml073540-S, ml187041-S, ml067255-S, and ml096680-S, respectively, were used for the analysis of PAO, CuAO, ADC, ODC, SPMS, and SPDS.

Determination of ROS and Degree of Membrane Lipid Peroxidation

The H_2O_2 in roots was extracted with 0.1% trichloroacetic acid solution and determined as per procedure put forward by Velikova et al. (2000). Root superoxide anion free radical (O_2^-) content was assayed using the procedure described by He et al. (2020). Root malonaldehyde (MDA, the degree of membrane lipid peroxidation) concentration was determined according to Sudhakar et al. (2001).

Statistical Analysis

The measurement of all variables was replicated four times. Experimental data (mean $\pm$ SD, $n = 4$) generated were performed by ANOVA according to SAS (SAS Institute, Cary, NC, United States). The percentage regarding root AMF colonization rate required arcsine transformation before ANOVA. Duncan's multiple range tests were used to compare the significant differences between treatments at the 5% level.

RESULTS

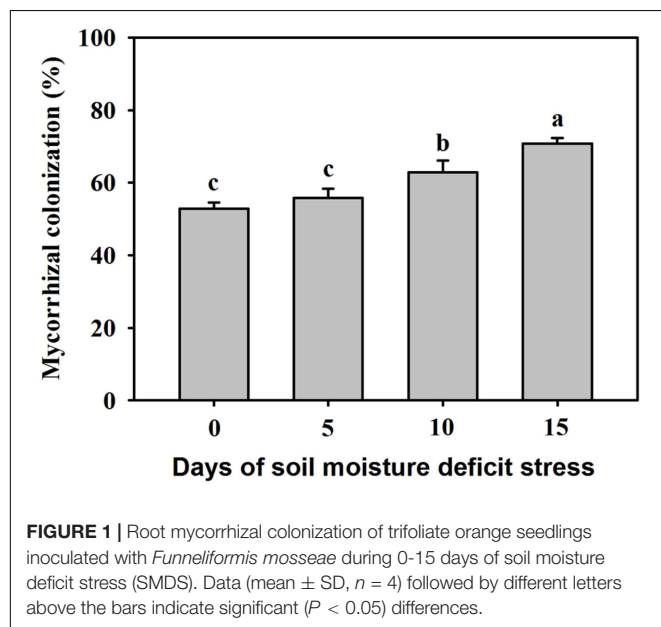
Changes in Plant Growth

Compared with non-AMF-inoculated seedlings, AMF-inoculated seedlings recorded significantly ($P < 0.05$) higher plant height,

TABLE 1 | Plant growth of trifoliate orange seedlings inoculated with *Funnelliformis mosseae* before soil moisture deficit stress (SMDS).

Treatments	Plant height (cm)	Stem diameter (cm)	Leaf number per plant	Shoot biomass (g FW/plant)	Root biomass (g FW/plant)
–AMF	15.30 ± 1.3b	0.31 ± 0.04b	16 ± 1b	1.02 ± 0.14b	1.62 ± 0.24b
+AMF	25.7 ± 4.4a	0.36 ± 0.04a	23 ± 3a	1.72 ± 0.30a	2.37 ± 0.32a

Data (mean ± SD, $n = 4$) followed by different letters in the same column indicate significant ($P < 0.05$) differences.



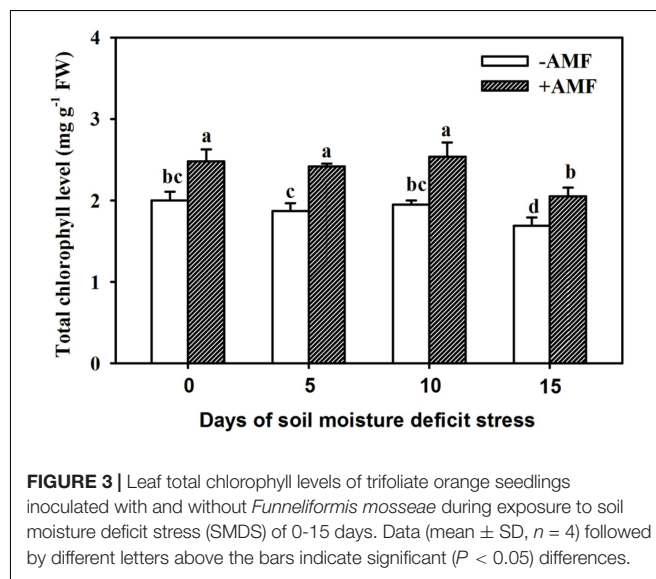
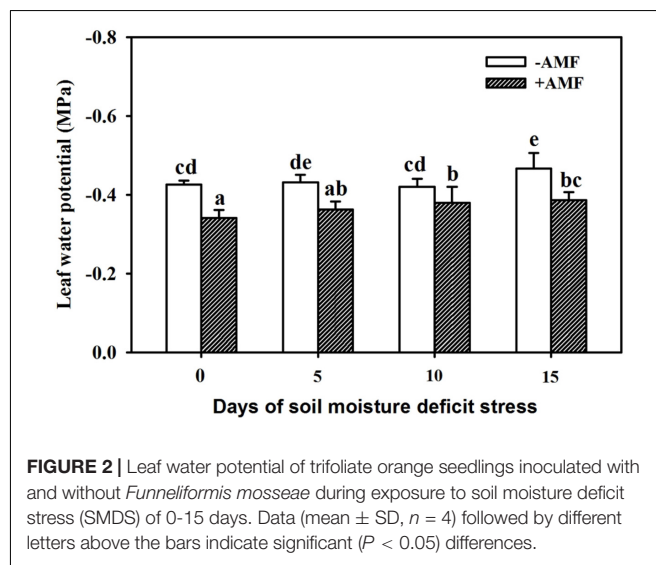
stem diameter, leaf number per plant, shoot biomass, and root biomass by 68%, 16%, 44%, 69%, and 46%, respectively, before SMDS began (Table 1).

Changes in Root Mycorrhizal Colonization

Mycorrhizal colonization is an indicator of a given mycorrhizal species to colonize the roots of host plants. In this study, no mycorrhizal structure was observed in the roots of non-AMF-inoculated plants. The root mycorrhizal colonization ranged from 52.81 to 70.74%, and the soil water deficit markedly affected the root mycorrhizal colonization (Figure 1). Compared with the inoculated plants at 0 SMDS, root AMF colonization showed no significant difference versus inoculated plants at 5 days of SMDS. Interestingly, root AMF colonization was 19% and 34% significantly ($P < 0.05$) higher in the inoculated seedlings exposed to 10 days and 15 days of SMDS than exposed to 0 days, respectively.

Changes in Leaf Ψ

With an increase in duration of SMDS, leaf Ψ of plants showed a decreasing trend (Figure 2). SMDS up to 5 days showed no significant effect on leaf Ψ in AMF- and non-AMF-inoculated plants, and thereafter up to 15 days of SMDS Ψ consistently decreased as compared with SMDS for 0 days. The AMF-treated plants recorded 20%, 16%, 9%, and 17% significantly ($P < 0.05$)



higher leaf Ψ than non-AMF-treated plants at 0, 5, 10, and 15 days of SMDS, respectively.

Changes in Leaf Total Chlorophyll Concentration

The process of drought stress in soil affected leaf total chlorophyll content, to some extent, and the 15-day drought stress strongly inhibited total leaf chlorophyll contents (Figure 3). Inoculation with AMF produced a significant ($P < 0.05$) increase in total

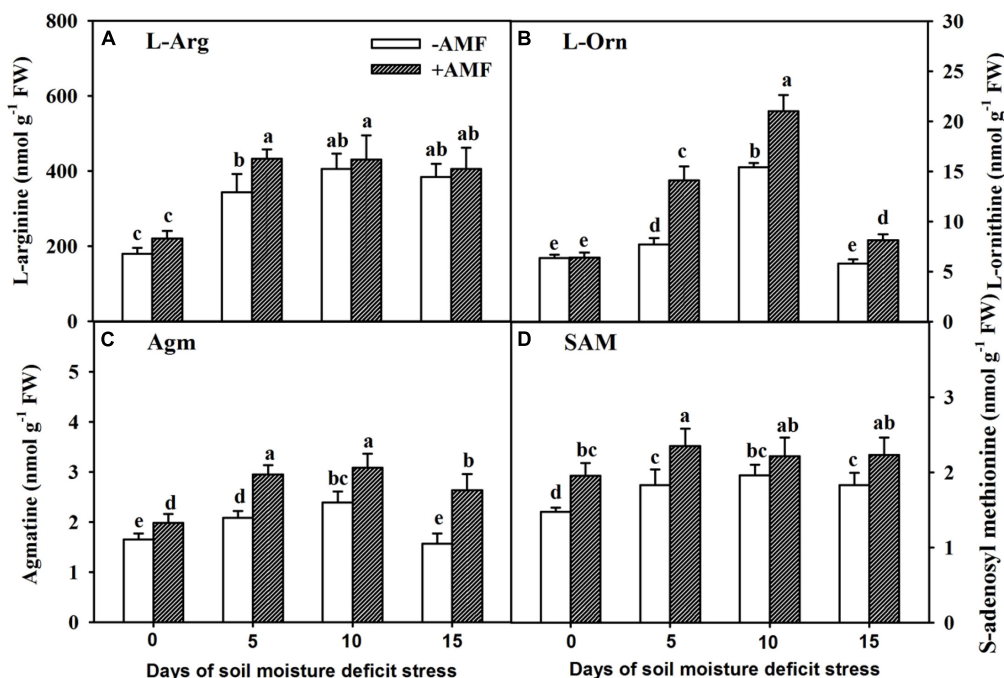


FIGURE 4 | Concentrations of root L-arginine (L-Arg) (A), L-ornithine (L-Orn) (B), agmatine (Agm) (C), and S-adenosyl methionine (SAM) (D) of trifoliate orange seedlings inoculated with and without *Funneliformis mosseae* during exposure to soil moisture deficit stress (SMDS) of 0-15 days. Data (mean $\pm$ SD, $n = 4$) followed by different letters above the bars indicate significant ($P < 0.05$) differences.

chlorophyll content by 24%, 29%, 30%, and 21%, respectively, at 0, 5, 10, and 15 days of SMDS compared with non-AMF treatment.

Changes in Concentration of Precursors of PAs in Roots

In the course of 15 days of SMDS, concentration of different precursors (L-Arg, L-Orn, Agm, and SAM) for the synthesis of PAs in roots showed an initial trend of increase followed by decreasing trend later (Figure 4). AMF inoculation did not change root L-Arg concentrations at 0, 10, and 15 days of SMDS, but increased root L-Arg concentration at 5 days of SMDS by 26% over non-AMF inoculation (Figure 4A). On the other hand, root L-Orn concentration remained unchanged between AMF- and non-AMF-inoculated plants at 0 days of SMDS, and was significantly ($P < 0.05$) higher in AMF-inoculated plants than in non-AMF-inoculated plants at 5, 10, and 15 days of SMDS by 83%, 36%, and 40%, respectively (Figure 4B). AMF-inoculated plants showed 21%, 42%, 29%, and 68% significantly ($P < 0.05$) higher root Agm concentration than non-AMF-inoculated plants at 0, 5, 10, and 15 days of SMDS, respectively (Figure 4C). Similarly, AMF-inoculated plants recorded 33%, 28%, and 22% significantly ($P < 0.05$) higher root SAM concentration over non-AMF-inoculated plants at 0, 5, and 15 days of SMDS, respectively (Figure 4D).

Changes in Root PA Concentration

The Put levels in roots of non-AMF-inoculated plants increased gradually with the prolongation of the SMDS, reached maximum

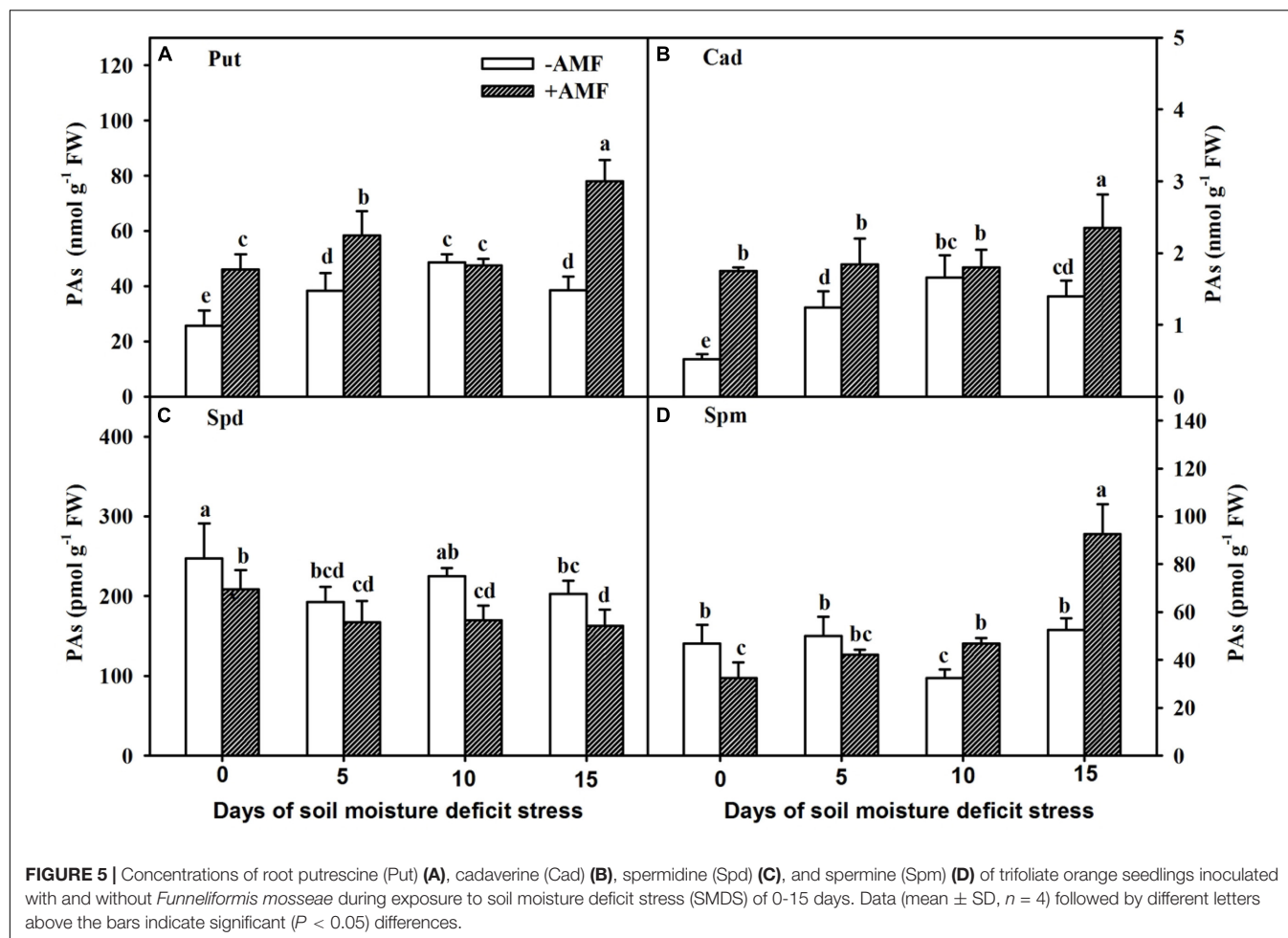
at 10 days, and then followed a declining trend (Figure 5A), while the Put levels in roots of AMF-inoculated plants reached maximum at 15 days of SMDS. Compared with non-AMF-inoculated plants, AMF-inoculated trifoliate orange seedlings showed 78%, 52%, and 103% significantly ($P < 0.05$) higher root Put level at 0, 5, and 15 days, respectively.

In non-AMF-inoculated plants, root Cad levels increased gradually with the SMDS and reached the peak at 10 days, followed by a reduction (Figure 5B). However, Cad concentration in roots of AMF-inoculated plants did not change between 0 and 10 days, and increased at 15 days. AMF inoculation significantly ($P < 0.05$) elevated the concentration of root Cad by 238%, 48%, and 68% at 0, 5, and 15 days of SMDS, respectively, compared with non-AMF treatment.

SMDS, to some degree, reduced the root Spd content but partly increased root Spm level (Figures 5C,D). AMF-inoculated plants recorded 16%, 24%, and 20% significantly ($P < 0.05$) lower root Spd content than non-AMF-inoculated plants at 0, 10, and 15 days, respectively (Figure 5C). AMF treatment significantly ($P < 0.05$) increased root Spm level by 44% and 76% at 10 and 15 days of SMDS, respectively, though reduced root Spm concentration by 30% at 0 days (Figure 5D).

Changes in PA Synthetases in Roots

A short 5-day SMDS induced an increase in root PA synthetase activities in non-AMF-inoculated plants and subsequently remained relatively stable (Figure 6). In AMF-inoculated plants, root ADC activity remained stable during SMDS, root SPMS activity increased slowly, root ODC activity slightly increased and



then gradually decreased, and root SPDS activity showed diverse changes. AMF-inoculated plants showed significantly ($P < 0.05$) higher root ADC (Figure 6A) and SPDS (Figure 6C) activities than non-AMF-inoculated plants at 0, 5, and 15 days of SMDS, respectively. On the other hand, AMF colonization significantly ($P < 0.05$) elevated root ODC (Figure 6B) and SPMS (Figure 6D) activities at 0, 5, 10, and 15 days of SMDS, respectively, compared with non-AMF inoculation.

Changes in PA-Degradated Enzymes in Roots

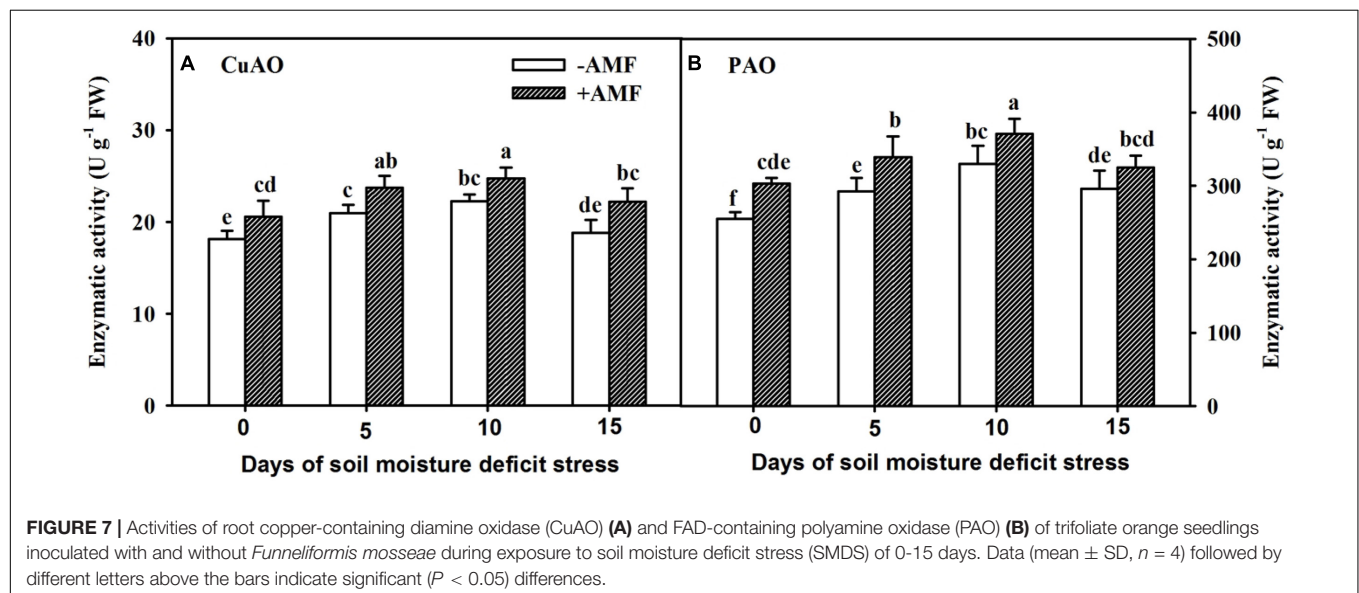
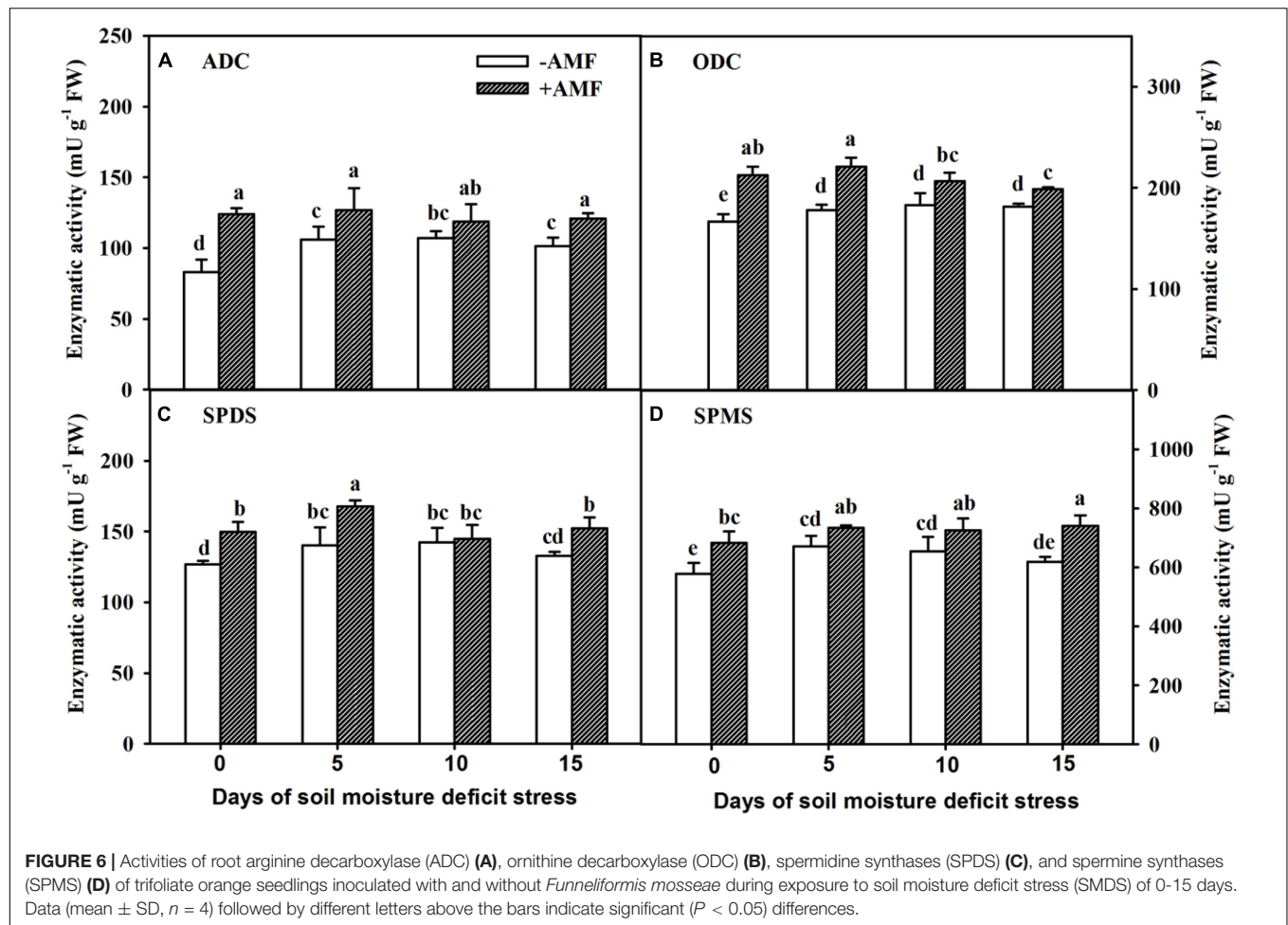
Root CuAO and PAO activities in AMF- and non-AMF-inoculated trifoliolate orange seedlings initially increased and then reduced, with the increasing duration of SMDS, and the highest peak was observed at 10 days (Figure 7). In the course of soil drought, AMF-inoculated plants showed 13%, 13%, 11%, and 18% significantly ($P < 0.05$) higher root CuAO activity than non-AMF-inoculated plants at 0, 5, 10, and 15 days, respectively (Figure 7A). AMF-inoculated plants registered 15%, 29%, and 16% significantly ($P < 0.05$) higher root PAO activities over non-AMF-inoculated plants at 0, 5, and 10 days, respectively (Figure 7B).

Changes in ROS and MDA Levels in Roots

Root H_2O_2 , O_2^- , and MDA concentrations in roots gradually increased with increasing span of SMDS, regardless of AMF- and non-AMF-colonized plants (Figures 8A–C). AMF-inoculated plants recorded significantly ($P < 0.05$) lower root H_2O_2 , O_2^- , and MDA concentrations than non-AMF-inoculated plants at 0, 10, and 15 days, but not at 5 days, suggesting a lowering of H_2O_2 , O_2^- , and MDA levels in AMF-inoculated trifoliolate seedlings on exposure to SMDS.

DISCUSSION

In the present study, root AMF colonization gradually increased with the time of SMDS; however, it is difficult to distinguish whether the increase in mycorrhizal colonization is due to soil drought or contributed by the growth of mycorrhizal fungi. Augé (2001) summarized that SMDS affected root AMF colonization, increasing root colonization more often than decreasing it. An increase of root mycorrhizal colonization under sustained soil drought may be caused by the reduction in P diffusion rate in soil, which led to the decrease of P levels in plants, thereby



stimulating the mycorrhizal fungal colonization (Augé, 2001). There was also an increase in Put content of roots during the SMDS process, indicating the relationship between mycorrhizal

fungal colonization and root Put level. Earlier studies have shown the importance of PAs in root mycorrhizal colonization and arbuscule formation (Wu et al., 2012b; Salloum et al., 2018).

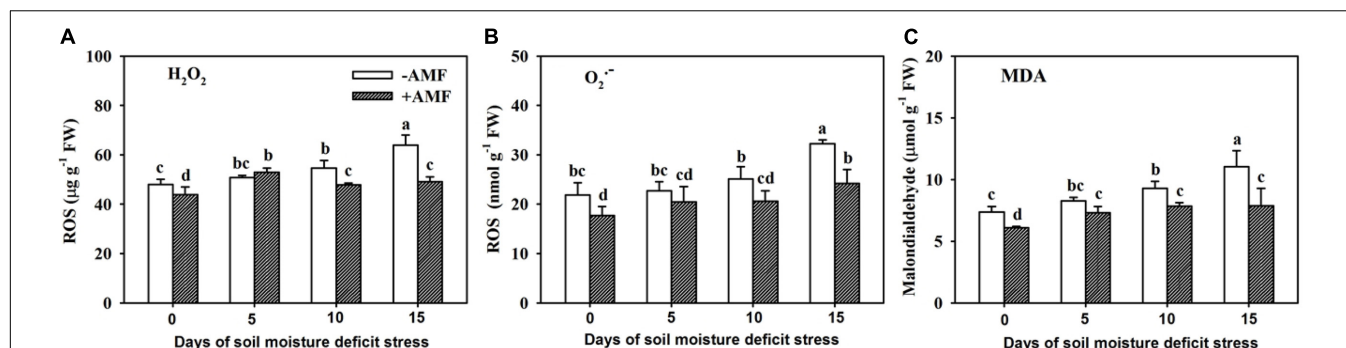


FIGURE 8 | Contents of root hydrogen peroxide (H_2O_2) (A), superoxide anion free radical (O_2^-) (B), and malondialdehyde (MDA) (C) of trifoliate orange seedlings inoculated with and without *Funnelliformis mosseae* during exposure to soil moisture deficit stress of 0–15 days. Data (mean $\pm$ SD, $n = 4$) followed by different letters above the bars indicate significant ($P < 0.05$) differences.

Among PAs, Put, but not Spm and Spd, is the key factor involved in regulating the root mycorrhizal establishment (Wu et al., 2010).

In this study, AMF-inoculated plants showed higher total chlorophyll content than non-AMF-inoculated plants exposed to SMDS. PAs are bound to the pattern of thylakoid, prevented chlorophyll loss, not integrity, thus, stimulating the photosystem II in plants, which is an important mechanism operating in enhancing drought tolerance of plants (Unal et al., 2008; Munzi et al., 2009; Talaat and Shawky, 2013). PAs (e.g., Spm and Put) act as photo-protectors of PS II to exert a protective role in photo-adaptation under abiotic stress (Yaakoubi et al., 2014). However, leaf PA contents were not measured in this study, so it is difficult to establish the link between root PAs and total chlorophyll. Future work may focus on the relationship between mycorrhizal enhancement of total chlorophyll and regulation of PAs in leaves of host plants.

The present study also observed the reduction in leaf Ψ of both AMF- and non-AMF-inoculated seedlings under SMDS, indicating the effect of SMDS in limiting the water absorption of plants. On the other hand, during the SMDS, AMF-inoculated plants recorded significantly higher leaf Ψ than non-AMF-inoculated plants. In fact, mycorrhizal extraradical hyphae directly absorb water from the soil and transfer it back to arbuscule-containing cortical cells of roots for unloading to the host (Zhang et al., 2018). AMF-treated plants also showed higher root hydraulic conductivity than non-AMF-treated plants under soil drought conditions (Sánchez-Romera et al., 2016). PAs (e.g., Put) like compatible osmolytes prevented water loss of plants exposed to polyethylene glycol stress (Kotakis et al., 2014).

The synthetic precursors of PAs in plants mainly include L-Arg, L-Orn, Agm, and SAM (Liu et al., 2015). Simultaneously, L-Arg has an important role in sensing environmental changes and later aiding in adaptation to such abiotic stress (Yang and Gao, 2007). In plants, L-Arg is first transformed into Agm and then into Put, and L-Arg and L-Orn can be transformed into each other (Liu et al., 2015). In our study, we observed that AMF did not increase root L-Arg content, but SMDS of 5–10 days, to some extent, stimulated the concentration of four PA precursors in AM and non-AM trifoliate orange seedlings. Also,

AMF inoculation substantially increased L-Orn, Agm, and SAM contents under SMDS, alongside higher PA synthase (e.g., ADC, ODC, SPDS, and SPMS) activity. These observations meant mycorrhizal fungi may accelerate the conversion of L-Arg into Agm by ADC, thereby resulting in an increased concentration of Agm without obvious changes in L-Arg. At the same time, AMF also accelerated the accumulation of other PA precursors (L-Orn, Agm, and SAM), later transformed into Put and Spd by combined effect of ODC, SPDS, and SPMS (Kusano et al., 2008; Vera-Sirera et al., 2010; Liu et al., 2015). Such changes in AMF-inoculated plants exposed to SMDS could be considered as SMDS responsive mechanism, beneficial for AMF to regulate PA homeostasis in roots, so that AMF-inoculated plants develop an elevated ability to tolerate SMDS than non-AMF-inoculated plants.

In our study, we analyzed the changes in concentration of diamine (Put and Cad), triamine (Spd), and tetramine (Spm) in roots in response to SMDS and AMF inoculation. The changed pattern of root Spd and Spm concentrations in AMF- and non-AMF-inoculated plants was different in response to SMDS. We further observed two contrasting trends in Put and Cad concentration between AMF- and non-AMF-inoculated plants. The results suggested that diamine of AMF-treated plants was not activated up to 10 days of SMDS, while non-AMF-treated plants experienced a reverse change, indicating that AMF-treated plants exhibited a relatively higher tolerance against SMDS than non-AMF-treated plants. Interestingly, AMF-treated plants possessed higher diamine content at the beginning of exposure to SMDS. AMF-colonized plants showed higher root Put and Cad content than non-AMF-colonized plants at 0, 5, and 15 days of SMDS. Wu et al. (2012a) earlier reported an increase in root Put level in red tangerine after inoculation with *F. mosseae* under ample water conditions. Zhang et al. (2020) also noted higher root Put and Cad concentration in trifoliate orange colonized by *F. mosseae* under both WW and drought stress conditions. Put is associated with stress tolerance by abscisic acid regulation and antioxidant system activation (Pál et al., 2015). It is concluded that higher diamine content in AM versus non-AM plants is considered beneficial to enhance the SMDS tolerance of AM plants. On the other hand, Spd, a downstream product of Put (Liu et al., 2015), was relatively lower in AMF- than in non-AMF-colonized

trifoliolate orange during SMDS. Root Spm was lower in AM plants than in non-AM plants in the beginning of the stress, but ended up with higher Spm. Higher Spm in AMF versus non-AMF plants under SMDS may increase stress-related gene expression level to protect against any potential stress damage (Fu et al., 2014; Pál et al., 2015). Roots are the main site for synthesis of Put (Moschou et al., 2008). Mycorrhizal fungi colonize the root system and, thus, stimulate the content of root Put and Cad, while Spd and Spm are downstream products of Put and synthesized mainly in shoots (Moschou et al., 2008), thus, exhibiting the diminishing response in AMF-inoculated plants.

Past studies have confirmed that AMF colonization alleviated the oxidative burst of host plants induced by drought stress, thus, maintaining low ROS levels (Gholinezhad et al., 2020; Langeroodi et al., 2020; Zou et al., 2020). In our study, we observed relatively higher CuAO and PAO activity in AMF-inoculated plants than non-AMF-inoculated plants under SMDS, which is in agreement with previous studies under varying stress conditions (Xue et al., 2009; Toumi et al., 2010; Zhang et al., 2020). Normally, drought stress causes PA catabolism to result in ROS (e.g., H_2O_2) burst (Gupta et al., 2013). However, our study still showed relatively lower root ROS (e.g., H_2O_2 and O_2^-) levels and a low degree of root membrane lipid peroxidation (e.g., MDA) in AMF-inoculated trifoliolate orange seedlings than in non-AMF-inoculated seedlings exposed to SMDS. He et al. (2020) earlier observed similar events in trifoliolate orange inoculated with *F. mosseae* under both WW and drought stress. In fact, inoculation with AMF activated the antioxidant defense system of both host plants and AMF to respond to drought-induced ROS burst (Zou et al., 2020). In our other study, colonization by *F. mosseae* significantly upregulated the expression levels of *PtMn-SOD*, *PtCu/Zn-SOD*, and *PtCAT1* genes in the roots of trifoliolate orange exposed to a 7-week SMDS (50% maximum water holding capacity) (Zhang et al., 2020). In addition, PA application is involved in the elicitation of antioxidant enzyme defense systems in stressed plants (Wu et al., 2020). It seems that PA-induced H_2O_2 could trigger the antioxidant defense system of plants, thereby bringing PAs into operation as scavengers of free radicals (Gupta et al., 2013). More in-depth studies are needed to elucidate the signal role of H_2O_2 induced by PA catabolism in response to SMDS under mycorrhization.

REFERENCES

- Alcázar, R., Fortes, A. M., and Tiburcio, A. F. (2020). Editorial: polyamines in plant biotechnology, food nutrition, and human health. *Front. Plant Sci.* 11:120. doi: 10.3389/fpls.2020.00120
- Augé, R. M. (2001). Water relations, drought and vesicular-arbuscular mycorrhizal symbiosis. *Mycorrhiza* 11, 3–42. doi: 10.1007/s005720100097
- Baslam, M., and Giocoechea, N. (2012). Water deficit improved the capacity of arbuscular mycorrhizal fungi (AMF) for inducing the accumulation of antioxidant compounds in lettuce leaves. *Mycorrhiza* 22, 347–359. doi: 10.1007/s00572-011-0408-9
- Cheng, H. Q., Ding, Y. E., Shu, B., Zou, Y. N., Wu, Q. S., and Kuča, K. (2020). Plant aquaporin responses to mycorrhizal symbiosis under abiotic stress. *Int. J. Agric. Biol.* 23, 786–794.
- Chiappello, M., Martino, E., and Perotto, S. (2015). Common and metal-specific proteomic responses to cadmium and zinc in the metal tolerant ericoid

CONCLUSION

In this study, AMF facilitated the accumulation of Put, Cad, and PA precursors and increased PA synthase activity, accompanied with low oxidative burst in trifoliolate orange in response to SMDS. Thus, AMF modulated the PA homeostasis of host plants to effectively cope with SMDS. Such results provide strong clues in understanding the physiological mechanisms imparting an enhanced SMDS tolerance in AMF-inoculated plants. Future studies should highlight the mechanisms regarding AMF-induced PA regulatory network at the molecular level and the involvement of associated signals.

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

FZ and Q-SW conceived and designed the experiments. FZ and Y-NZ performed the experiments. Y-NZ, FZ, AKS, Q-SW, and KK analyzed the data. FZ and Q-SW prepared the figures. Y-NZ wrote the manuscript. Y-NZ, AKS, Q-SW, and KK revised the manuscript. All authors contributed to the article and approved the submitted version.

FUNDING

This study was supported by the National Key Research and Development Program of China (2018YFD1000303) and the Plan in Scientific and Technological Innovation Team of Outstanding Young Scientists, Hubei Provincial Department of Education (T201604). This work was also supported by the UHK project VT2019-2021.

- mycorrhizal fungus *Oidiodendron maius* Zn. *Metallomics* 7, 805–815. doi: 10.1039/c5mt00024f
- Davies, W. J., Tardieu, F., and Trejo, C. L. (1994). How do chemical signals work in plants that grow in drying soil? *Plant Physiol.* 104, 309–314. doi: 10.1104/pp.104.2.309
- Ducros, V., Ruffieux, D., Belvabesnet, H., Defraipont, F., Berger, F., and Favier, A. (2009). Determination of dansylated polyamines in red blood cells by liquid chromatography–tandem mass spectrometry. *Anal. Biochem.* 390, 46–51. doi: 10.1016/j.ab.2009.04.007
- Fu, X. Z., Xing, F., Wang, N. Q., Peng, L. Z., Cao, L., Ling, L. L., et al. (2014). Exogenous spermine pretreatment confers tolerance to combined high-temperature and drought stress in vitro in trifoliolate orange seedlings via modulation of antioxidative capacity and expression of stress-related genes. *Biotechnol. Biotechnol. Equip.* 28, 192–198. doi: 10.1080/13102818.2014.909152
- Gholinezhad, E., Darvishzadeh, R., Moghaddam, S. S., and Popovic-Djordjevic, J. (2020). Effect of mycorrhizal inoculation in reducing water stress in sesame

- (*Sesamum indicum* L.): the assessment of agrobiological traits and enzymatic antioxidant activity. *Agric. Wat. Manag.* 238, 106234. doi: 10.1016/j.agwat.2020.106234
- Goicoechea, N., Szalai, G., Antolin, M. C., Sanchez-Diazl, M., and Paldi, E. (1998). Influence of arbuscular mycorrhizae and Rhizobium on free polyamines and proline levels in water-stressed alfalfa. *J. Plant Physiol.* 153, 706–711. doi: 10.1016/s0176-1617(98)80224-1
- Gupta, K., Dey, A., and Gupta, B. (2013). Plant polyamines in abiotic stress responses. *Acta Physiol. Plant* 35, 2015–2036. doi: 10.1007/s11738-013-1239-4
- He, J. D., Dong, T., Wu, H. H., Zou, Y. N., Wu, Q. S., and Kuča, K. (2019). Mycorrhizas induce diverse responses of root *TIP* aquaporin gene expression to drought stress in trifoliate orange. *Sci. Hortic.* 243, 64–69. doi: 10.1016/j.scienta.2018.08.010
- He, J. D., Zou, Y. N., Wu, Q. S., and Kuča, K. (2020). Mycorrhizas enhance drought tolerance of trifoliate orange by enhancing activities and gene expression of antioxidant enzymes. *Sci. Hortic.* 262, 108745. doi: 10.1016/j.scienta.2019.108745
- Hu, Y. B., and Chen, B. D. (2020). Arbuscular mycorrhiza induced putrescine degradation into γ -aminobutyric acid, malic acid accumulation, and improvement of nitrogen assimilation in roots of water-stressed maize plants. *Mycorrhiza* 30, 329–339. doi: 10.1007/s00572-020-00952-0
- Huang, Y. M., Zou, Y. N., and Wu, Q. S. (2017). Alleviation of drought stress by mycorrhizas is related to increased root H_2O_2 efflux in trifoliate orange. *Sci. Rep.* 7, 423–435.
- Kotakis, C., Theodoropoulou, E., Tassis, K., Oustamanolakis, C., Ioannidis, N. E., and Kotzabasis, K. (2014). Putrescine, a fast-acting switch for tolerance against osmotic stress. *J. Plant Physiol.* 171, 48–51. doi: 10.1016/j.jplph.2013.09.015
- Kusano, T., Berberich, T., Tateda, C., and Takahashi, Y. (2008). Polyamines: essential factors for growth and survival. *Planta* 228, 367–381. doi: 10.1007/s00425-008-0772-7
- Langeroodi, A. R. S., Osipitan, O. A., Radicetti, E., and Mancinelli, R. (2020). To what extent arbuscular mycorrhiza can protect chicory (*Cichorium intybus* L.) against drought stress. *Sci. Hortic.* 263, 109–119.
- Lichtenthaler, H. K., and Wellburn, A. R. (1983). Determinations of total carotenoids and chlorophyll a and b of leaf extracts in different solvents. *Biochem. Soc. Trans.* 11, 591–592. doi: 10.1042/bst0110591
- Liu, J. H., Wang, W., Wu, H., Gong, X. Q., and Moriguchi, T. (2015). Polyamines function in stress tolerance: from synthesis to regulation. *Front. Plant Sci.* 6:827. doi: 10.3389/fpls.2015.00827
- Lokhandwala, A., and Hoeksema, J. D. (2019). Priming by arbuscular mycorrhizal fungi of plant antioxidant enzyme production: a meta-analysis. *Annu. Plant Rev.* 2, 1069–1084. doi: 10.1002/9781119312994.apr0680
- Luo, Y. (2009). *Effects of AMF on Cell Membrane, Endogenous Polyamines, and Salicylic Acid in Citrus Under Drought Stress*. 1–42. Master Dissertation. Huazhong Agriculture University, Wuhan.
- Millar, N. S., and Bennett, A. E. (2016). Stressed out symbiotes: hypotheses for the influence of abiotic stress on arbuscular mycorrhizal fungi. *Oecologia* 182, 625–641. doi: 10.1007/s00442-016-3673-7
- Minocha, R., Majumdar, R., and Minocha, S. (2014). Polyamines and abiotic stress in plants: a complex relationship. *Front. Plant Sci.* 5:175. doi: 10.3389/fpls.2014.00175
- Moschou, P. N., Paschalidis, K. A., and Roubelakis-Angelakis, K. A. (2008). Plant polyamine catabolism: the state of the art. *Plant Signal. Behav.* 3, 1061–1066. doi: 10.4161/psb.3.12.7172
- Munzi, S., Pirtos, S. A., and Loppi, S. (2009). Chlorophyll degradation and inhibition of polyamine biosynthesis in the lichen *Xanthoria parietina* under nitrogen stress. *Ecotoxicol. Environ. Saf.* 72, 281–285. doi: 10.1016/j.ecoenv.2008.04.013
- Muthusamy, M., Uma, S., Backiyarani, S., and Saraswathi, M. S. (2014). Computational prediction, identification, and expression profiling of microRNAs in banana (*Musa* spp.) during soil moisture deficit stress. *J. Hortic. Sci. Biotechnol.* 89, 208–214. doi: 10.1080/14620316.2014.11513070
- Pál, M., Szalai, G., and Janda, T. (2015). Speculation: Polyamines are important in abiotic stress signaling. *Plant Sci.* 237, 16–23. doi: 10.1016/j.plantsci.2015.05.003
- Phillips, J. M., and Hayman, D. S. (1970). Improved procedures for clearing roots and staining parasitic and vesicular-arbuscular mycorrhizal fungi for rapid assessment of infection. *Trans. Br. Mycol. Soc.* 55, 158–161. doi: 10.1016/s0007-1536(70)80110-3
- Salloum, M. S., Menduni, M. F., Benavides, M. P., Larrauri, M., Luna, C. M., and Silvente, S. (2018). Polyamines and flavonoids: key compounds in mycorrhizal colonization of improved and unimproved soybean genotypes. *Symbiosis* 76, 265–275. doi: 10.1007/s13199-018-0558-z
- Sánchez-Romera, B., Ruiz-Lozano, J. M., Zamarreño, Á.M., García-Mina, J. M., and Aroca, R. (2016). Arbuscular mycorrhizal symbiosis and methyl jasmonate avoid the inhibition of root hydraulic conductivity caused by drought. *Mycorrhiza* 26, 111–122. doi: 10.1007/s00572-015-0650-7
- Šebela, M., Radová, A., Angelini, R., Tavladoraki, P., Frébort, I., and Peè, P. (2001). FAD-containing polyamine oxidases: a timely challenge for researchers in biochemistry and physiology of plants. *Plant Sci.* 160, 197–207. doi: 10.1016/s0168-9452(00)00380-0
- Srivastava, A. K., and Singh, S. (2008). Citrus nutrition research in India: Problems and prospects. *Ind. J. Agric. Sci.* 78, 3–16.
- Srivastava, A. K., and Singh, S. (2009). Citrus decline: soil fertility and plant nutrition. *J. Plant Nutri.* 32, 197–245. doi: 10.1080/01904160802592706
- Sudhakar, C., Lakshmi, A., and Giridharakumar, S. (2001). Changes in the antioxidant enzyme efficacy in two high yielding genotypes of mulberry (*Morus alba* L.) under NaCl salinity. *Plant Sci.* 161, 613–619. doi: 10.1016/s0168-9452(01)00450-2
- Takahashi, T. (2020). Plant polyamines. *Plants* 9, 511. doi: 10.3390/plants9040511
- Talaat, N. B., and Shawky, B. T. (2013). Modulation of nutrient acquisition and polyamine pool in salt-stressed wheat (*Triticum aestivum* L.) plants inoculated with arbuscular mycorrhizal fungi. *Acta Physiol. Plant* 35, 2601–2610. doi: 10.1007/s11738-013-1295-9
- Toumi, I., Moschou, P. N., Paschalidis, K. A., Bouamama, B., Salem-fnayou, A. B., Ghorbel, A. W., et al. (2010). Abscissic acid signals reorientation of polyamine metabolism to orchestrate stress responses via the polyamine exodus pathway in grapevine. *J. Plant Physiol.* 167, 519–525. doi: 10.1016/j.jplph.2009.10.022
- Unal, D., Tuney, I., and Sukatar, A. (2008). The role of external polyamines on photosynthetic responses, lipid peroxidation, protein and chlorophyll a content under the UV-A (352 nm) stress in *Physcia semipinnata*. *J. Photocem. Photobiol. B* 90, 64–68. doi: 10.1016/j.jphotobiol.2007.11.004
- Upadhyay, R. K., Fatia, T., Handa, A. K., and Mattoo, A. K. (2020). Polyamines and their biosynthesis/catabolism genes are differentially modulated in response to heat versus cold stress in tomato leaves (*Solanum lycopersicum* L.). *Cells* 9, 1749. doi: 10.3390/cells9081749
- Velikova, V., Yordanov, I., and Edreva, A. (2000). Oxidative stress and some antioxidant systems in acid rain-treated bean plants: protective role of exogenous polyamines. *Plant Sci.* 151, 59–66. doi: 10.1016/s0168-9452(99)00197-1
- Venice, F., de Pinto, M. C., Novero, M., Ghignone, S., Salvio, A., and Bonfante, P. (2017). *Gigaspora margarita* with and without its endobacterium shows adaptive responses to oxidative stress. *Mycorrhiza* 27, 747–759. doi: 10.1007/s00572-017-0790-z
- Vera-Sirera, F., Minguez, E. G., Singh, S. K., Ljung, K., Tuominen, H., Blazquez, M. A., et al. (2010). Role of polyamines in plant vascular development. *Plant Physiol. Biochem.* 48, 534–539. doi: 10.1016/j.plaphy.2010.01.011
- Wu, Q. S., He, J. D., Srivastava, A. K., Zou, Y. N., and Kuča, K. (2019). Mycorrhizas enhance drought tolerance of citrus by altering root fatty acid compositions and their saturation levels. *Tree Physiol.* 39, 1149–1158. doi: 10.1093/treephys/tpz039
- Wu, Q. S., He, X. H., Zou, Y. N., Liu, C. Y., Xiao, J., and Li, Y. (2012a). Arbuscular mycorrhizas alter root system architecture of *Citrus tangerine* through regulating metabolism of endogenous polyamines. *Plant Growth Regul.* 68, 27–35. doi: 10.1007/s10725-012-9690-6
- Wu, Q. S., Srivastava, A. K., and Zou, Y. N. (2013). AMF-induced tolerance to drought stress in citrus: a review. *Sci. Hortic.* 164, 77–87. doi: 10.1016/j.scienta.2013.09.010
- Wu, Q. S., Zou, Y. N., and He, X. H. (2010). Exogenous putrescine, not spermine or spermidine, enhances root mycorrhizal development and plant growth of trifoliate orange (*Poncirus trifoliata*) seedlings. *Int. J. Agric. Biol.* 12, 576–580.
- Wu, Q. S., Zou, Y. N., Liu, M., and Cheng, K. (2012b). Effects of exogenous putrescine on mycorrhiza, root system architecture, and physiological traits of *Glomus mosseae*-colonized trifoliate orange seedlings. *Not. Bot. Hort. Agrobot.* 40, 80–85. doi: 10.15835/nbha4027926

- Wu, Z. W., Wang, J. F., Yan, D. L., Yuan, H. W., Wang, Y., He, Y., et al. (2020). Exogenous spermidine improves salt tolerance of pecan-grafted seedlings via activating antioxidant system and inhibiting the enhancement of Na^+/K^+ ratio. *Acta Physiol. Plant* 42, 83.
- Xue, B., Zhang, A., and Jiang, M. (2009). Involvement of polyamine oxidase in abscisic acid-induced cytosolic antioxidant defense in leaves of maize. *J. Integr. Plant. Biol.* 51, 225–234. doi: 10.1111/j.1744-7909.2008.00766.x
- Yaakoubi, H., Hamdani, S., Bekalé, L., and Carpentier, R. (2014). Protective action of spermine and spermidine against photoinhibition of photosystem I in isolated thylakoid membranes. *PLoS One* 9:e112893. doi: 10.1371/journal.pone.0112893
- Yang, H. Q., and Gao, H. J. (2007). Physiological function of arginine and its metabolites in plants. *J. Plant Physiol. Mol. Biol.* 33, 1–8. doi: 10.1002/9781118783054.ch1
- Zhang, F., Zou, Y. N., and Wu, Q. S. (2018). Quantitative estimation of water uptake by mycorrhizal extraradical hyphae in citrus under drought stress. *Sci. Hortic.* 229, 132–136. doi: 10.1016/j.scienta.2017.10.038
- Zhang, F., Zou, Y. N., Wu, Q. S., and Kuča, K. (2020). Arbuscular mycorrhizas modulate root polyamine metabolism to enhance drought tolerance of trifoliate orange. *Environ. Exp. Bot.* 171, 103962. doi: 10.1016/j.envexpbot.2019.103926
- Zou, Y. N., Wu, H. H., Giri, B., Wu, Q. S., and Kuča, K. (2019). Mycorrhizal symbiosis down-regulates or does not change root aquaporin expression in trifoliate orange under drought stress. *Plant Physiol. Biochem.* 144, 292–299. doi: 10.1016/j.plaphy.2019.10.001
- Zou, Y. N., Wu, Q. S., and Kuča, K. (2020). Unravelling the role of arbuscular mycorrhizal fungi in mitigating the oxidative burst of plants under drought stress. *Plant Biol.* doi: 10.1111/plb.13161. [Epub ahead of print].

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.

Copyright © 2021 Zou, Zhang, Srivastava, Wu and Kuča. This is an open-access article distributed under the terms of the Creative Commons Attribution License (CC BY). The use, distribution or reproduction in other forums is permitted, provided the original author(s) and the copyright owner(s) are credited and that the original publication in this journal is cited, in accordance with accepted academic practice. No use, distribution or reproduction is permitted which does not comply with these terms.



Arbuscular Mycorrhiza Improves Photosynthesis and Restores Alteration in Sugar Metabolism in *Triticum aestivum* L. Grown in Arsenic Contaminated Soil

Samta Gupta, Sarda Devi Thokchom and Rupam Kapoor*

Department of Botany, University of Delhi, New Delhi, India

OPEN ACCESS

Edited by:

Juan Manuel Ruiz-Lozano,
Estación Experimental del Zaidín
(EEZ), CSIC, Spain

Reviewed by:

Gabriela Quiroga,
Estación Experimental del Zaidín
(EEZ), CSIC, Spain
Sara Adrián López de Andrade,
State University of Campinas, Brazil

*Correspondence:

Rupam Kapoor
kapoor_rupam@yahoo.com

Specialty section:

This article was submitted to
Plant Abiotic Stress,
a section of the journal
Frontiers in Plant Science

Received: 11 December 2020

Accepted: 11 February 2021

Published: 11 March 2021

Citation:

Gupta S, Thokchom SD and
Kapoor R (2021) Arbuscular
Mycorrhiza Improves Photosynthesis
and Restores Alteration in Sugar
Metabolism in *Triticum aestivum* L.
Grown in Arsenic Contaminated Soil.
Front. Plant Sci. 12:640379.
doi: 10.3389/fpls.2021.640379

Contamination of agricultural soil by arsenic (As) is a serious menace to environmental safety and global food security. Symbiotic plant-microbe interaction, such as arbuscular mycorrhiza (AM), is a promising approach to minimize hazards of As contamination in agricultural soil. Even though the potential of AM fungi (AMF) in redeeming As tolerance and improving growth is well recognized, the detailed metabolic and physiological mechanisms behind such beneficial effects are far from being completely unraveled. The present study investigated the ability of an AM fungus, *Rhizophagus intraradices*, in mitigating As-mediated negative effects on photosynthesis and sugar metabolism in wheat (*Triticum aestivum*) subjected to three levels of As, viz., 0, 25, and 50 mg As kg⁻¹ of soil, supplied as sodium arsenate. As exposure caused significant decrease in photosynthetic pigments, Hill reaction activity, and gas exchange parameters such as net photosynthetic rate, stomatal conductance, transpiration rate, and intercellular CO₂ concentration. In addition, As exposure also altered the activities of starch-hydrolyzing, sucrose-synthesizing, and sucrose-degrading enzymes in leaves. Colonization by *R. intraradices* not only promoted plant growth but also restored As-mediated impairments in plant physiology. The symbiosis augmented the concentration of photosynthetic pigments, enhanced Hill reaction activity, and improved leaf gas exchange parameters and water use efficiency of *T. aestivum* even at high dose of 50 mg As kg⁻¹ of soil. Furthermore, inoculation with *R. intraradices* also restored As-mediated alteration in sugar metabolism by modulating the activities of starch phosphorylase, α -amylase, β -amylase, acid invertase, sucrose synthase, and sucrose-phosphate synthase in leaves. This ensured improved sugar and starch levels in mycorrhizal plants. Overall, the study advocates the potential of *R. intraradices* in bio-amelioration of As-induced physiological disturbances in wheat plant.

Keywords: arbuscular mycorrhizal fungi, arsenic stress, wheat, photosynthesis, sucrose, starch

INTRODUCTION

Environmental arsenic (As) contamination is a global agricultural, environmental, and health issue owing to its highly carcinogenic and toxic nature. As is ingressed into the environment, through natural processes, viz., weathering of As-rich rocks, volcanic activity, or anthropogenic activities, namely mining, unwarranted use of As-based pesticides, and irrigation with groundwater

contaminated with As in agriculture (Khalid et al., 2017; Abbas et al., 2018). It is a non-essential metalloid and hence not required in any specific metabolic reactions in plants. Both organic and inorganic species of As are present in nature, with the latter being more mobile and toxic than the organic As species. While arsenate [As(V)] exists in oxidized environment, arsenite [As(III)] dominates in reduced environment (Khalid et al., 2017). Being an analog of inorganic phosphate (Pi), As(V) is transported across plasma membrane through the phosphate transport systems. Thus, it competes and interferes with Pi uptake and metabolism (Stoeva and Bineva, 2003) in plants. Once taken up by the plants, various Pi transporters enable easy movement of As(V) from one cellular compartment to another (Finnegan and Chen, 2012). In doing so, all parts of cellular metabolism get exposed to the toxicant. For instance, it replaces phosphate in ATP and forms an unstable ADP-As complex, thereby leading to interference of energy flow in cells (Meharg, 1994).

As can affect the growth and productivity of plants due to a surfeit of morphological, physiological, and biochemical alterations (Chandrakar et al., 2016; Srivastava et al., 2017). Production of reactive oxygen species (ROS) is one of the most perilous biochemical effects of As at the subcellular level, causing non-repairable damage to various macromolecules, such as lipids, proteins, carbohydrate, and DNA (Talukdar, 2013; Chandrakar et al., 2016).

As is reported to disrupt net photosynthetic rate (Pn) in plants (Gusman et al., 2013), due to disturbances either in the photochemical or biochemical steps or both. Light-harvesting apparatus of plants gets affected by As via reduction of chlorophyll (Chl) concentrations and photosynthetic activity (Anjum et al., 2011; Emamverdian et al., 2015). Rate of CO₂ fixation and activity of photosystem (PS) II also get reduced considerably under As exposure (Stoeva and Bineva, 2003). As also negatively influences photochemical efficiency and heat dissipation capacity, thereby upholding changes in gas exchange rate and fluorescence emission (Chandrakar et al., 2016; Debona et al., 2017). These toxic effects of As on photosynthetic parameters of plants are manifested in the forms of diminution in growth, wilting, and violet color development of leaves (Musil et al., 2014).

In addition to disturbing Pn in plants, As has also been demonstrated to influence carbon partitioning and sugar metabolism in plants (Jha and Dubey, 2004; Choudhury et al., 2010; Sil et al., 2019; Majumder et al., 2020). Metabolism of basic carbohydrates such as sugars and starch in plants is deleteriously

affected under As stress (Chandrakar et al., 2016). Sucrose and starch, the resultant products of photosynthesis, act as regulators of stress responses and play a principal role in gene expression under abiotic stresses (Rosa et al., 2009). Accumulation of soluble sugars can take place in response to the stress (Gramss, 2012) as a means to cope up with As-mediated oxidative stress with a discrepancy in the contents of reducing sugars (RSs) and non-RSs (NRSs) (Jha and Dubey, 2004). Conversion of NRSs, primarily sucrose, into RSs (hexoses) is generally observed under As stress, indicating the suppression of sucrose synthesis (Jha and Dubey, 2004). Inhibition of starch-degrading enzymes' activities as a consequence of As-mediated plant toxicity has also been reported (Jha and Dubey, 2004). Considering the aspect that plant directly exposed to As require more energy and carbon molecules to cope with the stress, studies focusing on photosynthesis and sugar metabolism under As stress could help in developing relevant strategies to confer plant tolerance toward As-instigated toxicity.

Among the cereals, wheat (*Triticum aestivum* L.) is the second most important crop consumed mainly as a source of carbohydrate as well as dietary protein and minerals. It ranks first in terms of global consumption (Food and Agriculture Organization (FAO), 2020). The major concern arising in wheat cultivation is the concurrence of As-contamination area with its cultivated area. For instance, in India, wheat is cultivated under six diverse agro-climatic zones, wherein Indo-Gangetic Plains comprising the two zones form major wheat-cultivating plain (Rasheed et al., 2018). Wheat grains harvested from this region also report high As accumulation resulting from unparalleled As biomagnification. The risks posed by the contaminant from wheat grains, however, do not outweigh the global demand of wheat and its products. Thus, with the increasing global population, production of wheat needs to meet the global demand as well as reduce the toxic content in its nutritional composition. In this context, use of arbuscular mycorrhizal fungi (AMF) in agriculture has been reported to sustainably improve plant's tolerance to various heavy metal stresses (Sharma et al., 2017; Zhan et al., 2018; Alam et al., 2019; Wu et al., 2020).

Arbuscular mycorrhizal fungi, belonging to the subphylum Glomeromycotina, are the most widespread root symbiotic fungi, reportedly developing mutualistic associations with approximately 80% of terrestrial plants (Smith and Read, 2008). It was reported that colonization by AMF not only improves growth and biomass of wheat plants but also aids the host to surmount As-induced P deficiency and also maintains higher P/As ratio when compared with non-colonized plants (Sharma et al., 2017). In previous study, it was found that AMF colonization can reduce uptake of As and its translocation to wheat grains. Altered mineral status and photosynthetic parameters mediated by AMF in stressed plants as mitigation strategy is also accountable to affect carbohydrate metabolism in plants. In addition, carbon handling is a fundamental aspect of plant-AMF interaction, as a significant fraction of the plant's photosynthates (sugars) is directed toward AMF (Bago et al., 2000) and thus can alter carbohydrate metabolism. In line with this, studies on the effect of As contamination and AMF inoculation on physiological processes such as photosynthesis and carbohydrate metabolism are restricted to

Abbreviations: AI, acid invertase; AMF, arbuscular mycorrhizal fungi; ANOVA, analysis of variance; As(III), arsenite; As(V), arsenate; As, arsenic; CaCl₂, calcium chloride; Chl, chlorophyll; C_i, intercellular CO₂ concentration; DCPIP, 2,6-dichlorophenolindophenol; DNSA, 3,5-dinitrosalicylic acid; DTT, dithiothreitol; E, transpiration rate; EDTA, ethylenediaminetetraacetic acid; Fo, minimal fluorescence; Fv/Fo, potential of PSII; Gs, stomatal conductance; HSD, honestly significant difference; INVAM, International Culture Collection of (Vesicular) Arbuscular Mycorrhizal Fungi; M, mycorrhizal; MgCl₂, magnesium chloride; MTI, metal tolerance index; NM, non-mycorrhizal; NRS, non-reducing sugar; Pi, inorganic phosphate; PMSF, phenylmethylsulfonyl fluoride; Pn, net photosynthetic rate; PS, photosystem; qP, quenching coefficient; ROS, reactive oxygen species; RS, reducing sugar; SP, starch phosphorylase; SPS, sucrose-phosphate synthase; SPSS, Statistical Package for the Social Sciences; SS, sucrose synthase; TCA, trichloroacetic acid; T-Chl, total chlorophyll; TSS, total soluble sugar.

assessment at seedling stage (Jha and Dubey, 2004; Choudhury et al., 2010; Sil et al., 2019). Keeping this in mind, the present study was performed to assess the ability of *Rhizophagus intraradices* to (i) promote photosynthesis-related activities (pigment concentration, Hill reaction activity, gas exchange parameters, and Chl *a* fluorescence) and (ii) stimulate the activities of enzymes involved in sucrose and starch metabolism, in wheat plants exposed to three levels of As.

MATERIALS AND METHODS

Plant Material and Fungal Inoculum

Seeds of wheat variety HD-2967 were procured from Agricultural Technology Information Centre, Indian Agricultural Research Institute (IARI), New Delhi, India. *R. intraradices* (accession number CMCCWep319) inoculum was provided by Center for Mycorrhizal Culture Collection, The Energy and Resources Institute, New Delhi, India. Proliferation of fungal spores was carried out in sterile soil using *Sorghum bicolor* L. as trap plant (Kapoor et al., 2002). After confirmation of colonization by mycorrhizal fungi (Phillips and Hayman, 1970), the plants were let to dry progressively to stimulate spore formation under shade (INVAM, 2014). The inoculum prepared consisted of finely chopped *R. intraradices* colonized roots and dried soil containing approximately 120–150 spores 10 g^{-1} of soil.

Experimental Layout

A pot-based (3 kg soil per pot) experiment was set up in the Botanical Garden, Department of Botany, University of Delhi. Three As levels (0, 25, and 50 mg As kg^{-1} of soil) and two mycorrhizal treatments [inoculated with *R. intraradices* (M) and non-mycorrhizal (NM) ones] were the factors under consideration. In total, there were six treatments structured in complete randomized block design.

Soil Treatment and Plant Growth Conditions

Soil was collected from the Botanical Garden (0–15 cm in depth) of the Department of Botany, University of Delhi, and air-dried for use for the experiment. The soil was sieved through 2-mm sieve and was maintained in a 3:1 (v:v) ratio by mixing thoroughly with sand (henceforth termed as soil). Analysis of available nutrients in soil was done prior to As addition at the Division of Soil Science and Agricultural Chemistry, IARI, New Delhi, India. The soil contained adequate levels of N (2,144.82 mg kg^{-1} of soil), P (1,868.74 mg kg^{-1} of soil), K (5,703.82 mg kg^{-1} of soil), Ca (268.27 mg kg^{-1} of soil), and Mg (103.56 mg kg^{-1} of soil). The soil was autoclaved at 121°C and 15 psi for 1 h, twice, to eradicate any existing microbes, followed by treatment with three As concentrations, namely, 0, 25, and 50 mg As kg^{-1} of soil, prepared using sodium arsenate ($\text{Na}_2\text{HAsO}_4 \cdot 7\text{H}_2\text{O}$). Selection of these As concentrations was based on a previous study done in wheat (Sharma et al., 2017) and concentrations of As reported in agricultural soils of Southeast Asia (Alam and Sattar, 2000; Rahman et al., 2013; Tong et al., 2014; Shrivastava et al., 2017). These soil treatments are hereafter referred to as 0, 25, and 50As.

The required quantity of $\text{Na}_2\text{HAsO}_4 \cdot 7\text{H}_2\text{O}$ for the three different As levels was dissolved in distilled water (50 ml) and then mixed with soil thoroughly in plastic trays to ensure homogenous As distribution. The soil was left in trays to equilibrate with recurrent cycles of saturation and air-drying for a period of 1 month and dispensed in pots (Cox and Kovar, 2001). Air-dried soil (100 mg) of each treatment was microwave digested using HNO_3 and H_2O_2 . Volume of the digest was made up to 40 ml with Milli-Q water and later filtered using 0.2- μm membrane filter and analyzed for total As, using inductively coupled plasma mass spectrometer (ICP-MS 7900, Agilent Technologies, Japan). The total As in soil of 0, 25, and 50As was 15.2 $\mu\text{g kg}^{-1}$, 23.6 mg kg^{-1} , and 48.9 mg kg^{-1} , respectively.

Ten surface-sterilized (using 1% sodium hypochlorite solution) wheat seeds were sown per pot. Mycorrhizal plants were provided with 20 g of *R. intraradices* inoculum dispensed at a depth of 2 cm in each pot. NM plants were supplied with 20 ml of soil washing of an equal amount of soil filtered through Whatman No. 1. This guaranteed introduction of microbial populations other than any other propagules, along with an equal amount of autoclaved soil mix to exclude any other variables. Pots were placed outside in open ground at the Botanical Garden, Department of Botany, University of Delhi, under natural conditions (rabi season; 9–16°C), humidity (56–90%), and natural light. Plants were watered to 60% field capacity of soil to avoid drainage of As.

Plant Sampling

Just before the initiation of florets (i.e., 42 days after sowing), six plants from each treatment were harvested along with roots. The plants were washed with water to remove adhering soil particles. Three plants from each treatment were oven dried at 60°C till a constant weight was recorded. The remaining three were used for determining AMF colonization in roots and biochemical estimation in leaves.

Mycorrhizal Colonization and Metal Tolerance Index

Colonization of root cortex by *R. intraradices* was confirmed after clearing and staining roots with 5% KOH and 0.05% trypan blue in lactoglycerol following the modified protocol of Phillips and Hayman (1970). Percent root colonization was calculated following the gridline intersect method (Giovannetti and Mosse, 1980). For this, one hundred 1 cm root segments were placed in petri dish with gridlines having 1 cm^2 boxes. Roots were observed under stereoscope, and horizontal and vertical intersects having mycorrhizal structures were counted. Root colonization was calculated as per the following formula:

$$\text{Mycorrhizal colonization(\%)} = \frac{\text{No. of roots colonized}}{\text{Total no. of roots}} \times 100$$

Metal tolerance index (MTI) of wheat to As in soil was determined according to Rabie (2005) using the following formula:

$$\text{MTI (\%)} = \frac{\text{Plant DW}_{(\text{at particular level of As})}}{\text{Plant DW}_{(\text{in control soil})}} \times 100$$

where plant DW indicates dry weight of plant (roots and shoots) determined after 42 days of sowing.

Concentration of As, Mg, P, and N

Oven-dried leaves were analyzed for their nutrient composition. Leaves (100 mg) were finely ground and sieved through a 0.5-mm sieve. Sieved samples were then subjected to acid digestion in a microwave reaction system (Anton Paar make, model: Multiwave PRO) using concentrated nitric acid. The internal temperature limit of the digester was maintained at 200°C for 30 min. The volume of the digest was made to 40 ml with Milli-Q water and later filtered using 0.2- μ m membrane filter and analyzed for the estimation of As and other nutrient concentrations. As and Mg concentrations were quantified using ICP-MS (ICP-MS 7900, Agilent Technologies, Japan). While a concentration of P was quantified following the ammonium molybdate blue method described by Allen (1989), N concentration was measured by CHNS analyzer (Elementar Analysis System, Vario Micro Cube, Germany).

Concentration of Total Protein

The trichloroacetic acid (TCA)–acetone method described by Jia et al. (2019) was used for extraction of total protein. The leaves (100 mg) were ground in liquid nitrogen with pestle and mortar. Five-time volume of TCA/acetone (1:9) was added to the homogenized powder and mixed using a vortex. The mixture was incubated at –20°C for 4 h, followed by centrifugation at 6,000 g for 40 min at 4°C. The supernatant was discarded, and the resultant pellet was washed three times with chilled acetone. The precipitate was air-dried and reconstituted in buffer (1:30, v/v) containing sodium dodecyl sulfate (4%), dithiothreitol (DTT; 100 mM) and Tris–HCl (150 mM; pH 8). The mixture was sonicated (80 W for 10 s, intermittent for 15 s) for 10 cycles and boiled for 5 min. After that, the lysate was re-centrifuged for 40 min at 14,000 g. The resulting supernatant was filtered with 0.22- μ m filter, and total protein was estimated following the Bradford (1976) assay using bovine albumin serum as standard.

Photosynthesis

Photosynthetic Pigments

Intact leaf tissues (100 mg) were dipped in 7 ml of dimethyl sulfoxide and heated at 65°C for 30 min to extract photosynthetic pigments (Hiscox and Israelstam, 1979). The extract was then transferred to a graduated tube. With the use of dimethyl sulfoxide, the final volume of the extract was made up to 10 ml. Absorbance of the extract was read at 453, 645, and 663 nm. Concentration of total Chl (T-Chl), Chl *a*, Chl *b*, and total carotenoids was calculated using the formula of Arnon (1949).

Hill Reaction Activity

Hill reaction activity was assayed following the protocol of Vishniac (1957). Leaf samples (1 g) were homogenized in 5 ml of sucrose-phosphate buffer (0.5 M of sucrose in 0.05 M of sodium phosphate buffer; pH 6.2). The homogenate was then centrifuged at 1,000 g at 4°C for 10 min. The supernatants collected were re-centrifuged for 15 min at 5,000 g at 4°C. Suspensions of chloroplast were made by dissolving the pellets

after centrifugation in 5 ml of sucrose-phosphate buffer; 1 ml of chloroplast suspension was then mixed with 4 ml of sucrose-phosphate buffer and 0.5 ml 2,6-dichlorophenolindophenol (DCPIP) (0.03%). Following this, the reaction sets were kept under bright light (irradiance 800–1,000 μ mol m^{–2} s^{–1}) for 30 min after taking initial absorbance at 610 nm. After discoloration of the reaction mixture, absorbance was again recorded. The differences in the absorbances were estimated, and Hill reaction activity was calculated referring to a standard curve prepared with DCPIP and was expressed as μ g DCPIP reduced mg^{–1} Chl min^{–1}.

Gas Exchange Parameters

Leaf gas exchange parameters such as leaf Pn, transpiration rate (E), stomatal conductance (Gs), and intercellular CO₂ concentration (Ci) were analyzed on fully expanded young leaves between 9:00 and 11:00 am on a clear sunny day using a portable infrared gas analyzer (Gas Exchange Fluorescence System, GFS-3000). The analyzer was adjusted for leaf surface area (3.00 cm²), ambient CO₂ concentration (ca) (398 ppm), photosynthetic photon flux density (1,000 μ mol m^{–2} s^{–1}), impeller at 7, and relative humidity inside the cuvette maintained at 35%. Water use efficiency (WUE) was calculated by dividing the value of Pn by E.

Chlorophyll *a* Fluorescence

Chl *a* fluorescence was also monitored in terms of minimal fluorescence (Fo), potential efficiency of PSII (Fv/Fo), maximum efficiency of PSII (Fv/Fm), and photochemical quenching coefficient (qP), where Fv is variable fluorescence (Fm – Fo), Fo is minimal fluorescence, and Fm is maximal fluorescence on the adaxial leaf surface, using a portable infrared gas analyzer (Gas Exchange Fluorescence System, GFS-3000). For this, leaf was dark adapted for 30 min and later irradiated by a saturating pulse of 2,000 μ mol m^{–2} s^{–1}, sufficient for complete oxidation of the reaction centers.

Sugar Metabolism

Total Soluble Sugar

Concentration of total soluble sugar (TSS) was quantified following the phenol sulfuric acid reagent method (Dubois et al., 1956). Samples were homogenized using 80% ethanol. The extracts were then centrifuged for 20 min at 2,000 rpm. Reaction mixture contained 1 ml of supernatant, 0.05 ml of phenol (5%), and sulfuric acid (98%). The mixtures were then incubated in water bath for 20 min at 30°C. Absorbance was measured at 490 nm. With the use of standard curve of glucose, content of TSS was quantified and expressed as mg g^{–1} fresh weight (FW).

Reducing Sugar, Non-reducing Sugar, and Reducing Sugar/Non-reducing Sugar Ratio

Concentration of RS was measured following the protocol of Miller (1972). Plant samples (1 g) were extracted in 5 ml of 80% ethanol and later centrifuged at 2,000 g for 20 min. The collected supernatants were then mixed with 1% 3,5-dinitrosalicylic acid reagent (0.5 ml) followed by incubation for 5 min in boiling water bath. Absorbance of the mixture was read at 515 nm. Concentration of RS was estimated from a standard curve of

glucose and expressed as mg g^{-1} FW. Quantification of NRS was done by subtracting the values of RS from that of TSS and expressed as mg g^{-1} FW.

Sucrose-Phosphate Synthase Activity

Sucrose-phosphate synthase (SPS) activity was assessed according to Miron and Schaffer (1991). Plant enzyme extract was prepared using the extraction buffer that contained HEPES–NaOH buffer (50 mM; pH 7.5) containing MgCl_2 (5 mM), EDTA (1 mM), DTT (2.5 mM), and Triton X-100 (0.05%; v/v). The extracts were then centrifuged at 4°C at 10,000 rpm for 10 min. The reaction mixture consisted of HEPES–NaOH buffer (50 mM; pH 7.5), MgCl_2 (15 mM), fructose-6-phosphate (25 mM), glucose-6-phosphate (25 mM), UDP-glucose (25 mM), and enzyme extract. The mixtures were then incubated at 37°C for 30 min. Termination of the reaction was brought by addition of 30% KOH. Sucrose formed during SPS catalyzed reaction was then estimated, and its activity was expressed as $\text{nmol sucrose formed mg}^{-1}$ protein min^{-1} .

Sucrose Synthase Activity

Activity of SS was estimated following the protocol of Miron and Schaffer (1991). Plant enzyme extract was prepared using HEPES–NaOH buffer (50 mM; pH 7.5) containing MgCl_2 (5 mM), EDTA (1 mM), DTT (2.5 mM), and Triton X-100 (0.05%; v/v). The obtained extract was then centrifuged at 4°C at 10,000 rpm for 10 min. Reaction mixture included HEPES–NaOH buffer (50 mM; pH 7.5), MgCl_2 (15 mM), fructose (25 mM), UDP-glucose (25 mM), and enzyme extract. The mixture was then incubated at 37°C for 30 min. The reaction was terminated by adding 30% KOH. Sucrose hydrolyzed during SS catalyzed reaction was estimated, and the enzyme activity was expressed as $\mu\text{mol sucrose hydrolyzed mg}^{-1}$ protein min^{-1} .

Acid Invertase Activity

Activity of acid invertase (AI) was estimated following the method of Borkowska and Szczerha (1991). Plant samples were homogenized in sodium acetate buffer (10 mM; pH 4.6) containing MgCl_2 (3.3 mM), EDTA (1 mM), and phenylmethylsulfonyl fluoride (PMSF) (1 mM). Homogenates were then centrifuged for 20 min at 10,000 rpm at 4°C . Assay mixture consisted of sodium acetate buffer (10 mM; pH 4.6), sucrose (0.4 M), and the enzyme extract. The final volume of 1.0 ml was made up. After incubation for 30 min at 30°C , termination of the reaction was brought by addition of Na_2HPO_4 (0.5 M). Resulting RSs were then estimated by Nelson–Somogyi method (Nelson, 1944), and activity was expressed as $\mu\text{mol sucrose hydrolyzed mg}^{-1}$ protein min^{-1} .

Starch Metabolism

Starch Concentration

Starch concentration was estimated following the protocol of McCready et al. (1950). Residual mass collected after centrifugation (for TSS extraction) was dissolved in distilled water. Later, perchloric acid was added and stirred, followed by centrifugation of the mixture for 20 min at 2,000 rpm. Supernatants were collected and then poured in conical flasks.

The total volume was later made up to 100 ml with the addition of distilled water. Starch concentration was measured in 1 ml of filtrate following the same procedure as that of TSS. Starch quantity was then estimated in terms of glucose, and the factor 0.9 was used to convert the values of glucose to starch. Starch concentration was expressed in mg g^{-1} FW.

α -Amylase and β -Amylase Activities

Following the method of Bush et al. (1989), the activity of α -amylase was determined. Plant samples were homogenized in sodium acetate buffer (0.1 M; pH 4.8) containing cysteine (5 μM) and centrifuged for 10,000 rpm 15 min at 4°C . The obtained supernatants were then heated at 70°C for 5 min in the presence of CaCl_2 (3 mM). Reaction mixture consisted of sodium acetate buffer (0.1 M; pH 4.8), soluble starch (1%) in NaCl (0.15 M), and the enzyme extract. The final volume of the reaction mixture was made up to 4 ml and left for incubation at 30°C for 5 min. Termination of reaction was brought by addition of HCl (6 M). Aliquots (1 ml) were then transferred to conical flasks in which 0.5 ml of IKI solution (0.2% I_2 in 2% KI) was later added. The final volume was then made up to 25 ml with distilled water. Absorbance was read at 660 nm. Activity of enzyme was expressed as $\mu\text{g of starch hydrolyzed mg}^{-1}$ protein min^{-1} .

Activity of β -amylase was estimated following the protocol of Bernfeld et al. (1965). The enzyme was extracted from plant samples in phosphate buffer (pH 7.0) that contained NaCl (0.5 M). Starch solution (1 ml) and properly diluted enzyme (1 ml) were pipetted out and incubated at 25°C for 15 min. The reaction was later stopped by adding 2 ml of 3,5-dinitrosalicylic acid reagent. The reaction mixture was then heated in a water bath (60°C) for 5 min. While the tubes were warm, 1 ml of potassium sodium tartrate solution was added, followed by cooling of the mixture under running tap water. The final volume was made up to 10 ml using distilled water. The absorbance was recorded at 570 nm. Activity of the enzyme was expressed as $\mu\text{g of maltose hydrolyzed mg}^{-1}$ protein min^{-1} .

Starch Phosphorylase Activity

The activity of starch phosphorylase (SP) was determined following the protocol of Dubey and Singh (1999). Plant samples were homogenized in 50 mM of citrate buffer containing EDTA (1 mM; pH 6.0), β -mercaptoethanol (5 mM), and PMSF (1 mM). Homogenized samples were then centrifuged for 20 min at 10,000 rpm at 4°C . Assay mixture was prepared containing citrate buffer (50 mM), soluble starch (5%; w/v), glucose-1-phosphate (0.1 mM), and enzyme extract; and the total volume was made up to 4.0 ml. With the addition of 5% TCA, the reaction was stopped after 10 min. Reaction mixture was then centrifuged, and the phosphorus content in the supernatant was determined following the method of Fiske and Subbarow (1925). Enzyme activity was calculated as $\text{nmol of Pi liberated mg}^{-1}$ protein min^{-1} .

Statistical Analysis

Statistical Package for the Social Sciences Statistics software version 21.0 (SPSS Inc., IBM Corporation, Armonk, NY, United States) was used to analyze the results. Multivariate analysis of variance (MANOVA) was used to evaluate statistical

differences among treatments. One-way analysis of variance (ANOVA) was done for comparing the differences between individual means using Tukey's honestly significant difference (HSD) *post hoc* test. All the values were represented as means of three biological replicates $\pm$ standard deviation (SD).

RESULTS

Root Colonization by *Rhizophagus intraradices*

Histochemical staining of the roots showed successful colonization of *Triticum aestivum* roots by *R. intraradices*. The presence of As in soil increased per cent colonization. However, the extent of increase varied in the two As concentrations. In comparison with 0As, root colonization increased by 10.1 and 4.2% in 25 and 50As, respectively.

Metal Tolerance Index and Concentrations of As, Mg, P, N, and Total Protein

As level, mycorrhizal status, and their interaction significantly affected MTI of wheat plants (Table 1). A significant ($p \leq 0.05$) reduction in MTI was observed in response to As amendments in soil. In NM plants, an increase in As level from 25 to 50 mg resulted in a decline of MTI by 38.9%. However, plants colonized by *R. intraradices* increased MTI by 91.7% at 25As and 131.5% at 50As when compared with their corresponding NM plants (Table 1).

Two-way ANOVA showed that As additions in soil and *R. intraradices* inoculation independently as well as interactively affected concentration of As in wheat leaves (Table 1). With increase in As level in soil, there was a concomitant increase in leaf As concentration as substantiated by a 94.1% increase in plants of 50As when compared with that of 25As. Nevertheless, colonization by *R. intraradices* decreased leaf As concentrations at all As levels with respect to their corresponding NM plants.

Concentration of P declined in wheat leaves in response to presence of As in soil (Table 1). M plants possessed higher P concentration in leaves over NM plants at all As levels. When compared with NM plants, P concentrations increased by 78.1, 30.6, and 87.8% at 0, 25, and 50As, respectively, in M plants. A similar effect was observed on concentrations of other nutrients and total protein. Concentrations of Mg, N, and total protein declined in wheat leaves in response to As stress (Table 1). Colonization by AMF improved the antagonistic effect inflicted by As and increased their concentration significantly when compared with their NM counterparts at all As levels. At 50As, their concentrations were adversely affected, and the ameliorative effect of *R. intraradices* was evident with 37.5, 24.4, and 30.7% increase in Mg, N, and total protein concentrations, respectively.

Photosynthesis

Photosynthetic Pigments

Presence of As in soil, mycorrhizal status, and interaction of both the factors had significant influence on T-Chl, Chl

a, Chl *b*, total carotenoids, and Chl *a/b* ratio (Table 2). With an increase in As level in soil, leaves of wheat plants showed decline in concentration of T-Chl, Chl *a*, Chl *b*, and total carotenoids. When compared with 0As, plants of 25As treatment showed decline of 34.1, 29.6, 50.0, and 27.4% in concentrations of T-Chl, Chl *a*, Chl *b*, and total carotenoid, respectively. A similar but more severe effect was observed on photosynthetic pigments at high As level. Plants grown at 50As showed 56.1, 49.0, 80.0, and 38.35% decline in concentrations of T-Chl, Chl *a*, Chl *b*, and total carotenoid, respectively, over plants grown at 0As. Colonization by *R. intraradices* augmented the concentrations of these photosynthetic pigments at all As levels (Table 2). Chl *a/b* ratio also increased with increasing As concentration in soil in NM plants. In M plants, the ratio was significantly ($p \leq 0.05$) lower than that of NM plants at 25 and 50As. In response to mycorrhizal colonization, the concentration of T-Chl increased by 12.2, 40.7, and 83.3% at 0, 25, and 50As, respectively. Similarly, total carotenoid concentration at the abovementioned As levels increased by 12.3, 26.4, and 33.3%, respectively, in M plants over NM plants.

Hill Reaction Activity

Hill reaction activity of plant significantly ($p \leq 0.05$) decreased with increased As concentration in soil (Figure 1). Colonization by *R. intraradices* showed increase in the activity at all As levels when compared with NM plants, with a maximum of 40% increase reported at 50As.

Gaseous Exchange

All the gas exchange parameters, except WUE, showed decline with corresponding increase in As concentration in soil (Figure 2). Mycorrhizal colonization assisted wheat plants in maintaining better gaseous exchange when compared with NM plants. At 0As, M plants significantly increased Pn and Ci by 6.0 and 15.9%, respectively, over their NM counterparts. The influence of *R. intraradices* in improving gaseous exchange was more evident when plants were exposed to low As stress, wherein M plants maintained 55.1, 24.1, 5.7, 2.6, and 46.9% higher Pn, Gs, E, Ci, and WUE, respectively, when compared with NM plants. Likewise, at high As level, the abovementioned parameters increased by 40.9, 45.7, 3.2, 13.3, and 36.5%, respectively, in M plants over their NM counterparts.

Chlorophyll *a* Fluorescence

While As level, mycorrhizal status, and their interaction showed a significant ($p \leq 0.001$) effect on Fo, Fv/Fo, and Fv/Fm, a non-significant effect was reported on qP (Table 3). Exposure to As stress led to decrease of Fo, Fv/Fo, and Fv/Fm in a dose-dependent manner (Figure 3). Colonization by *R. intraradices* led to an overall increase in Fv/Fm and Fv/Fm as compared with NM plants, although the extent of increase varied with each As level. Contrary to this, *R. intraradices* colonization decreased Fo at 0As by 7.7%; however, it increased by 22.3 and 70.1% at 25 and 50As, respectively, when compared with NM plants.

TABLE 1 | Arbuscular mycorrhizal colonization; MTI; and concentrations of As, P, Mg, N, and total proteins in leaves of *Triticum aestivum* in response to *Rhizophagus intraradices* inoculation (M, mycorrhizal; NM, non-mycorrhizal) and As addition to the soil.

As level (mg As kg ⁻¹ soil)	AMF status	Mycorrhizal colonization (%)	MTI (%)	As (μg g ⁻¹ DW)	P (mg g ⁻¹ DW)	Mg (mg g ⁻¹ DW)	N (mg g ⁻¹ DW)	Total protein (mg g ⁻¹ FW)
0	NM	—	—	0.48 ± 0.01e	3.24 ± 0.08b	5.46 ± 0.002d	44.56 ± 0.98b	112.54 ± 1.45b
	M	56.00 ± 1.00c	—	0.04 ± 0.01f	5.77 ± 0.14a	6.95 ± 0.003a	52.63 ± 0.97a	119.99 ± 2.56a
25	NM	—	59.82 ± 6.95c	12.54 ± 1.36c	2.15 ± 0.12d	5.05 ± 0.004e	38.46 ± 0.61d	80.72 ± 1.06e
	M	61.66 ± 1.53a	114.67 ± 10.35a	7.12 ± 1.05d	2.81 ± 0.07c	6.70 ± 0.10b	41.30 ± 0.91c	92.78 ± 2.50c
50	NM	—	36.50 ± 4.95d	24.35 ± 0.77a	1.48 ± 0.09e	4.37 ± 0.001f	27.76 ± 1.15f	67.72 ± 2.93f
	M	58.33 ± 0.58b	84.49 ± 5.48b	14.07 ± 0.11b	2.78 ± 0.05c	6.01 ± 0.004c	34.53 ± 0.60e	88.50 ± 1.96d
Significance As		***	*	***	***	***	***	***
AMF		—	***	***	***	***	***	***
As × AMF		—	***	*	***	***	***	***

Values represent means of three biological replicates ± SD. Different letters within the column represent significant difference among the treatments at $p \leq 0.05$, derived from Tukey HSD.

* and *** represent significance at $p \leq 0.05$ and $p \leq 0.001$, respectively, derived from two-way ANOVA.

As, arsenic; AMF, arbuscular mycorrhizal fungi; Mg, magnesium; MTI, metal tolerance index; N, nitrogen; P, phosphorus; HSD, honestly significant difference.

TABLE 2 | Concentrations of total T-Chl, Chl a, Chl b, total carotenoids, and Chl a/b ratio in leaves of *Triticum aestivum* in response to *Rhizophagus intraradices* inoculation (M, mycorrhizal; NM, non-mycorrhizal) and As addition to soil.

As level (mg As kg ⁻¹ soil)	AMF status	T-Chl (mg g ⁻¹ FW)	Chl a (mg g ⁻¹ FW)	Chl b (mg g ⁻¹ FW)	Chl a/b ratio	Total carotenoids (mg g ⁻¹ FW)
0	NM	2.05 ± 0.04b	1.55 ± 0.07a,b	0.50 ± 0.02a	3.09 ± 0.31c	0.73 ± 0.06a
	M	2.30 ± 0.05a	1.72 ± 0.09a	0.58 ± 0.04a	2.98 ± 0.40c	0.82 ± 0.09a
25	NM	1.35 ± 0.06c	1.09 ± 0.06c	0.25 ± 0.01c	4.29 ± 0.34b	0.53 ± 0.03d
	M	1.90 ± 0.17b	1.51 ± 0.02b	0.39 ± 0.006b	3.83 ± 0.12b,c	0.67 ± 0.07b
50	NM	0.90 ± 0.09d	0.79 ± 0.08d	0.10 ± 0.01d	7.32 ± 0.36a	0.45 ± 0.05e
	M	1.65 ± 0.11c	1.26 ± 0.04c	0.38 ± 0.07b	3.31 ± 0.54b,c	0.60 ± 0.02c
Significance						
As		***	***	***	***	***
AMF		***	***	***	***	**
As × AMF		***	**	***	***	***

Values represent means of three biological replicates ± SD. Different letters within the column represent significant difference among the treatments at $p \leq 0.05$, derived from Tukey HSD.

** and *** represent significance at $p \leq 0.01$ and $p \leq 0.001$, respectively, derived from two-way ANOVA.

As, arsenic; AMF, arbuscular mycorrhizal fungus; T-Chl, total chlorophyll; Chl a, chlorophyll a; Chl b, chlorophyll b; HSD, honestly significant difference.

Sugar Metabolism

Total Soluble Sugar, Reducing Sugar, and Non-reducing Sugar Concentrations

Two-way ANOVA revealed that As level, mycorrhizal status, and interaction of both these factors significantly affected concentrations of TSS and NRS in wheat leaves. Exposure of plants to As increased concentrations of TSS and RS at both As levels and decreased NRS concentration with increased As level in soil. However, mycorrhizal colonization showed a varied effect on sugar concentrations (Table 4). While mycorrhizal colonization significantly ($p \leq 0.05$) increased TSS and NRS concentration at 25 and 50As, RS concentration increased significantly ($p \leq 0.05$) at 50As only. In response to mycorrhizal colonization, wheat plants showed increase in TSS concentration by 41.1 and 35.3% at 25 and 50As, respectively, when compared with NM plants. A similar trend was observed in case of NRS. When compared with NM plants, the concentration of NRS increased in M plants by 106.5 and 117.2% at 25 and 50As, respectively. However, a positive effect of *R. intraradices* colonization on RS concentration

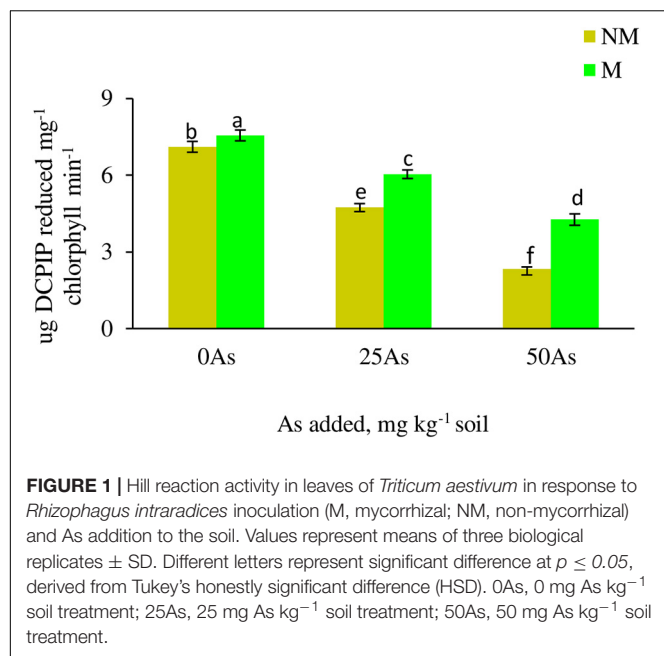
was evident only at 50As level, as RS concentration significantly increased by 6.6% in M plants when compared with NM plants exposed to a similar As level.

Sucrose-Phosphate Synthase Activity

With increased concentration of As in soil, the activity of SPS increased significantly ($p \leq 0.05$), indicating sensitivity of the enzyme toward As toxicity. When compared with 0As plants, SPS activity increased significantly by 124.7 and 298.9% at 25 and 50As, respectively (Figure 4). Colonization by *R. intraradices* enhanced the enzyme activity by 16.5, 62.4, and 8.3% at 0, 25, and 50As, respectively, in M plants over their corresponding NM plants.

Sucrose Synthase and Acid Invertase Activities

Both the activities of SS and AI increased in plants exposed to As in a concentration-dependent manner. At 25As, SS and AI activities of NM plants were enhanced by 35.7 and 25%, respectively, over 0As plants (Figure 4). When compared with 0As level, an increment of 8.9 and 31.0% was observed in SS



and AI activities, respectively, in plants grown at 50As. At all As levels, mycorrhizal colonization increased the activities of these enzymes with respect to their NM counterparts. *R. intraradices* colonization increased SS activity by 27.1 and 8.6% and AI activity by 20.0 and 9.7% at 25 and 50As, respectively, over 0As plants.

Starch Metabolism

Starch Concentration

As stress resulted in decrease in starch concentration with the lowest concentration reported at 50As. The same trend of decline in the concentration was observed upon mycorrhizal colonization at 25 and 50As. Nevertheless, in comparison with their respective NM counterparts, M plants showed significant ($p \leq 0.05$) increase in starch concentration by 23.1% at 0As. However, this AMF-mediated increase was non-significant at 25 and 50As (Figure 5).

α -Amylase and β -Amylase Activities

Activities of α - and β -amylase increased in a dose-dependent manner when exposed to low and high concentrations of As. When compared with 0As plants, an increase of 28.6 and 51.4% was observed on α -amylase activity in leaves of NM plants exposed to 25 and 50As, respectively. No influence of *R. intraradices* on α -amylase activity was reported, as indicated by a non-significant effect of mycorrhizal status on its activity at 0As. However, its activity decreased significantly at 25 and 50As by 26.1 and 35.2%, respectively, in comparison with NM plants (Figure 5). Following a similar trend of α -amylase activity, β -amylase showed increased activity with increased As level. In NM plants, increments of 73.9 and 86.4% were observed over control when plants were exposed to 25 and 50As, respectively. However, the degree of increase in β -amylase activity was less in M plants when compared with NM plant of the same As level.

Starch Phosphorylase Activity

Statistically significant ($p \leq 0.05$) increments in SP activity was observed when plants were exposed to As. SP activity increased by 41.5 and 89.9% at 25 and 50As, respectively, when compared with 0As plants. Inoculation with *R. intraradices* decreased its activity at all As levels when compared with NM plants; nevertheless, with increase in As concentration, its activity increased (Figure 5).

DISCUSSION

Presence of As in soil increased *R. intraradices* colonization in wheat roots. Higher colonization of wheat roots by *R. intraradices* in the presence of As suggests the tolerance of *R. intraradices* to As. This observation is in congruence with higher spore density of *R. intraradices* in As-contaminated sites over non-contaminated soil reported by Schneider et al. (2013) and Krishnamoorthy et al. (2015). Higher root colonization may be an adaptive strategy of *R. intraradices* to As in soil.

The findings of the present study showed that colonization of *T. aestivum* with *R. intraradices* alleviates the detrimental effects of As stress on the photosynthetic parameters such as pigment concentrations, Hill reaction activity, leaf gaseous exchange, and Chl *a* fluorescence. The effect of As stress on the above parameters has been studied in several plants, including wheat; however, most of these studies are limited to seedling stage (Chen et al., 2017; Sil et al., 2019; Majumder et al., 2020). While seedlings depend on reserves stored in seed endosperm (Ferreira et al., 2009), mature plants primarily meet their nutritional need through photosynthesis and display a more complex source-sink relationship (Yu et al., 2015). Further, it is known that formation of AM in roots creates a strong carbon sink and thus influence photosynthesis of plants (Gavito et al., 2019). Study on the long-term effect of As on plants and the role of AMF in amelioration of As stress requires assessment on mature plants. To the best of our knowledge, this is the first study to provide evidence for amelioration of As-induced perturbation in carbohydrate metabolism by AMF inoculation.

The physiological and biochemical changes in plants due to As contamination in soil can be attributed to As toxicity, ionic imbalance, and replacement of essential elements with As in various enzymatic reactions (Sharma et al., 2017; Alam et al., 2019). The first and foremost reason for mitigation of As-induced damage in photosynthetic machinery in M plants can be attributed to reduced uptake and translocation of As in leaves of *T. aestivum* observed in the study and improved antioxidant potential (Sharma et al., 2017).

Roots get directly exposed to As present in the soil that inhibits its growth and proliferation, resulting in compromised nutrient uptake (Alam et al., 2019). Being a structural analog of inorganic P, As(V) is transported across plasma membrane through the phosphate transport systems, where it competes and interferes with P uptake and metabolism (Stoeva and Bineva, 2003). This caused an increase in As and a decrease in P concentrations in leaves of wheat grown in As-contaminated soil in the present study. Further, wheat plants when exposed to As in soil showed decline in concentrations of N and Mg in

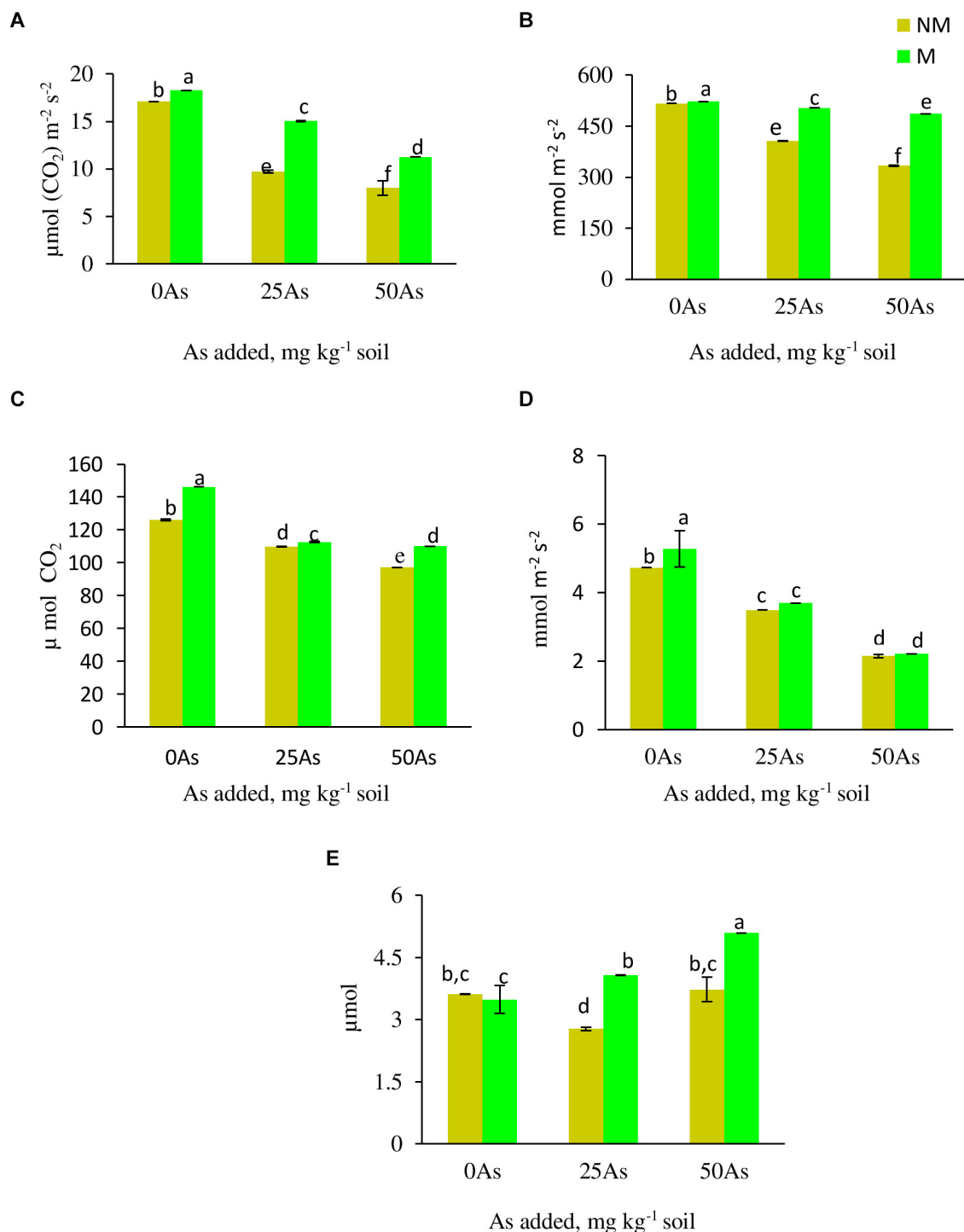


FIGURE 2 | Gas exchange parameters **(A)** net photosynthetic rate, P_n; **(B)** stomatal conductance, G_s; **(C)** intercellular CO₂ concentration, C_i; **(D)** transpiration rate, E; and **(E)** water use efficiency, WUE, of *Triticum aestivum* in response to *Rhizophagus intraradices* inoculation (M, mycorrhizal; NM, non-mycorrhizal) and As addition to the soil. Values represent means of three biological replicates ± SD. Different letters represent significant difference at $p \leq 0.05$, derived from Tukey's honestly significant difference (HSD). 0As, 0 mg As kg⁻¹ soil treatment; 25As, 25 mg As kg⁻¹ soil treatment; 50As, 50 mg As kg⁻¹ soil treatment.

leaves. The negative effects of As on the uptake of these nutrients can be directly linked to reduced root growth due to alteration in morphological and physiological characteristics of roots by

As (Khudsar et al., 2000). A lower N concentration under As stress can also be due to the disturbances in activities as well as affinities of key enzymes involved in N uptake and metabolism

TABLE 3 | Two-way analysis of variance showing the effect of As level, mycorrhizal status, and their interaction on various physiological attributes of *Triticum aestivum*.

Parameter	As level	AMF status	As × AMF
Hill reaction activity	***	***	***
Pn	***	***	***
E	***	*	ns
Gs	***	***	***
Ci	***	***	***
WUE	***	***	***
Fo	***	***	***
Fv/Fo	***	***	***
Fv/Fm	***	***	***
qP	ns	ns	ns
SPS	***	***	***
SS	***	***	***
AI	***	***	***
Starch	***	***	*
SP	***	***	*
α-Amylase	***	***	**
β-Amylase	***	*	ns

* $p \leq 0.05$; ** $p \leq 0.01$ *** $p \leq 0.001$; ns, not significant.

As, arsenic; AMF, arbuscular mycorrhizal fungi; Pn, net photosynthetic rate; E, transpiration rate; Gs, stomatal conductance; Ci, intercellular CO₂ concentration; WUE, water use efficiency; Fo, minimal fluorescence; Fv/Fo, potential efficiency of PSII; Fv/Fm, maximum efficiency of PSII; qP, photochemical quenching coefficient; SPS, sucrose-phosphate synthase; SS, sucrose synthase; AI, acid invertase; SP, starch phosphorylase.

(Ghosh et al., 2013; Sil et al., 2019). Decreased concentration of As in M plants over NM plants in the present study substantiates the potential of *R. intraradices* in mitigating As accumulation in wheat leaves. AMF compensates for the As-mediated root toxicity in plant by providing another route for nutrient uptake by virtue of its extraradical hyphae (Ezawa et al., 2002; Smith and Read, 2008). The extraradical hyphae of AMF ensure access to larger soil volume for acquisition of nutrients (Smith and Read, 2008), augment N assimilation by influencing enzymes of N metabolism (Govindarajulu et al., 2005; Zhu et al., 2016), mobilize immobile nutrients by lowering the pH of rhizosphere (Smith and Read, 2008), and also rapidly transfer Pi as polyphosphate (Ezawa et al., 2002). However, there is no report on transport of As(V) as polyarsenate by an AMF hypha. There are reports that deprivation in concentrations of these nutrients causes closure of reaction centers of PSII, disrupts electron transport chain, and compromises synthesis of photosynthetic pigment that ultimately limits photosynthesis (Singh et al., 2017). An augmentation in concentrations of these nutrients by AMF in present study supports its ameliorative role under As stress.

One of the most consequential responses of plants to As stress is the decline in concentrations of photosynthetic pigments (Gusman et al., 2013; Emamveridian et al., 2015). Concentrations of Chl *a*, Chl *b*, and total carotenoids decreased in NM as well as M plants when subjected to As stress. Concentrations of all pigments were significantly higher in M plants than NM plants at each level of As contamination in soil. Increase in concentrations of Chl *a* and Chl *b* in M over NM plants has been

reported in citrus, cucumber, and chickpea (Li et al., 2013; Chen et al., 2017; Garg and Cheema, 2020). Chl contains tetrapyrrole with Mg in the center and proteins (Fiedor et al., 2008). The decrease in Chl concentrations due to As stress may be due to decrease in the protein, N, and Mg concentrations observed in the present study. The concentration of total proteins was higher in M plants compared with NM plants at all levels of As including control (0As), and this may be attributed to improved N uptake in M plants over NM plants. Kitajima and Hogan (2003) proposed that adjustment of Chl *a/b* ratio is an integral feature of acclimatization to low N availability. The Chl *a/b* ratio is expected to increase with decline in N concentrations. Carotenoids are accessory pigments that protect Chl *a* and Chl *b* from oxidative damage (Hou et al., 2007). Several studies have related decline in Chl pigments to reduced level of carotenoids (Zhou et al., 2018; Sil et al., 2019; Majumder et al., 2020). Higher concentration of total carotenoids in M than NM plants at all levels of As stress suggests protection of Chl from As-induced oxidative damage.

Among all the photosynthetic pigments, Chl *b* concentration was most affected under As stress, followed by Chl *a* and total carotenoids. An enhancement in Chl *a/b* ratio in present study indicates higher degradation of Chl *b* over Chl *a* in the presence of As. There are reports by Sil et al. (2019) and Majumder et al. (2020) stating higher sensitivity of Chl *b* over Chl *a* under As stress. However, the exact mechanism for such differential behavior under As stress needs further investigation. Higher Chl *a/b* ratio indicates higher distress on thylakoids (Zhou et al., 2018), which reflects a plant's inadequacy to transfer electron and excitation energy to the PSII core complex (Xu et al., 1995). Dose-dependent increase in Chl *a/b* ratio in *T. aestivum* in present study is in line with the reports on maize, wheat, and rice (Dresler et al., 2014; Sil et al., 2019; Majumder et al., 2020). The increase in Chl *a/b* ratio can be ascribed to As-mediated decrease in photosynthetic pigments and Hill reaction activity (Allakhverdiev et al., 2000; Sil et al., 2019). Low Hill reaction activity in plants results in decline in NADP reduction, phosphorylation inactivation, and CO₂ assimilation (Yang et al., 2009). The decline in CO₂ assimilation is corroborated in terms of decline in Pn and starch concentration in As-stressed plants observed in this study.

The lower Chl *a/b* ratio in M plants over NM plants reflects higher efficiency to transfer excitation energy to the PSII core complex, consequently resulting in more CO₂ assimilation, higher Pn, and augmented starch concentration in M plants when compared with NM plants. These results show that M plants are superior to NM plants in counterbalancing As-mediated limitation in photosynthetic pigment and Hill reaction activity.

It is well documented that heavy metals affect gas exchange parameters in plants (Tian et al., 2014; Majumder et al., 2020). Contamination of As in soil resulted in reduced Pn and Gs in present study. Normally, RuBisCo (ribulose-1,5-bis-phosphate carboxylase/oxygenase) activity positively affects assimilation of CO₂ in plants. The decline in Pn under As stress has been ascribed to inactivation of RuBisCo involved in carbon fixation (Finnegan and Chen, 2012). Additionally, As-mediated P deficiency in present study is also a possible reason for lower Pn under As stress. P is involved in synthesis of ATP and

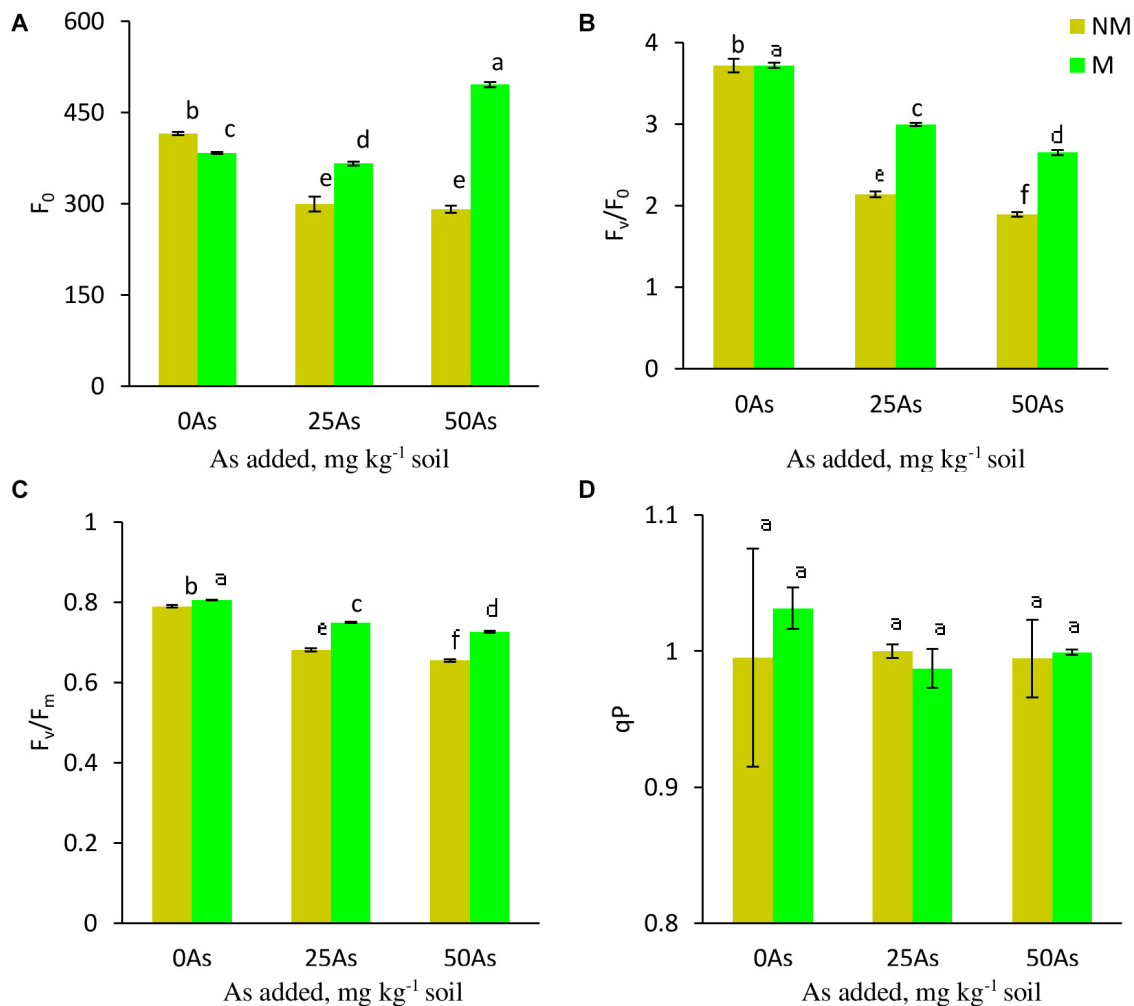


FIGURE 3 | Chl a fluorescence (A) minimal fluorescence, F_o ; (B) potential efficiency of PSII, F_v/F_o ; (C) maximum efficiency of PSII, F_v/F_m ; and (D) photochemical quenching coefficient, qP , in leaves of *Triticum aestivum* in response to *Rhizophagus intraradices* inoculation (M, mycorrhizal; NM, non-mycorrhizal) and As addition to the soil. Values represent means of three biological replicates $\pm$ SD. Different letters represent significant difference at $p \leq 0.05$, derived from Tukey's honestly significant difference (HSD). 0As, 0 mg As kg⁻¹ soil treatment; 25As, 25 mg As kg⁻¹ soil treatment; 50As, 50 mg As kg⁻¹ soil treatment.

TABLE 4 | Concentrations of TSS, RS, NRS, and NRS/RS ratio in leaves of *Triticum aestivum* in response to *Rhizophagus intraradices* inoculation (M, mycorrhizal; NM, non-mycorrhizal) and As addition to the soil.

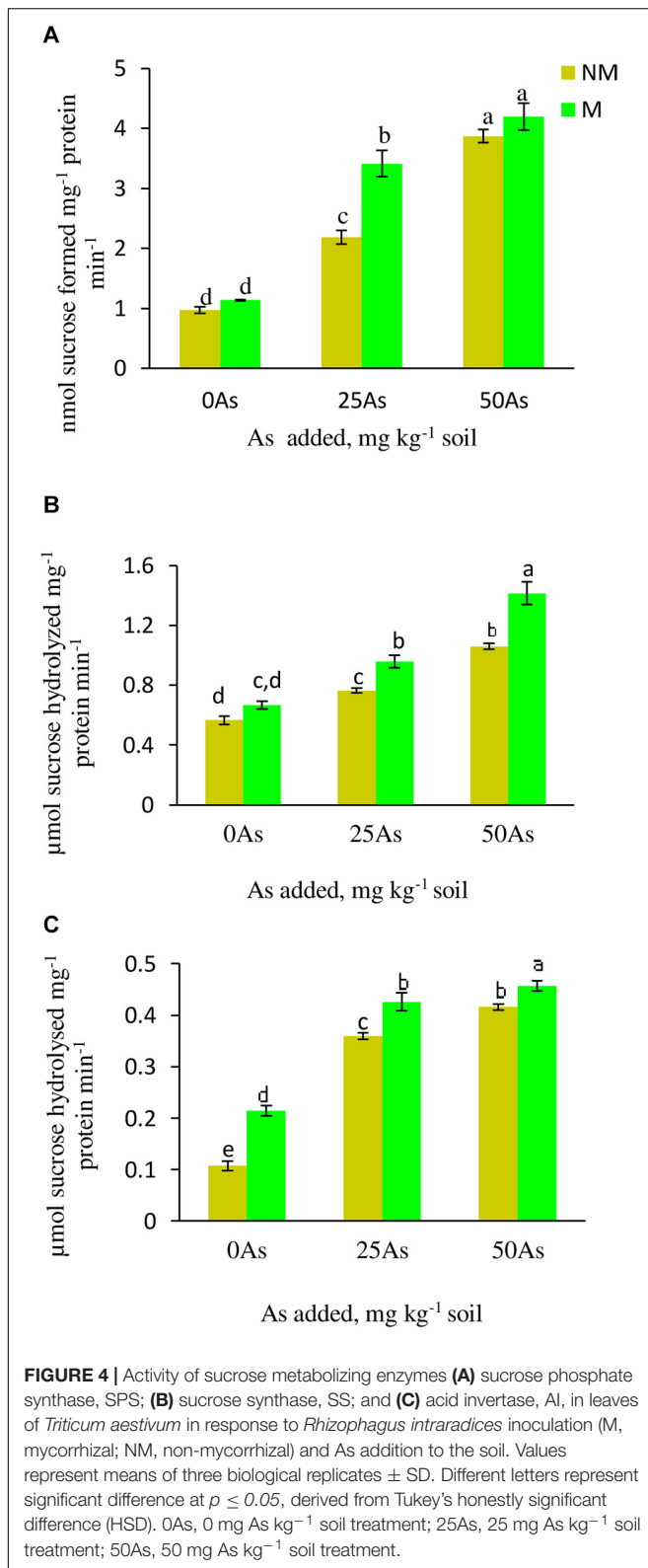
As level (mg As kg ⁻¹ soil)	AMF status	TSS (mg g ⁻¹ FW)	RS (mg g ⁻¹ FW)	NRS (mg g ⁻¹ FW)	NRS/RS ratio
0	NM	8.59 $\pm$ 0.17d	3.27 $\pm$ 0.33d	5.32 $\pm$ 0.49b	1.62
	M	9.74 $\pm$ 0.77d	4.04 $\pm$ 0.55d	5.69 $\pm$ 0.41b	1.40
25	NM	13.20 $\pm$ 1.12c	8.72 $\pm$ 0.17c	4.47 $\pm$ 1.12b	0.51
	M	18.62 $\pm$ 0.12b	9.39 $\pm$ 0.46c	9.23 $\pm$ 0.34a	0.98
50	NM	20.54 $\pm$ 0.47b	16.13 $\pm$ 0.37b	4.41 $\pm$ 0.68b	0.27
	M	27.79 $\pm$ 2.70a	17.20 $\pm$ 0.23a	9.58 $\pm$ 1.24a	0.55
Significance As		***	***	*	
AMF		***	***	***	
As $\times$ AMF		***	ns	***	

Values represent means of three biological replicates $\pm$ SD. Different letters within the columns represent significant difference among the treatments at $p \leq 0.05$, derived from Tukey HSD.

*** represent significance at $p \leq 0.05$, $p \leq 0.01$, and $p \leq 0.001$, respectively, derived from two-way ANOVA.

ns, not significant.

As, arsenic; AMF, arbuscular mycorrhizal fungus; NRS, non-reducing sugars; RSS, reducing sugar; TSS, total soluble sugars; HSD, honestly significant difference.



other phosphorylated metabolites of photosynthesis. Deficiency of P has been reported to cause closure of PSII reaction centers and to inhibit transfer of electrons from PSII to PSI (Singh

et al., 2017). AMF colonization promotes photosynthesis by increasing RuBisCo carboxylation and RuBP (ribulose-1,5-bis-phosphate) regeneration (Chen et al., 2017), and augmenting P uptake. Rai et al. (2014) showed that the presence of As reduces CO_2 assimilation and subsequently decreases CO_2 demand that accounts for decline in Gs observed in present study.

Arbuscular mycorrhizal fungi are obligate symbionts that obtain all the carbon needed for their growth and activities from the host plant. Approximately, one-fifth of the carbon fixed as photosynthates is used in sustenance of AM symbiosis (Bago et al., 2000). This increase in carbon demand in combination with higher concentration of photosynthetic pigments, lower Chl *a/b* ratio, and enhanced Hill reaction activity triggers Pn in M plants, leading to higher stomatal conductance and consequently increasing E and Ci.

Among the two energy harvesting centers in plants, PSII is more sensitive to stress than PSI (Björkman and Demmig, 1987; Stoeva and Bineva, 2003; Iriel et al., 2015). PSII photochemistry is represented by Chl *a* fluorescence attributes, namely, Fo, Fv/Fo, and Fv/Fm (Zhu et al., 2014). Decline in these features due to As in *T. aestivum* indicates compromised functionality of PSII, damage to photosynthetic apparatus, and photoinhibition (Stoeva and Bineva, 2003; Wang et al., 2016). Depreciation in PSII efficiency due to As stress has also been reported in *Oryza sativa* and *Glycine max* (de Andrade et al., 2015; Piršelová et al., 2016). Nevertheless, colonization of wheat plants by *R. intraradices* protected PSII reaction center from As-mediated damage. Under As stress, M plants showed lesser decrease in Fv/Fm than did NM plants. It can be inferred that adverse effects of As on photochemistry of PSII of wheat plants can be alleviated by inoculation with *R. intraradices*, which aid in improving As tolerance.

Sugars generated by photosynthesis, besides serving as substrates in cellular respiration that fuel metabolism, also play a pivotal role in the maintenance of growth, osmotic homeostasis, and membrane stabilization of plant cells (Muller et al., 2011; Bouthour et al., 2012; Sami et al., 2016). On the other hand, starch is the main storage carbohydrate in plants. Under stressful conditions, breakdown of starch results in accumulation of soluble sugars to carry out basal metabolism to sustain plant's growth and development (Stitt and Zeeman, 2012; Begum et al., 2019; Gupta and Thind, 2019). The increment in concentration of TSS and decline in starch concentration with increase in As stress in the present study is in agreement with the above statements. This is further supported by increase in the activities of the starch-degrading enzymes.

Enhanced concentration of TSS generates a feedback inhibition of Pn (Goldschmidt and Huber, 1992). The limitation in Pn activates remobilization of starch as observed in the present study, as indicated by enhanced activities of starch-hydrolyzing enzymes. Additionally, decline in starch concentration can also be credited to As-mediated restrain on starch synthesizing enzymes as reported by Yadav (2010). Findings of the study indicate that when subjected to As-treated soil, plants sustain basic metabolism by enhancing sugars accumulation, limiting Pn, and augmenting starch degradation. Contrary to this, inoculation of wheat plants with *R. intraradices* mitigated effects

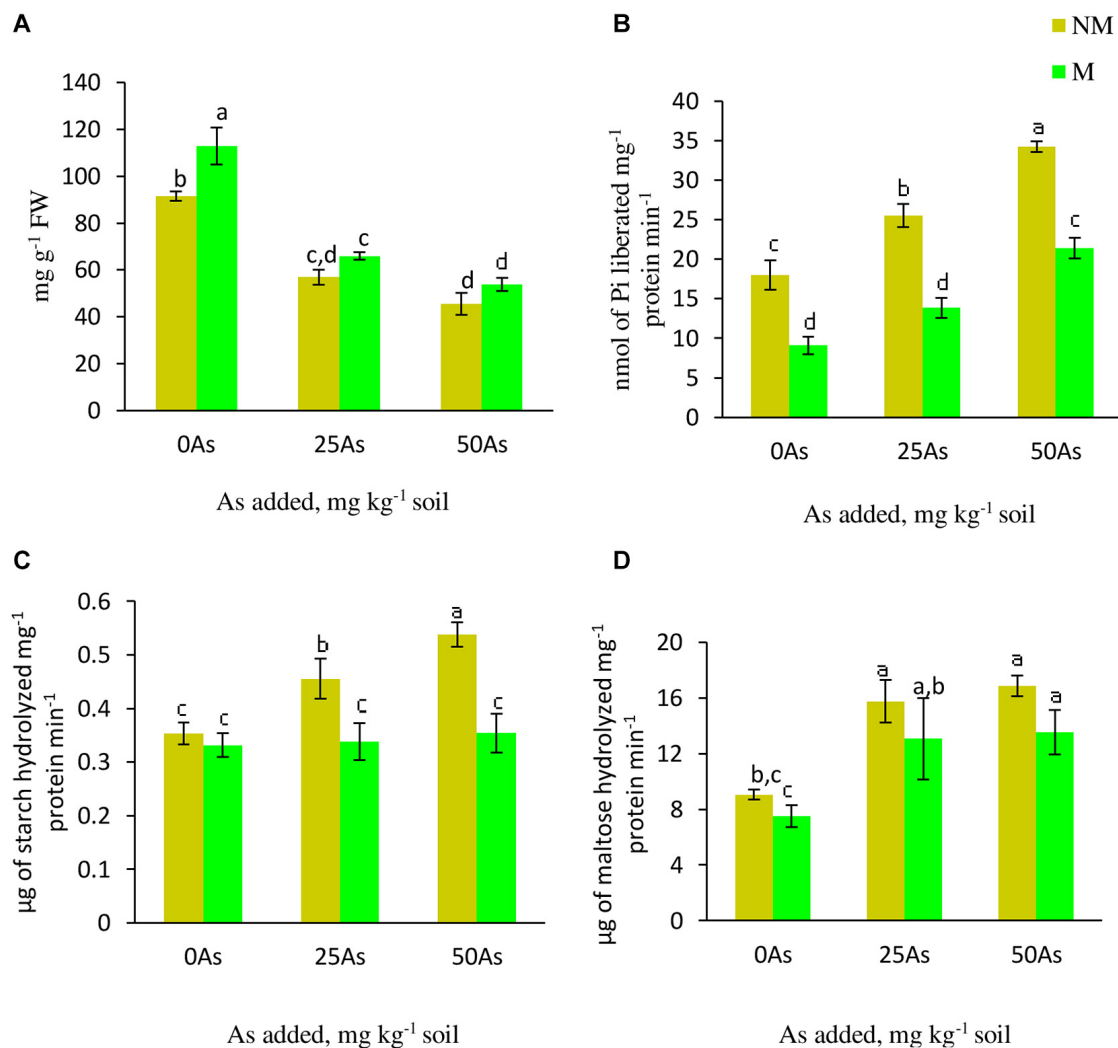


FIGURE 5 | Concentration of (A) starch and activities of starch metabolizing enzymes; (B) starch phosphorylase, SP; (C) α -amylase, and (D) β -amylase in leaves of *Triticum aestivum* in response to *Rhizophagus intraradices* inoculation (M, mycorrhizal; NM, non-mycorrhizal) and As addition to the soil. Values represent means of three biological replicates $\pm$ SD. Different letters represent significant difference at $p \leq 0.05$, derived from Tukey's honestly significant difference (HSD). 0As, 0 mg As kg^{-1} soil treatment; 25As, 25 mg As kg^{-1} soil treatment; 50As, 50 mg As kg^{-1} soil treatment.

of As on starch-hydrolyzing enzymes, as indicated by enhanced starch concentration as a consequence of decreased activities of starch-degrading enzymes. This can be explained by (i) lower requirement of sugar due to lower intensity of As stress (low As concentration) in M plants and (ii) higher Pn that contributes to sugar requirement under As stress and further reduces starch degradation.

The increase in RS and NRS together contributed to increase in concentrations of TSS in NM and M plants. The increase in TSS and RS concentrations in response to As can be linked to increment in activities of sucrose-metabolizing enzymes and breakdown of starch as indicated by elevated activities of starch-degrading enzymes in the present study. Sucrose-synthesizing enzyme, SPS, is reported to be influenced by abiotic as well as biotic stress conditions (Krause et al., 1998). Increase in activity of SPS at 25 and 50As was also observed

in wheat plants under As stress. Soon after its synthesis, sucrose is degraded by sucrose-metabolizing enzymes to produce RS as observed in this study as well. The observed changes are consistent with the report of Choudhury et al. (2010). Increase in AI activity under heavy metal stress facilitates production of hexoses that aid in quenching free radicals and also ensure instigation of ROS metabolism via oxidative pentose phosphate pathway (Peshev et al., 2013). Interestingly, while in the absence of As, the ratio of NRS/RS was higher in NM plants; under increasing As stress, NRS/RS ratio was higher in M plants. This implies that under As stress, RS was synthesized more than NRS in NM plants, and NRS contributed more to the increase in concentration of TSS in M plants. The variations in relative proportion of sugars between NM and M plants can be explained on the basis that while NM plants require RS to scavenge As-induced ROS, M plants

are already endowed with high enzymatic and non-enzymatic antioxidants (Sharma et al., 2017). On the other hand, M plants require higher concentration of sucrose (NRS) for long-distance transport to meet the high demand of sugars to maintain AM symbiosis (Ward et al., 1997).

CONCLUSION

As-mediated perturbations in wheat plants resulted in a dose-dependent decline in gas exchange parameters, namely, Pn, Gs, Ci, and E. Apparent decrease in pigment concentration along with Hill reaction activity was also reported under As stress. Several factors contribute to ameliorative effects of AM on photosynthesis such as higher concentration of photosynthetic pigments, favorable Chl *a/b* ratio, higher Hill reaction activity, and PSII efficiency that ultimately depend upon As concentration in the leaf tissue and uptake of mineral nutrients such as P, N, and Mg. Additionally, increased carbon demand as a result of formation of AM prevents feedback inhibition of photosynthesis due to As-induced reduction of CO₂ assimilation. Higher Pn in M plants reduced the need for starch degradation to form sugars. Furthermore, proportion of NRS (sucrose) was higher in M plants that endowed better ability to tolerate As stress. Therefore, deployment of AMF as a biofertilizer in As-contaminated regions to refurbish physiological as well as biochemical impediments in wheat for improved growth is highly suggested.

REFERENCES

- Abbas, G., Murtaza, B., Bibi, I., Shahid, M., Niazi, N. K., Khan, M. I., et al. (2018). Arsenic uptake, toxicity, detoxification, and speciation in plants: physiological, biochemical, and molecular aspects. *Int. J. Environ. Res. Public Health* 15, 59–64. doi: 10.3390/ijerph15010059
- Alam, M. B., and Sattar, M. A. (2000). Assessment of arsenic contamination in soils and waters in some areas of Bangladesh. *Water Sci. Technol.* 42, 185–192. doi: 10.2166/wst.2000.0568
- Alam, M. Z., McGee, R., Hoque, M., Ahammed, G. J., and Carpenter-Boggs, L. (2019). Effect of arbuscular mycorrhizal fungi, selenium and biochar on photosynthetic pigments and antioxidant enzyme activity under arsenic stress in mung bean (*Vigna radiata*). *Front. Physiol.* 10:193. doi: 10.3389/fphys.2019.00193
- Allakhverdiev, S. I., Sakamoto, A., Nishiyama, Y., Inaba, M., and Murata, N. (2000). Ionic and osmotic effects of NaCl-induced inactivation of photosystems I and II in *Synechococcus* sp. *Plant Physiol.* 123, 1047–1056. doi: 10.1104/pp.123.3.1047
- Allen, S. E. (1989). *Chemical Analysis of Ecological Materials*, 2nd Edn. Oxford: Blackwell Scientific Publications.
- Anjum, S. A., Xie, X. Y., Wang, L. C., Saleem, M. F., Man, C., and Lei, W. (2011). Morphological, physiological and biochemical responses of plants to drought stress. *Afr. J. Agric. Res.* 6, 2026–2032. doi: 10.5897/AJAR10.027
- Arnon, D. I. (1949). Copper enzymes in isolated chloroplasts. Polyphenoloxidase in *Betavulgaris*. *Plant Physiol.* 24, 1–15. doi: 10.1104/pp.24.1.1
- Bago, B., Pfeffer, P. E., and Shachar-Hill, Y. (2000). Carbon metabolism and transport in arbuscular mycorrhizas. *Plant Physiol.* 124, 949–958.
- Begum, N., Qin, C., Ahanger, M. A., Raza, S., Khan, M. I., Ahmed, N., et al. (2019). Role of arbuscular mycorrhizal fungi in plant growth regulation: implications in abiotic stress tolerance. *Front. Plant Sci.* 10:1068. doi: 10.3389/fpls.2019.01068
- Bernfeld, P., Berkeley, B. J., and Bieber, R. E. (1965). Reversible dissociation of enzymes at high dilutions and their inhibition by polyanions. *Arch. Biochem. Biophys.* 111, 31–38.

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.

AUTHOR CONTRIBUTIONS

RK designed and planned the experiment. SG executed the experiments and analyzed the results. RK, SG, and ST jointly wrote the manuscript. All the authors have collectively reviewed the manuscript and approved it.

FUNDING

We gratefully acknowledge the Science and Engineering Research Board (CRG/2019/000836), Department of Science and Technology, Government of India, for providing financial support in undertaking the research work. The authors also gratefully acknowledge the Research Council, University of Delhi, New Delhi, India, and Institution of Eminence (Grant no. IoE/FRP/LS/2020/27), University of Delhi, India, for continued financial support. SG and ST are grateful to the Council of Scientific and Industrial Research for providing Senior Research fellowships.

- Björkman, O., and Demmig, B. (1987). Photon yield of O₂ evolution and chlorophyll fluorescence characteristics at 77 K among vascular plants of diverse origins. *Planta* 170, 489–504. doi: 10.1007/BF00402983
- Borkowska, B., and Szczerha, J. (1991). Influence of different carbon sources on invertase activity and growth of sour cherry (*Prunuscerasus* L.) shoot cultures. *J. Exp. Bot.* 42, 911–915. doi: 10.1023/A:1002160618700
- Bouthour, D., Hajjaji-Nasraoui, A., Saafi, L., Gouia, H., and Chaffei-Haouari, C. (2012). Effects of NaCl on growth and activity of enzymes involved in carbon metabolism in leaves of tobacco (*Nicotiana rustica*). *Afric. J. Biotechnol.* 11, 12619–12629. doi: 10.5897/AJB11.1103
- Bradford, M. M. (1976). A rapid and sensitive method for the quantitation of microgram quantities of protein utilizing the principle of protein-dye binding. *Anal. Biochem.* 72, 248–254. doi: 10.1016/0003-2697(76)90527-3
- Bush, D. S., Sticher, L., Van Huystee, R., Wagner, D., and Jones, R. L. (1989). The calcium requirement for stability and enzymatic activity of two isoforms of barley aleurone alpha-amylase. *Jio. Biol. Chem.* 264, 19392–19398.
- Chandrakar, V., Naithani, S. C., and Keshavkant, S. (2016). Arsenic-induced metabolic disturbances and their mitigation mechanisms in crop plants: a review. *Biologia* 71, 367–377. doi: 10.1515/biolog-2016-0052
- Chen, S., Zhao, H., Zou, C., Li, Y., Chen, Y., Wang, Z., et al. (2017). Combined inoculation with multiple arbuscular mycorrhizal fungi improves growth, nutrient uptake and photosynthesis in cucumber seedlings. *Front. Microbiol.* 8:2516. doi: 10.3389/fmicb.2017.02516
- Choudhury, B., Mitra, S., and Biswas, A. K. (2010). Regulation of sugar metabolism in rice (*Oryzasativa* L.) seedlings under arsenate toxicity and its improvement by phosphate. *Physiol. Mol. Biol. Plants* 16, 59–68. doi: 10.1007/s12298-010-0008-8
- Cox, M. S., and Kovar, J. L. (2001). Soil arsenic effects on canola seedling growth and ion uptake. *Commun. Soil Sci. Plan.* 32, 107–117.
- de Andrade, S. A. L., Domingues, A. P. Jr., and Mazzafera, P. (2015). Photosynthesis is induced in rice plants that associate with arbuscular mycorrhizal fungi and are grown under arsenate and arsenite stress. *Chemosphere* 134, 141–149. doi: 10.1016/j.chemosphere.2015.04.023

- Debona, D., Rodrigues, F. A., and Datnoff, L. E. (2017). Silicon's role in abiotic and biotic plant stresses. *Annu. Rev. Phytopathol.* 55, 85–107. doi: 10.1146/annurev-phyto-080516-035312
- Dresler, S., Hanaka, A., Bednarek, W., and Maksymiec, W. (2014). Accumulation of low-molecular-weight organic acids in roots and leaf segments of *Zeamays* plants treated with cadmium and copper. *Acta Physiol. Plant* 36, 1565–1575. doi: 10.1007/s11738-014-1532-x
- Dubey, R. S., and Singh, A. K. (1999). Salinity induces accumulation of soluble sugars and alters the activity of sugar metabolising enzymes in rice plants. *Biol. Plant* 42, 233–239.
- Dubois, M., Gilles, K. A., Hamilton, J. K., Rebers, P. A., and Smith, F. (1956). Colorimetric method for the determination of sugars and related substances. *Anal. Chem.* 28, 350–356.
- Emamveridian, A., Ding, Y., Mokherdoran, F., and Xie, Y. (2015). Heavy metal stress and some mechanisms of plant defense response. *Sci. World J.* 2015:756120. doi: 10.1155/2015/756120
- Ezawa, T., Smith, S. E., and Smith, F. A. (2002). P metabolism and transport in AM fungi. *Plant Soil* 244, 221–230. doi: 10.1023/A:1020258325010
- Ferreira, C. D. S., Piedade, M. T. F., Tiné, M. A. S., Rossatto, D. R., Parolin, P., and Buckeridge, M. S. (2009). The role of carbohydrates in seed germination and seedling establishment of *Himatanthus sucuuba*, an Amazonian tree with populations adapted to flooded and non-flooded conditions. *Ann. Bot.* 104, 1111–1119.
- Fiedor, L., Kania, A., Myśliwa-Kurczel, B., Orzeł, Ł., and Stochel, G. (2008). Understanding chlorophylls: central magnesium ion and phytyl as structural determinants. *(BBA) Bioenergetics* 1777, 1491–1500. doi: 10.1016/j.bbabi.2008.09.005
- Finnegan, P., and Chen, W. (2012). Arsenic toxicity: the effects on plant metabolism. *Front. Physiol.* 3:182. doi: 10.3389/fphys.2012.00182
- Fiske, C. H., and Subbarow, Y. (1925). The colorimetric determination of phosphorus. *J. Biol. Chem.* 66, 375–400.
- Food and Agriculture Organization (FAO) (2020). *FAO Cereal Supply and Demand Brief*. [Online]. Rome: FAO.
- Garg, N., and Cheema, A. (2020). Relative roles of Arbuscular Mycorrhizae in establishing a correlation between soil properties, carbohydrate utilization and yield in *Cicerarietinum* L. under As stress. *Ecotoxicol. Environ. Saf.* 207, 111–196. doi: 10.1016/j.ecoenv.2020.111196
- Gavito, M. E., Jakobsen, I., Mikkelsen, T. N., and Mora, F. (2019). Direct evidence for modulation of photosynthesis by an arbuscular mycorrhiza-induced carbon sink strength. *New Phytol.* 223, 896–907. doi: 10.1111/nph.15806
- Ghosh, S., Saha, J., and Biswas, A. K. (2013). Interactive influence of arsenate and selenate on growth and nitrogen 771 metabolism in wheat (*Triticum aestivum* L.) seedlings. *Acta Physiol. Plant* 35, 1873–1885.
- Giovannetti, M., and Mosse, B. (1980). An evaluation of techniques for measuring vesicular arbuscular mycorrhizal infection in roots. *New. Phytol.* 84, 489–500.
- Goldschmidt, E. E., and Huber, S. C. (1992). Regulation of photosynthesis by end-product accumulation in leaves of plants storing starch, sucrose, and hexose sugars. *Plant Physiol.* 99, 1443–1448. doi: 10.1104/pp.99.4.1443
- Govindarajulu, M., Philip, E., Pfeffer, H., Jin, H., Jehad, A., David, D., et al. (2005). Nitrogen transfer in the arbuscular mycorrhizal symbiosis. *Nature* 435, 819–823. doi: 10.1038/nature03610
- Gramss, G. (2012). Potential contributions of oxidoreductases from alfalfa plants to soil enzymology and biotechnology: a review. *J. Nat. Sci. Sustain. Technol.* 6, 169–173.
- Gupta, N., and Thind, S. K. (2019). Foliar application of glycine betaine alters sugar metabolism of wheat leaves under prolonged field drought stress. *Proc. Natl. Acad. Sci. India Sec. B Biol. Sci.* 89, 877–884. doi: 10.1007/s40011-018-1000-2
- Gusman, G. S., Oliveira, J. A., Farnese, F. S., and Cambraia, J. (2013). Arsenate and arsenite: the toxic effects on photosynthesis and growth of lettuce plants. *Acta Physiol. Plant* 35, 1201–1209. doi: 10.1007/s11738-012-1159-8
- Hiscox, J. D., and Israelstam, G. F. (1979). A method for the extraction of chlorophyll from leaf tissue without maceration. *Can. J. Bot.* 57, 1332–1334. doi: 10.1139/b79-163
- Hou, W., Chen, X., Song, G., Wang, Q., and Chang, C. C. (2007). Effects of copper and cadmium on heavy metal polluted waterbody restoration by duckweed (*Lemnaminor*). *Plant Physiol. Biochem.* 45, 62–69. doi: 10.1016/j.plaphy.2006.12.005
- INVAM (2014). *International Culture Collection of (Vesicular) Arbuscular Mycorrhizal Fungi*. Morgantown, WV: INVAM.
- Iriel, A., Dundas, G., Cirelli, A. F., and Lagorio, M. G. (2015). Effect of arsenic on reflectance spectra and chlorophyll fluorescence of aquatic plants. *Chemosphere* 119, 697–703. doi: 10.1016/j.chemosphere.2014.07.066
- Jha, A. B., and Dubey, R. S. (2004). Carbohydrate metabolism in growing rice seedlings under arsenic toxicity. *J. Plant Physiol.* 161, 867–872. doi: 10.1016/j.jplph.2004.01.004
- Jia, T., Wang, J., Chang, W., Fan, X., Sui, X., and Song, F. (2019). Proteomics analysis of *E. angustifolia* seedlings inoculated with arbuscular mycorrhizal fungi under salt stress. *Int. J. Mol. Sci.* 20, 788–801. doi: 10.3390/ijms20030788
- Kapoor, R., Giri, B., and Mukerji, K. G. (2002). *Glomus macrocarpum*: a potential bioinoculant to improve essential oil quality and concentration in Dill (*Anethumgraveolens* L.) and Carum (*Trachyspermumammi* (Linn.) Sprague). *World J. Microb. Biot.* 18, 459–463.
- Khalid, S., Shahid, M., Niazi, N. K., Rafiq, M., Bakhat, H. F., Imran, M., et al. (2017). *Enhancing Cleanup of Environmental Pollutants. Arsenic Behaviour in Soil-Plant System: Biogeochemical Reactions and Chemical Speciation Influences*. Berlin: Springer, 97–140.
- Khudsar, T., Mahmooduzzafar Soh, W. Y., and Iqbal, M. (2000). Morphological and anatomical variations of *Cajanus cajan* (Linn.) Huth raised in cadmium-rich soil. *J. Plant. Biol.* 43, 149–157. doi: 10.1007/BF03030492
- Kitajima, K., and Hogan, K. P. (2003). Increases of chlorophyll a/b ratios during acclimation of tropical woody seedlings to nitrogen limitation and high light. *Plant Cell Environ.* 26, 857–865. doi: 10.1046/j.1365-3040.2003.01017.x
- Krause, K. P., Hill, L., Reimholz, R., Hamborg Nielsen, T., Sonnewald, U., and Stitt, M. (1998). Sucrose metabolism in cold-stored potato tubers with decreased expression of sucrose phosphate synthase. *Plant Cell Environ.* 21, 285–299. doi: 10.1046/j.1365-3040.1998.00271.x
- Krishnamoorthy, R., Kim, C. G., Subramanian, P., Kim, K. Y., Selvakumar, G., and Sa, T. M. (2015). Arbuscular mycorrhizal fungi community structure, abundance and species richness changes in soil by different levels of heavy metal and metalloids concentration. *PLoS One* 10:e0128784. doi: 10.1371/journal.pone.0128784
- Li, Y., Ren, B., Ding, L., Shen, Q., Peng, S., and Guo, S. (2013). Does chloroplast size influence photosynthetic nitrogen use efficiency? *PLoS One* 8:e0062036. doi: 10.1371/journal.pone.0062036
- Majumder, B., Das, S., Biswas, S., Mazumdar, A., and Biswas, A. K. (2020). Differential responses of photosynthetic parameters and its influence on carbohydrate metabolism in some contrasting rice (*Oryzasativa* L.) genotypes under arsenate stress. *Ecotoxicology* 29, 912–931.
- McCready, R. M., Guggolz, J., Silveira, V., and Owens, H. S. (1950). Determination of starch and amylose in vegetables. *Anal. Chem.* 22, 1156–1158.
- Meharg, A. A. (1994). Integrated tolerance mechanisms: constitutive and adaptive plant responses to elevated metal concentrations in the environment. *Plant Cell Environ.* 17, 989–993. doi: 10.1111/j.1365-3040.1994.tb02032.x
- Miller, G. L. (1972). Use of dinitrosalicylic acid reagent for determination of reducing sugars. *Anal. Chem.* 31, 426–428.
- Miron, D., and Schaffer, A. A. (1991). Sucrose phosphate synthase, sucrose synthase, and invertase activities in developing fruit of *Lycopersiconesculentum* Mill. and the sucrose accumulating *Lycopersiconhirsutum* Humb. and Bonpl. *Plant Physiol.* 95, 623–627. doi: 10.1104/pp.95.2.623
- Muller, B., Pantin, F., Génard, M., Turc, O., Freixes, S., Piques, M., et al. (2011). Water deficits uncouple growth from photosynthesis, increase C content, and modify the relationships between C and growth in sink organs. *J. Exp. Bot.* 62, 1715–1729. doi: 10.1093/jxb/erq438
- Musil, S., Matoušek, T., Currier, J. M., Styblo, M., and Dědina, J. (2014). Speciation analysis of arsenic by selective hydride generation-cryotrapping-atomic fluorescence spectrometry with flame-in-gas-shield atomizer: achieving extremely low detection limits with inexpensive instrumentation. *Anal. Chem.* 86, 10422–10428. doi: 10.1021/ac502931k
- Nelson, N. (1944). A photometric adaptation of the somogyis method for the determination of reducing sugar. *Anal. Chem.* 31, 426–428.
- Peshev, D., Vergauwen, R., Moglia, A., Hideg, É., and Van den Ende, W. (2013). Towards understanding vacuolar antioxidant mechanisms: a role for fructans? *J. Exp. Bot.* 64, 1025–1038. doi: 10.1093/jxb/ers377

- Phillips, J. M., and Hayman, D. S. (1970). Improved procedures for clearing roots and staining parasitic and vesicular-arbuscular mycorrhizal fungi for rapid assessment of infection. *Trans. Brit. Mycol. Soc.* 55, 158–168.
- Piršelová, B., Kuna, R., Lukáč, P., and Havrlentová, M. (2016). Effect of cadmium on growth, photosynthetic pigments, iron and cadmium accumulation of Faba Bean (*Vicia faba* cv. Aštar). *Agriculture (Polnohospodárstvo)* 62, 72–79. doi: 10.1515/agri-2016-0008
- Rabie, G. H. (2005). Contribution of arbuscular mycorrhizal fungus to red kidney and wheat plants tolerance grown in heavy metal-polluted soil. *Afr. J. Biotechnol.* 4, 332–345.
- Rahman, M. M., Asaduzzaman, M., and Naidu, R. (2013). Consumption of arsenic and other elements from vegetables and drinking water from an arsenic-contaminated area of Bangladesh. *J. Hazard. Mater.* 262, 1056–1063. doi: 10.1016/j.jhazmat.2012.06.045
- Rai, R., Pandey, S., Shrivastava, A. K., and Pandey Rai, S. (2014). Enhanced photosynthesis and carbon metabolism favor arsenic tolerance in *Artemisia annua*, a medicinal plant as revealed by homology-based proteomics. *Int. J. Proteom.* 2014, 2014–2035. doi: 10.1155/2014/163962
- Rasheed, H., Kay, P., Slack, R., and Gong, Y. Y. (2018). Arsenic species in wheat, raw and cooked rice: exposure and associated health implications. *Sci. Total Environ.* 634, 366–373.
- Rosa, M., Hilal, M., González, J. A., and Prado, F. E. (2009). Low-temperature effect on enzyme activities involved in sucrose–starch partitioning in salt-stressed and salt-acclimated cotyledons of quinoa (*Chenopodium quinoa* Willd.) seedlings. *Plant Physiol. Biochem.* 47, 300–307. doi: 10.1016/j.plaphy.2008.12.001
- Sami, F., Yusuf, M., Faizan, M., Faraz, A., and Hayat, S. (2016). Role of sugars under abiotic stress. *Plant Physiol. Biochem.* 109, 54–61. doi: 10.1016/j.plaphy.2016.09.005
- Schneider, J., Sturmer, S. L., Guilherme, L. R., de Souza Moreira, F. M., and Soares, C. R. (2013). Arbuscular mycorrhizal fungi in arsenic-contaminated areas in Brazil. *J. Hazard. Mater.* 262, 1105–1115.
- Sharma, S., Anand, G., Singh, N., and Kapoor, R. (2017). Arbuscular mycorrhiza augments arsenic tolerance in wheat (*Triticum aestivum* L.) by strengthening antioxidant defense system and thiol metabolism. *Front. Plant Sci.* 8:906. doi: 10.3389/fpls.2017.00906
- Shrivastava, A., Barla, A., Singh, S., Mandraha, S., and Bose, S. (2017). Arsenic contamination in agricultural soils of Bengal deltaic region of West Bengal and its higher assimilation in monsoon rice. *J. Hazard. Mater.* 324, 526–534. doi: 10.1016/j.jhazmat.2016.11.022
- Sil, P., Das, P., Biswas, S., Mazumdar, A., and Biswas, A. K. (2019). Modulation of photosynthetic parameters, sugar metabolism, polyamine and ion contents by silicon amendments in wheat (*Triticum aestivum* L.) seedlings exposed to arsenic. *Environ. Sci. Pollut. Res.* 26, 13630–13648. doi: 10.1007/s11356-019-04896-7
- Singh, S. K., Reddy, V. R., Fleisher, D. H., and Timlin, D. J. (2017). Relationship between photosynthetic pigments and chlorophyll fluorescence in soybean under varying phosphorus nutrition at ambient and elevated CO₂. *Photosynthetica* 55, 421–433. doi: 10.1007/s11099-016-0657-0
- Smith, S. E., and Read, D. J. (2008). *Mycorrhizal Symbiosis*. San Diego, CA: Academic Press.
- Srivastava, S., Sinha, P., and Sharma, Y. K. (2017). Status of photosynthetic pigments, lipid peroxidation and anti-oxidative enzymes in *Vigna mungo* in presence of arsenic. *J. Plant Nutr.* 40, 298–306. doi: 10.1080/01904167.2016.1240189
- Stitt, M., and Zeeman, S. C. (2012). Starch turnover: pathways, regulation and role in growth. *Curr. Opin. Plant Biol.* 15, 282–292. doi: 10.1016/j.pbi.2012.03.016
- Stoeva, N., and Bineva, T. (2003). Oxidative changes and photosynthesis in oat plants grown in As-contaminated soil. *Bulg. J. Plant Physiol.* 29, 87–95.
- Talukdar, D. (2013). Arsenic-induced changes in growth and antioxidant metabolism of fenugreek. *Russ. J. Plant Physiol.* 60, 652–660. doi: 10.1134/S1021443713050130
- Tian, T., Ali, B., Qin, Y., Malik, Z., Gill, R. A., Ali, S., et al. (2014). Alleviation of lead toxicity by 5-aminolevulinic acid is related to elevated growth, photosynthesis, and suppressed ultrastructural damages in oilseed rape. *BioMed. Res. Intl.* 2014:530642. doi: 10.1155/2014/530642
- Tong, J., Guo, H., and Wei, C. (2014). Arsenic contamination of the soil–wheat system irrigated with high arsenic groundwater in the Hetao Basin, Inner Mongolia, China. *Sci. Total Environ.* 496, 479–487. doi: 10.1016/j.scitotenv.2014.07.073
- Vishniac, W. (1957). “Methods for the study of Hill reaction,” in *Methods in Enzymology*, Vol. 4, eds S. F. S. F. Colowick and N. O. Kaplan (New York, NY: Academic Press), 324.
- Wang, J., Lu, W., Tong, Y., and Yang, Q. (2016). Leaf morphology, photosynthetic performance, chlorophyll fluorescence, stomatal development of lettuce (*Lactuca sativa* L.) exposed to different ratios of red light to blue light. *Front. Plant Sci.* 7:250. doi: 10.3389/fpls.2016.00250
- Ward, J. M., Kühn, C., Tegeder, M., and Frommer, W. B. (1997). *Sucrose Transport in Higher Plants. In International Review of Cytology*, Vol. 178. Cambridge, MA: Academic Press, 41–71.
- Wu, J. T., Wang, L., Zhao, L., Huang, X. C., and Ma, F. (2020). Arbuscular mycorrhizal fungi effect growth and photosynthesis of *Phragmites australis* (Cav.) Trin. ex. Steudel under copper stress. *Plant Biol.* 22, 62–69. doi: 10.1111/plb.13039
- Xu, Q., Paulsen, A. Q., Guikema, J. A., and Paulsen, G. M. (1995). Functional and ultrastructural injury to photosynthesis in wheat by high temperature during maturation. *Environ. Exp. Bot.* 35, 43–54. doi: 10.1016/0098-8472(94)00030-9
- Yadav, S. K. (2010). Heavy metals toxicity in plants: an overview on the role of glutathione and phytochelatins in heavy metal stress tolerance of plants. *S. Afr. J. Bot.* 76, 167–179.
- Yang, C. W., Xu, H. H., Wang, L. L., Liu, J., Shi, D. C., and Wang, D. L. (2009). Comparative effects of salt-stress and alkali-stress on the growth, photosynthesis, solute accumulation, and ion balance of barley plants. *Photosynthetica* 47, 79–86. doi: 10.1007/s11099-009-0013-8
- Yu, S. M., Lo, S. F., and Ho, T. H. D. (2015). Source–sink communication: regulated by hormone, nutrient, and stress cross-signaling. *Trends Plant Sci.* 20, 844–857.
- Zhan, F., Li, B., Jiang, M., Yue, X., He, Y., Xia, Y., et al. (2018). Arbuscular mycorrhizal fungi enhance antioxidant defense in the leaves and the retention of heavy metals in the roots of maize. *Environ. Sci. Pollut. Res. Int.* 25, 24338–24347. doi: 10.1007/s11356-018-2487-z
- Zhou, X., Sun, C., Zhu, P., and Liu, F. (2018). Effects of antimony stress on photosynthesis and growth of *Acorus calamus*. *Front. Plant Sci.* 9:579. doi: 10.3389/fpls.2018.00579
- Zhu, X. Q., Wang, C. Y., Chen, H., and Tang, M. (2014). Effects of arbuscular mycorrhizal fungi on photosynthesis, carbon content, and calorific value of black locust seedlings. *Photosynthetica* 52, 247–252. doi: 10.1007/s11099-014-0031-z
- Zhu, X., Song, F., Liu, S., and Liu, F. (2016). Arbuscular mycorrhiza improve growth, nitrogen uptake, and nitrogen use efficiency in wheat grown under elevated CO₂. *Mycorrhiza* 26, 133–140. doi: 10.1007/s00572-015-0654-3

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.

Copyright © 2021 Gupta, Thokchom and Kapoor. This is an open-access article distributed under the terms of the Creative Commons Attribution License (CC BY). The use, distribution or reproduction in other forums is permitted, provided the original author(s) and the copyright owner(s) are credited and that the original publication in this journal is cited, in accordance with accepted academic practice. No use, distribution or reproduction is permitted which does not comply with these terms.



Soybean Inoculated With One Bradyrhizobium Strain Isolated at Elevated [CO₂] Show an Impaired C and N Metabolism When Grown at Ambient [CO₂]

OPEN ACCESS

Edited by:

Luisa M. Sandalio,
Departamento de Bioquímica,
Biología Celular y Molecular
de Plantas, Estación Experimental del
Zaidín (EEZ), Spain

Reviewed by:

Juan Sanjuan,
Estación Experimental de Zaidín,
Consejo Superior de Investigaciones
Científicas, Spanish National
Research Council, Spain
Ana Laura Furlan,
National University of Río Cuarto,
Argentina

*Correspondence:

Maite Lacuesta
maite.lacuesta@ehu.es
Alvaro Sanz-Saez
azs0223@auburn.edu

† These authors have contributed
equally to this work

Specialty section:

This article was submitted to
Plant Abiotic Stress,
a section of the journal
Frontiers in Plant Science

Received: 21 January 2021

Accepted: 31 March 2021

Published: 20 May 2021

Citation:

Soba D, Aranjuelo I, Gakière B,
Gilard F, Pérez-López U,
Mena-Petite A, Muñoz-Rueda A,
Lacuesta M and Sanz-Saez A (2021)
Soybean Inoculated With One
Bradyrhizobium Strain Isolated at
Elevated [CO₂] Show an Impaired C
and N Metabolism When Grown at
Ambient [CO₂].
Front. Plant Sci. 12:656961.
doi: 10.3389/fpls.2021.656961

David Soba¹, Iker Aranjuelo¹, Bertrand Gakière², Françoise Gilard², Usue Pérez-López³, Amaia Mena-Petite⁴, Alberto Muñoz-Rueda⁴, Maite Lacuesta^{4†} and Alvaro Sanz-Saez^{5†}

¹ Instituto de Agrobiotecnología (IdAB), Consejo Superior de Investigaciones Científicas (CSIC)-Gobierno de Navarra, Pamplona, Spain, ² Plateforme Métabolisme-Métabolome, Institut de Biologie des Plantes, Université Paris-Sud, Orsay, France, ³ Department of Plant Biology and Ecology, Faculty of Science and Technology, University of the Basque Country (UPV/EHU), Bilbao, Spain, ⁴ Department of Plant Biology and Ecology, Faculty of Pharmacy, University of the Basque Country (UPV/EHU), Vitoria-Gasteiz, Spain, ⁵ Department of Crop, Soil, and Environmental Sciences, Auburn University, Auburn, AL, United States

Soybean (*Glycine max* L.) future response to elevated [CO₂] has been shown to differ when inoculated with *B. japonicum* strains isolated at ambient or elevated [CO₂]. Plants, inoculated with three *Bradyrhizobium* strains isolated at different [CO₂], were grown in chambers at current and elevated [CO₂] (400 vs. 700 ppm). Together with nodule and leaf metabolomic profile, characterization of nodule N-fixation and exchange between organs were tested through ¹⁵N₂-labeling analysis. Soybeans inoculated with SFJ14-36 strain (isolated at elevated [CO₂]) showed a strong metabolic imbalance, at nodule and leaf levels when grown at ambient [CO₂], probably due to an insufficient supply of N by nodules, as shown by ¹⁵N₂-labeling. In nodules, due to shortage of photoassimilate, C may be diverted to aspartic acid instead of malate in order to improve the efficiency of the C source sustaining N₂-fixation. In leaves, photorespiration and respiration were boosted at ambient [CO₂] in plants inoculated with this strain. Additionally, free phytol, antioxidants, and fatty acid content could be indicate induced senescence due to oxidative stress and lack of nitrogen. Therefore, plants inoculated with *Bradyrhizobium* strain isolated at elevated [CO₂] may have lost their capacity to form effective symbiosis at ambient [CO₂] and that was translated at whole plant level through metabolic impairment.

Keywords: *Bradyrhizobium* strains, C and N metabolism, elevated [CO₂], metabolomics, N-fixation, nodule, soybean

INTRODUCTION

Atmospheric carbon dioxide concentration ([CO₂]) has increased strongly since preindustrial times (~280 ppm) to 412.8 ppm registered in November 2020 (¹CO₂.earth, 2020), and a further substantial increase is expected during this century. Carbon dioxide is the major greenhouse gas of anthropogenic activity origin and has been demonstrated to participate in climate change

¹ www.Co2.earth

whereby global temperature and precipitation patterns will be altered (IPCC, 2013). At the plant level, increasing [CO₂] leads to increased photosynthesis while reducing photorespiration and, as a consequence, increasing growth and seed yield (Ainsworth et al., 2002).

Soybean (*Glycine max* L.) is the fourth most important food crop and the most cultivated legume, with 349 million tons produced in 2018 (FAOSTAT, 2020) being the most traded agricultural commodity, accounting for over 10% of the total value of the global exchange. This legume is a rich source of high-quality proteins and oil and contains a considerable amount of carbohydrates, amino acids, and minerals that contribute to its nutritional value (Medic et al., 2014). Soybean physiological responses to elevated [CO₂] (e[CO₂]) have been extensively studied in both controlled and in field environments (Ainsworth et al., 2002; Bishop et al., 2015). Increased photosynthesis due to CO₂ fertilization leads to increases in leaf carbohydrate content (Ainsworth et al., 2004; Rogers et al., 2006), radiation use efficiency (RUE) (Sanz-Sáez et al., 2017), biomass production, and seed yield (Morgan et al., 2005), while decreasing stomatal conductance (Ainsworth and Rogers, 2007; Soba et al., 2020b) and leaf respiration (Tcherkez et al., 2008).

In some crops, sink limitation and photosynthesis downregulation is sometimes observed in plants grown at e[CO₂] as a consequence of sugar overaccumulation in the leaves (Ainsworth and Rogers, 2007; Gutiérrez et al., 2009). However, soybeans, as the rest of legume crops, forms symbiotic relationships with *Rhizobiaceae* family bacteria, specifically with *Bradyrhizobium japonicum*, which provide access to atmospheric N₂, through biological nitrogen fixation (BNF). According to Kaschuk et al. (2009), *Bradyrhizobium* bacteria can consume between 4 and 11% of carbohydrates fixed through photosynthesis and, therefore, increase plant sink capacity and stimulate legume growth under e[CO₂] avoiding C sink limitation (Ainsworth et al., 2004). On the other hand, BNF and carbohydrate consumption by the nodule is influenced in part by the strain of *Bradyrhizobium* (Kaschuk et al., 2009; Sanz-Sáez et al., 2015). Besides, the microbial population structure in the rhizosphere has been shown to be altered by e[CO₂] (Wang et al., 2017). Therefore, there is great interest in the isolation and selection for *Bradyrhizobium* strains adapted to future environments, such as e[CO₂], which hypothetically could be more N₂-fixation efficient ones compared with unselected or native strains. This hypothesis is reinforced by some studies in alfalfa (Bertrand et al., 2007; Sanz-Sáez et al., 2012) and soybean (Bertrand et al., 2007, 2011; Prévost et al., 2010; Sanz-Sáez et al., 2015) which showed that selected strains could improve legume productivity in response to e[CO₂] by fixing more N₂ and hence consuming more C.

Sugawara and Sadowsky (2013) studied different native *B. japonicum* strains from soybean nodules of plants grown and isolated at ambient [CO₂] (a[CO₂]) (390 ppm, strain SFJ4-24) and e[CO₂] (550 ppm, strain SFJ14-36), under fully open-field conditions at a FACE site at the University of Illinois at Urbana-Champaign. They observed that the strain SFJ14-36, isolated at e[CO₂], significantly overexpressed genes encoding for N₂-fixation and nodulation in comparison with the strain

isolated at a[CO₂] and the control strain (USDA 110). More recently, Sanz-Sáez et al. (2019) tested if the same strain isolated under e[CO₂] conditions (SFJ14-36) showed higher BNF and nodule number at e[CO₂] compared with plants inoculated with USDA110 as it was suggested by the expression profiles published by Sugawara and Sadowsky (2013); however, no statistical differences were observed between plants inoculated with different strains when grown at e[CO₂]. Therefore, the overexpression of genes observed by Sugawara and Sadowsky (2013) were not matched by a higher BNF. In addition, when the plants were grown at a[CO₂], those inoculated with the strain isolated at e[CO₂] showed lower plant fitness in comparison with USDA110. The authors hypothesized that the strains isolated at e[CO₂] may be attracted only by root exudates produced at e[CO₂] or that at a[CO₂] there is some metabolic restrictions in the nodules that reduce the fitness of the symbiotic relationship.

With the objective of better understanding the C and N metabolism interaction in the plants inoculated with different *B. japonicum* strains and grown under e[CO₂] conditions, that favor yield under climate change conditions, it is needed to study why some rhizobium strains respond better to e[CO₂] than others, and how this affects plant fitness.

Together and closely related with these physiological responses, plant metabolome is also perturbed under e[CO₂] (Aranjuelo et al., 2015; Tcherkez et al., 2020). The metabolome of the plant is the link between genotype and phenotype, allowing to study changes in gene expression in response to the environment (Saito and Matsuda, 2008). This makes metabolomic profiling an attractive tool for phenotyping, providing a comprehensive perspective of the environmental changes that influence plants (Obata and Fernie, 2012). In opposition to a traditional metabolic analysis, in which the researcher focuses on a specific class of metabolites related with a metabolic route, the simultaneous profiling of metabolites from biosynthetically unrelated pathways has been demonstrated to increase our understanding of the molecular mechanism that underlies plant response to different stresses (Li et al., 2015). While previous studies have explored the *Rhizobium*-legume specificity and their physiological response to e[CO₂] (Das et al., 2017; Rabara et al., 2017), to our knowledge, no previous works have analyzed the metabolic profile of soybean nodules and leaves inoculated with different *B. japonicum* strains grown under e[CO₂] conditions.

The aim of this work was to elucidate the metabolic features involved in *Bradyrhizobium*-soybean specificity under contrasting CO₂ conditions. With this purpose, metabolic profiling analysis were carried out under two contrasting levels of CO₂ using the same three *B. japonicum* strains isolated at different [CO₂] (Sugawara and Sadowsky, 2013) and whose physiologic and photosynthetic parameters were studied by Sanz-Sáez et al. (2019).

MATERIALS AND METHODS

Plant and Bacterial Material

For this study, the same *B. japonicum* strains isolated by Sugawara and Sadowsky (2013) were used: SFJ4-24 (serogroup 123) was

a strain isolated from nodules of soybean grown at a[CO₂] (390 ppm of CO₂), meanwhile SFJ14-36 (serogroup 38) was a strain isolated from soybean nodules grown at e[CO₂] (550 ppm of CO₂). As a control, we used USDA110 strain because it has demonstrated high soybean performance at ambient and elevated [CO₂] at SoyFACE facility at the University of Illinois at Urbana-Champaign² (Sanz-Sáez et al., 2015). Soybean cultivar (*Glycine max* cv. 93B15; Pioneer Hi-Bred) was used in the same way as being used by Sugawara and Sadowsky (2013) in order to avoid problems of compatibility between the soybean cultivar and the *Bradyrhizobium japonicum* strains. These strains were provided by Prof. Michael Sadowsky (University of Minnesota; SFJ4-24, and SFJ14-36) and by USDA-ARS Rhizobium Germplasm Resource Collection in Beltsville, MD (USDA110). The different *B. japonicum* cultures were made exactly as explained in Sanz-Sáez et al. (2019).

Plant Growth Conditions, Treatments, and Sampling

Soybean seeds were surface sterilized with sodium hypochlorite (1%) for 10 min and rinsed with sterile water until the smell of bleach disappeared. For the seed inoculation, 200 ml of medium liquid culture containing $\approx 5 \times 10^9$ cell ml⁻¹ of each individual *B. japonicum* strain was centrifuged for 15 min at $5,100 \times g$ to separate the bacteria from the media. The pellet containing the bacteria was resuspended in 2 ml of sterile deionized water containing 2% polyvinylpyrrolidone (PVPP) reaching a concentration of $\approx 10^{11}$ cel ml⁻¹. Then 200 seeds were placed in a 500-ml sterile beaker containing 2 ml of concentrated inoculum on a rotatory shaker overnight, and later immediately planted. For the liquid inoculation, 1 L of liquid culture containing $\approx 5 \times 10^9$ cell ml⁻¹ was centrifuged and resuspended as above to a final concentration of 10^8 cell ml⁻¹.

Five inoculated soybean seeds were planted in 10 L pots containing a mixture of peat moss, perlite, and vermiculite of 1:1:1 v/v/v that was previously sterilized as described in Sanz-Sáez et al. (2015). One week after emergence, plants were thinned to one plant. After plants emerged from the pot, they were inoculated three times coinciding with 2, 9, and 16 days after emergence (DAE) with the liquid inoculum from each *B. japonicum* strain (USDA110, SFJ4-24, and SFJ14-36). All plants were watered alternatively with Evans N-free solution and distilled water to avoid salt accumulation (Moore, 1974). Soybean plants were grown in two growth chambers (Phytotron Service, SGIker) at the University of the Basque Country (UPV/EHU), from the beginning of the experiment, one maintained at a[CO₂] (≈ 400 ppm CO₂), and other maintained at e[CO₂] (700 ppm CO₂). Both chambers were maintained at 60/70% day/night relative humidity and 25/22°C day/night temperature, and a photosynthetic photon flux density (PPFD) of 1,200 $\mu\text{mol m}^{-2} \text{s}^{-1}$ from 7:00 to 22:00 h, until developmental stage V5 (Fehr et al., 1971), when the day length was decreased by 2 h to induce flowering. Every 2 weeks, plants and CO₂ treatments were rotated among and within chambers in order to reduce potential chamber effects. Harvest was carried out when plants

reached full flowering developmental stage (R2). This stage was selected because flowering is the period when N₂ fixation is supposed to be in its peak, and nodules have not started to senesce (Rogers et al., 2006). At this moment, six plants per inoculation and CO₂ treatment were harvested and separated into organ samples; leaves, stems, roots, and nodules. Each organ sample was oven dried at 65°C for at least 72 h and then weighed. The data is presented as weight of each organ separately (g of dry weight plant⁻¹), and stacked by total dry weight. For metabolic analysis, samples of leaves and nodules from three plants were collected and immediately frozen in liquid nitrogen and stored at -80°C until analysis.

Nitrogen Isotopic Composition Analyses

Twenty-four hours before harvest, the underground zone was enriched with ¹⁵N₂, and after harvest, the labeled ¹⁵N incorporation [measured as stable ¹⁵N isotope composition ($\delta^{15}\text{N}$)] in nodule tissue was measured. This parameter has been recently used as a measure of nodule performance in soybean and other legumes (Soba et al., 2020a) showing that the higher the nodule $\delta^{15}\text{N}$ the greater the BNF. Three plants (there was one plant per pot) per inoculation and CO₂ treatment were labeled, while three plants were used as unlabeled controls and harvested at the same time as the labeled plants. The ¹⁵N₂ labeling was accomplished by injecting labeled gas into the root zone using handmade labeling pots following the procedure of Sanz-Sáez et al. (2015). Plants were grown in these pots for the duration of the experiment. On the night preceding the labeling experiment, the pots were sealed with plastic lids in order to avoid the escape of the labeled gas. To perform the ¹⁵N₂ labeling, 10% ¹⁵N₂-enriched gas was prepared in Supelco-Inert Foil Gas Sampling Bags (Sigma-Aldrich, St Louis, MO, United States) by mixing the ¹⁵N₂-labeled gas enriched at 99% with ambient air ($\delta^{15}\text{N}_2$ at 0‰). Two hundred milliliters of ¹⁵N₂ (10%) mixed gas was injected into the labeling pots using a gas syringe (SGE; Sigma-Aldrich) 2 and 4 h after the lights were turned on, coinciding with the period of greatest N₂-fixing activity (Molero et al., 2019). After 24 h, the plants were harvested and separated into nodules, roots, and leaves, then dried at 65°C for at least 72 h. The dried organs were weighed and ground to 1 mm particle size. The samples were analyzed in a Costech 4010 elemental analyzer coupled in continuous flow with a Thermo Fisher Delta V Advantage Isotope Ratio Mass Spectrometer (IRMS Thermo Scientific, Waltham, MA, United States). The ¹⁵N/¹⁴N ratio in soybean nodule, root, and leaf material was expressed in δ notation ($\delta^{15}\text{N}$) following the equation as described in Sanz-Sáez et al. (2019). The amount of ¹⁵N fixed during the day of the labeling experiment was calculated as ¹⁵N fixed in each organ following the equation, as described in Bei et al. (2013).

Estimation of Rate of RuBP Oxygenation From Gas Exchange Measurements

Photorespiratory rates were estimated as the rate of ribulose-1,5-bisphosphate (RuBP) oxygenation (v_o) derived from the measured rates of CO₂ uptake by the leaves according to

²<https://soyface.illinois.edu>

Sharkey (1988) and von Caemmerer (2000), as described in Noctor et al. (2002):

$$v_o = (A - R_d)/(1/O : C - 0.5),$$

where A is the measured rate of net CO₂ uptake, R_d is non-photorespiratory CO₂ release in the light (taken as 50% of the rate measured in the dark in each experiment) and $O:C$ is the ratio of RuBP oxygenation to carboxylation. The ratio $O:C$ was calculated as:

$$O : C = (1/S_{rel})(O_c/C_c)$$

where S_{rel} is the specificity factor of Rubisco (taken as 110; Keys, 1999), and C_c and O_c are the chloroplastic concentrations of CO₂ and O₂, respectively. O_c was assumed to be that of water in equilibrium with air at 20°C (276 μM) and C_c was derived from C_i by taking a CO₂ transfer conductance through the mesophyll (g_i) of 0.32 mol m⁻² s⁻¹ (Gillon and Yakir, 2000) and assuming that the rate of CO₂ uptake affects C_c relative to C_i as in Ruuska et al. (2000):

$$C_c = C_i - A/g_i$$

where C_c was converted to a molar concentration by applying a CO₂ solubility constant at 20°C of 0.0392 mol L⁻¹ (von Caemmerer, 2000). The gas exchange parameters A , R in the dark, and C_c were measured exactly as described in Sanz-Sáez et al. (2019).

Metabolic Analyses

Leaf and nodule samples (20 mg of powder from freeze-dried material) were ground in a mortar in liquid nitrogen, and then in 2 ml of 80% methanol, in which ribitol (100 μmol L⁻¹) was added as an internal standard. After centrifugation at 15,000 rpm for 15 min at 4°C, the supernatant was collected and centrifuged again. Then, the supernatants were spin-dried under vacuum and stored at -80°C until analysis. Relative metabolite content was determined by gas chromatography coupled to time-of-flight mass spectrometry (GC-MS) using a LECO Pegasus III coupled to an Agilent 6890N GC system. Sample derivatization and GC-MS analyses were carried out as described in Aranjuelo et al. (2015). Peak identity was established by comparison of the fragmentation pattern with MS-available databases (NIST). The integration of peaks was performed using the LECO Pegasus software and the automated peak integration was verified manually for each compound in all analyses. The quantification was normalized to dry weight (DW) to avoid any discrepancy due to changes in relative water content.

Statistical Analysis

Statistical analyses were performed with IBM SPSS Statistics for Windows, Version 20.0 (IBM Co., Armonk, NY, United States). Differences among the three *Bradyrhizobium* strains and the two CO₂ levels were evaluated by two-way analyses of variance (ANOVA), with the strain and CO₂ as fixed factors. All data were tested for normality (Kolmogorov-Smirnov test) and homogeneity of variances (Levene's test). For ANOVA analysis,

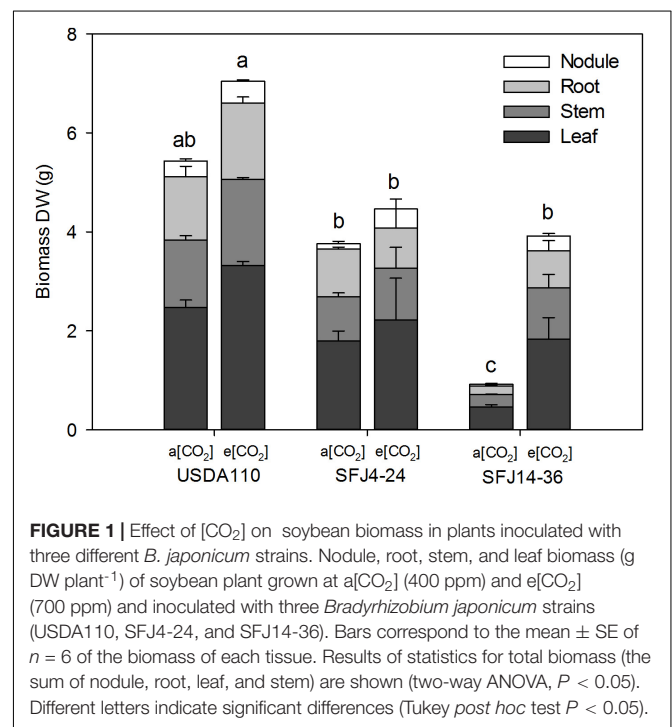
the results were considered to be significant when $p < 0.05$. In order to reduce the multivariate data complexity and identify patterns between samples, principal component analysis (PCA) was performed for nodules and leaves and the 121 metabolites were taken into account. Heat map and PCA for the two organs were conducted using XLSTAT 2008 (Addinsoft, Paris, France) software. Heat maps were done independently for leaves and nodules with metabolites showing significant differences between *B. japonicum* strain, [CO₂], and/or interactions in each tissue. Clustering was based on the Pearson's correlation coefficients among the metabolites. In this manuscript, the red color is proportional to a lower concentration; conversely, the intensity of the green color is proportional to higher concentration values.

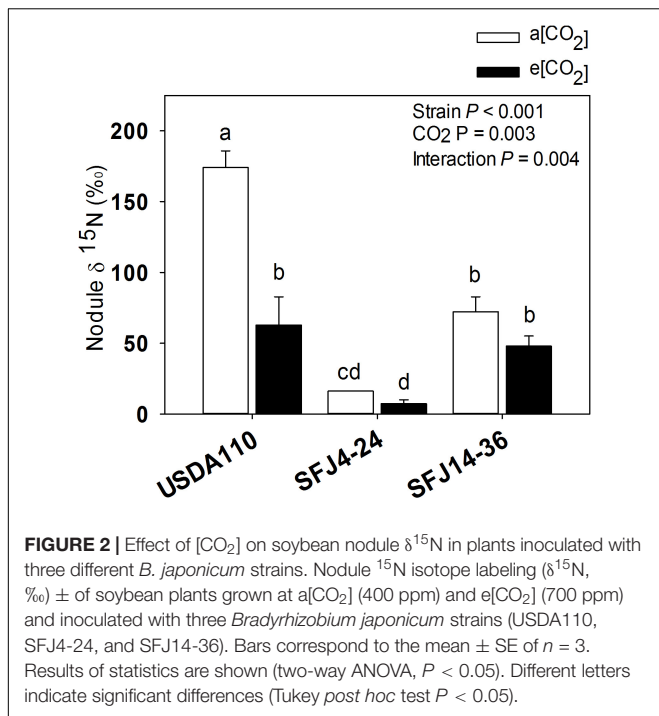
RESULTS

Biomass and Physiologic Measurements

At the R2 stage, strain, CO₂ level, and their interaction were found to have a significant effect on biomass of leaves, stems, and roots (data not shown). In contrast, nodule weight was only significantly affected by CO₂ level. Elevated [CO₂] significantly increased total biomass in all studied organs for the plants inoculated with *B. japonicum* strain isolated at e[CO₂] (SFJ14-36) but not for the reference strain (USDA110) or in the strain isolated at a[CO₂] (SFJ4-24) (Figure 1). However, the final value at e[CO₂] of SFJ14-36 strain was similar to the SFJ4-24 strain. Plants inoculated with USDA110, the reference strain, showed the highest values at both CO₂ concentrations (Figure 1).

For nodule ¹⁵N isotope labeling (δ¹⁵N), greatest value was found in nodules of plants inoculated with USDA110 strain





grown at a[CO₂] which was significantly higher than plants grown at e[CO₂] (Figure 2). Interestingly, nodules of SFJ4-24 and SFJ14-36 did not show significant differences between CO₂ levels, and the $\delta^{15}\text{N}$ was significantly greater in nodules of SFJ14-36 when compared with SFJ4-24 (Figure 2). For a more holistic vision, we calculated the amount of $\delta^{15}\text{N}$ per organ (mg ^{15}N organ⁻¹) (Figure 3). The results showed a clear significant effect of CO₂ for strain SFJ14-36 in the three studied tissues (leaf, root, and nodule) in contrast with USDA110 and SFJ4-24 strains.

The estimated rate of photorespiration (v_o) from gas exchange measures showed significant differences between CO₂ levels. Whereas both SFJ4-24 and SFJ14-36 showed a significant decrease of photorespiration under e[CO₂], USDA 110 did not (Figure 4).

Metabolite Patterns

Metabolite profiling was performed by gas chromatography coupled with time-of-flight mass spectrometry (GC-MS), and 121 different metabolites were identified by reference to their MS data. These metabolites were classified in eight chemical groups (organic acids, amino acids, sugars, fatty acids, polyols, nucleotides, secondary metabolites, and others), and their relative abundance is shown in Figure S1. The statistical analysis (PCA and heat map hierarchical clustering) of leaf and nodule metabolomic profile indicates that a clear differentiation between organs could be done (Figures 5, 6). While in the nodule metabolome, *B. japonicum* strain was found to have the main effect, with little impact of CO₂ level (except for SFJ14-36) (Figure 5A), in leaf metabolome, CO₂ level had the main effect (Figure 5C). Nodule PCA analysis revealed that the two principal components explained 73.5% of total variation between strains

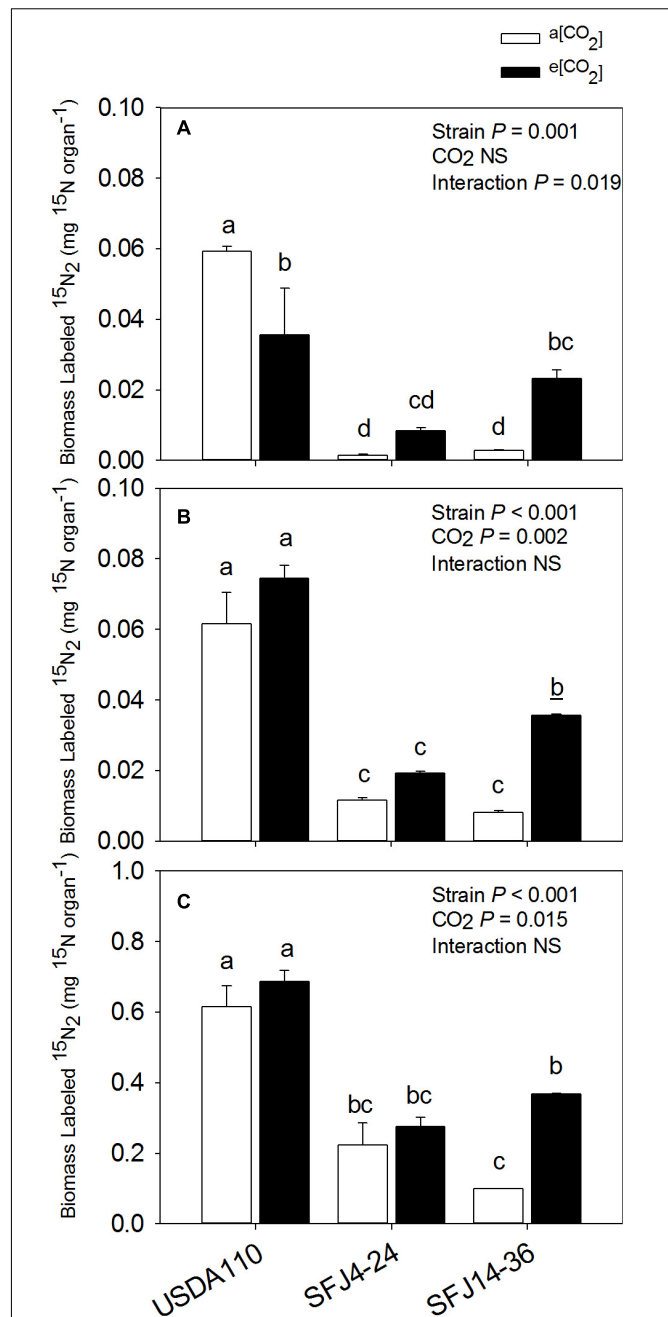


FIGURE 3 | Effect of [CO₂] on biomass labeled ^{15}N in three organs of soybean plants inoculated with three different *B. japonicum* strains. Biomass labeled ^{15}N (mg ^{15}N organ⁻¹) in (A) nodules, (B) roots, and (C) leaves of soybean grown at a[CO₂] (400 ppm) and e[CO₂] (700 ppm) and inoculated with three *Bradyrhizobium japonicum* strains (USDA110, SFJ4-24, and SFJ14-36). Bars correspond to the mean $\pm$ SE of $n = 3$. Results of statistics are shown (two-way ANOVA, $P < 0.05$). Different letters indicate significant differences (Tukey post hoc test $P < 0.05$).

and CO₂ levels (Figure 5A). The six combinations of strains and CO₂ levels were grouped by strains without differences between treatments except for the SFJ14-36, which showed significant differences between CO₂ levels. The loading plot

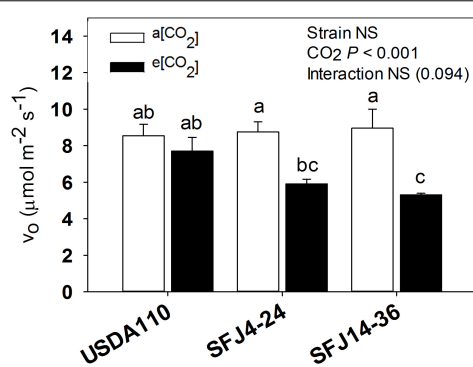


FIGURE 4 | Photorespiratory estimations from gas exchange measures. Estimated rate of RuBP Oxygenation (v_o) in soybean leaves grown at a[CO₂] (400 ppm) and e[CO₂] (700 ppm) and inoculated with three *Bradyrhizobium japonicum* strains (USDA110, SFJ4-24, and SFJ14-36). Bars correspond to the mean $\pm$ SE of $n = 6$. Results of statistics are shown (two-way ANOVA, $P < 0.05$). Different letters indicate significant differences (Tukey post hoc test $P < 0.05$).

revealed that the discrimination of samples by PC1 (48.2% of the total variance) was, in part due to sugars such as maltose and trehalose, whereas amino acids like lysine, methionine, threonine, leucine, glycine, and glutamine contributed to the separation of samples by PC2 (25.3% of the total variance). It is interesting to note that Krebs cycle related metabolites are grouped in the lower-left quadrant of the loading plot (Figure 5B). On the other hand, leaf PCA analysis showed a more unrelated distribution of strains and treatments, but a clear separation between *Bradyrhizobium* strains grown at ambient and elevated [CO₂] could be traced (Figure 5C). The two principal components explained 60.6% of total variation between strains and CO₂ levels. No distinctive pattern in metabolites were detected to explain this variation; nevertheless, important metabolites such as D-glucose-6-phosphate, glycine, serine, the polyamines putrescine and spermidine, and shikimic acid were important contributors to the separation of samples by PC2 (Figure 5D).

Of the 121 analyzed metabolites, 64 were significantly affected by CO₂, strain or their interaction in nodules and 54 in leaves (Figure 6). In order to provide a better understanding of this variation, metabolite profiling representation (heat map) and hierarchical clustering analysis was undertaken separately for each organ (Figure 6). In nodules, the hierarchical clustering of the six combinations (two levels of CO₂ and three *Bradyrhizobium* strains) formed three clusters, one for each strain with little differences between CO₂ treatments, except for the strain SFJ14-36 where differences between CO₂ levels were bigger (Figure 6A), as we have seen in the PCA analysis (Figure 5A). On the other hand, the hierarchical clustering of the 64 significant metabolites allowed the grouping of metabolites into two major clusters. Cluster 1 was mostly made of metabolites in higher concentration in plants inoculated with USDA110 strain as compared with other strains and could be subdivided in two subclusters; 1A: sugars (glycolysis pathway: sucrose, glucose, fructose, mannose, etc.) and 1B: N-related compounds such as

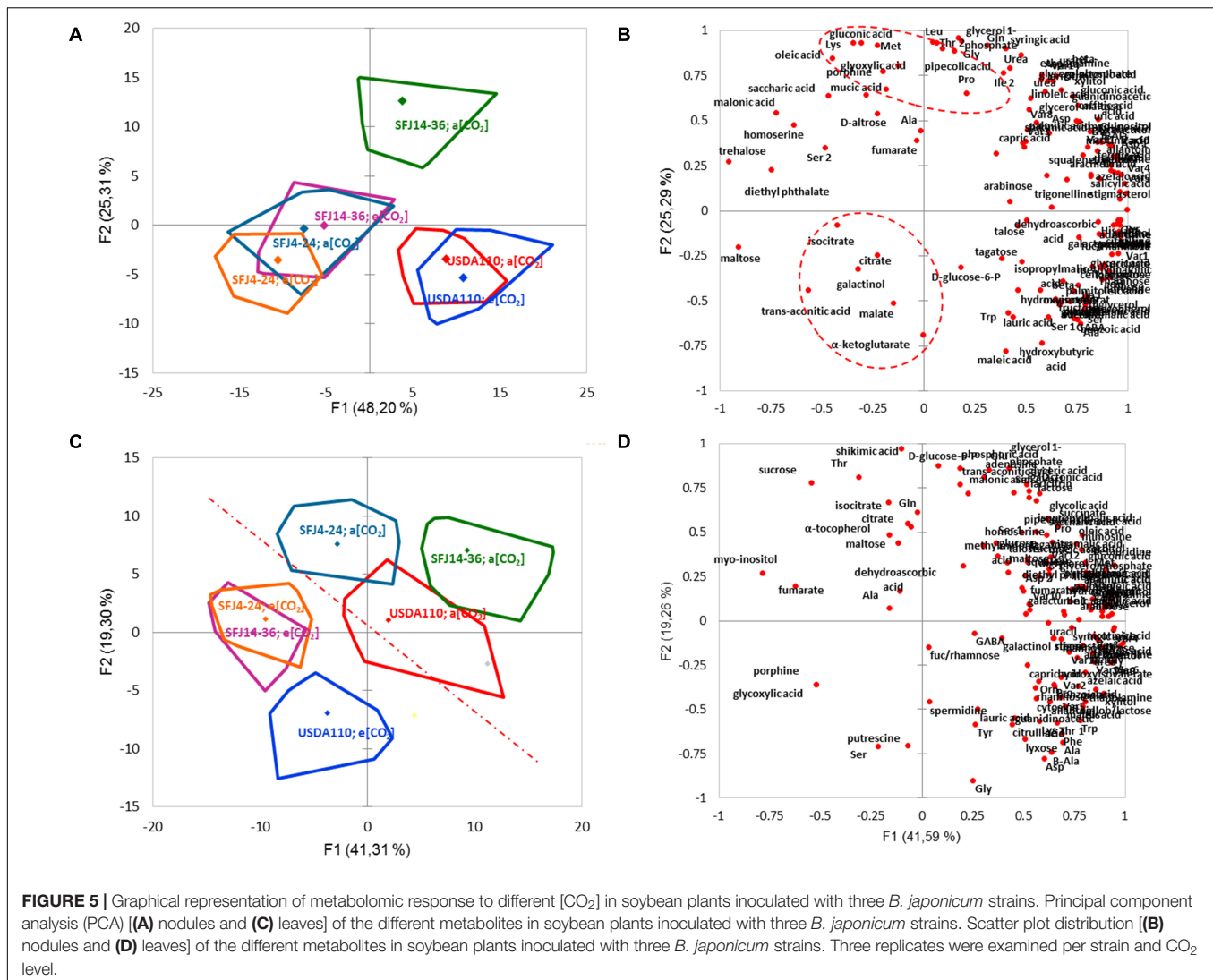
ureides and urea cycle metabolites (allantoin, uric acid, ornithine, aspartic acid, citrulline, urea, etc.). On the contrary, cluster 2 was formed of metabolites in lower concentration in USDA110 strain and mostly included Krebs cycle metabolites (fumarate, malate, citrate, isocitrate, α -ketoglutarate, etc.) (Figure 6A).

Contrary to nodules, in leaves, the hierarchical clustering of CO₂ levels and *Bradyrhizobium* strain combinations was grouped by CO₂ level in two clusters (Figure 6B). Cluster 1 formed by plants grown at e[CO₂] and cluster 2 by plants grown at a[CO₂]. In general, the metabolites were accumulated in greater quantity at a[CO₂]. The hierarchical clustering of the 54 significant metabolites showed also two different clusters. Cluster 1 was formed by two subclusters mainly composed by metabolites related with photorespiration (glycolate, glycine, serine, glycerate) and Krebs cycle (glutamate, citrate, succinate, etc.) and sugar metabolites (fructose, glucose, tagatose, talose, sorbitol, etc.). On the other hand, cluster 2 showed a greater accumulation of metabolites in plants inoculated with SFJ14-36 and lower in SFJ4-24 strain at a[CO₂]. This cluster was mainly composed of lipid-related metabolites (free fatty acids and sterols). Figures 7, 8 represent metabolites that were significantly affected by *B. japonicum* strain, [CO₂], or their interaction either on soybean nodule and leaf.

DISCUSSION

Physiologic Parameters

Our study showed that [CO₂] effect on soybean biomass was tightly dependent on the *B. japonicum* strain analyzed. While in SFJ4-24 and USDA110 changes between CO₂ levels were not significant, soybeans inoculated with SFJ14-36 strain showed an increase of 322% in their total biomass under e[CO₂] (Figure 1). Both, N₂ fixation and photosynthetic performance were involved in such different responses. When we analyze $\delta^{15}\text{N}$ in nodules of SFJ14-36, used as a measure of BNF-specific rate, no significant differences were found. However, significant differences, in nodule, root, and leaf, were found when the amount of total biomass labeled ¹⁵N₂ in this strain was calculated relating with the total N fixed by BNF and their translocation to other tissues (Figures 2, 3). The greater ¹⁵N content under e[CO₂] in SFJ14-36 compared with a[CO₂] was in contrast with the lack of significant differences between CO₂ level for the other two strains in roots and leaves. This difference may be due to a better translocation of fixed N to the aboveground tissues as shown by nodule metabolite data (greater accumulation of aspartic acid (Asp) and allantoin in nodules of plants inoculated with SFJ14-36 at a[CO₂]) (Figure 7). Briefly, in the plants inoculated with the strain isolated at e[CO₂] (SFJ14-36), even with a similar BNF-specific rate at both CO₂ levels, the greater nodule biomass at e[CO₂] leads to a greater amount of fixed N and this together with a better translocation to the aerial parts, allows a better biomass growth under e[CO₂]. However, nodulation in soybean that were inoculated with SFJ14-36 strain seems to be restricted or delayed when grown at a[CO₂] as previously showed by Sanz-S  ez et al. (2019) compromising the whole plant fitness due to a lower N availability. None of these



facts seems to occur in the model strain (USDA110) or in the one isolated at a[CO₂] (SFJ4-24).

In addition to BNF, gas exchange measures also contributed to explain the different responses of plants inoculated with different *B. japonicum* strains to e[CO₂]. As previously showed by Sanz-Sáez et al. (2019), photosynthesis was reduced when plants grew at a[CO₂], but this reduction was significantly greater in SFJ14-36 strain when compared with the other two. One of the most important parameters affecting C fixation through photosynthesis is photorespiration. Zhu et al. (2008) estimated a reduction of gross C3 photosynthesis efficiency by 48% at current [CO₂] and temperature conditions, mainly associated with the consumption of fixed C and energy in the glycolate recycling process. As observed in **Figure 4**, both SFJ4-24 and SFJ14-36 reduced the estimated v_o (32.5 and 40.7%, respectively) when grown at e[CO₂]; in contrast, values in USDA110 did not change. Therefore, in SFJ4-24 and SFJ14-36, the increase in the photosynthetic rate previously observed under e[CO₂] could be in part due to a reduction in the photorespiratory rate, especially

in SFJ14-36 as validated below with the metabolic data that shows a decrease in intermediates of the glycolate cycle (glycolic acid, glycine, and serine among others) (**Figure 6**) in leaves grown under e[CO₂].

The decreased nodulation and reduction of the symbiotic fitness of the plants inoculated with SFJ-14-36 and grown at a[CO₂] could be due to changes in the quantity and/or quality of phenolic substances excreted by the roots (Sugawara and Sadowsky, 2013; Wang et al., 2017). At e[CO₂], roots excrete more and different phenolic compounds that attract rhizobium species (Sugawara and Sadowsky, 2013). As SFJ-14-36 was isolated under e[CO₂], an effective nodulation may have been dependent of phenolic substances only emitted under these circumstances whereas at a[CO₂], these compounds may have changed (Sugawara and Sadowsky, 2013), reducing the nodule formation and the amount of N that was fixed and fed to the plant. Another reason for the lower nodulation of plants inoculated with SFJ14-36 strain at ambient [CO₂] could be the lack of synergy between *Bradyrhizobium* strain and soybean

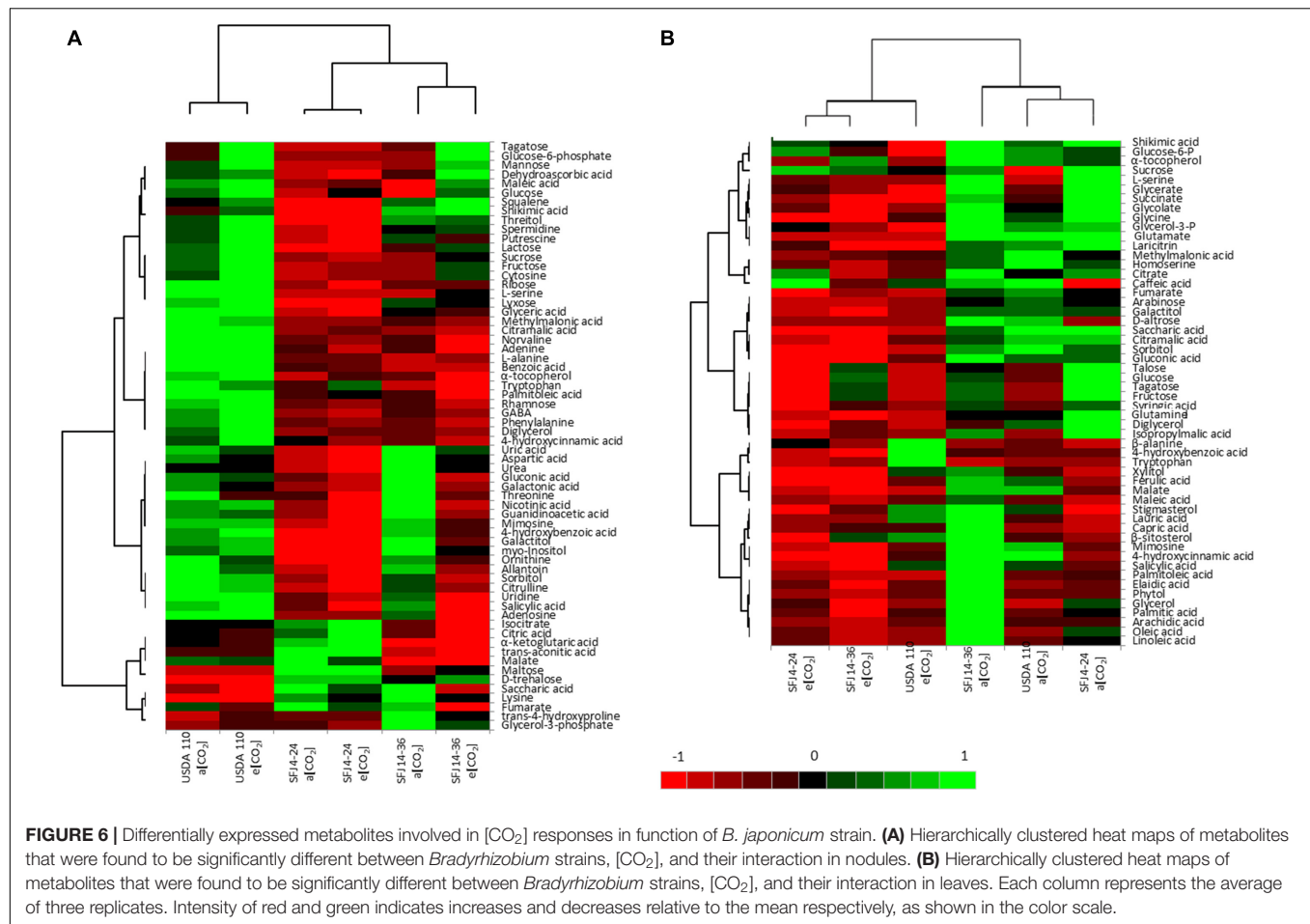


FIGURE 6 | Differentially expressed metabolites involved in [CO₂] responses in function of *B. japonicum* strain. **(A)** Hierarchically clustered heat maps of metabolites that were found to be significantly different between *Bradyrhizobium* strains, [CO₂], and their interaction in nodules. **(B)** Hierarchically clustered heat maps of metabolites that were found to be significantly different between *Bradyrhizobium* strains, [CO₂], and their interaction in leaves. Each column represents the average of three replicates. Intensity of red and green indicates increases and decreases relative to the mean respectively, as shown in the color scale.

cultivar. This is not likely because this study used the same soybean cultivar (cv. 93B15; Pioneer Hi-Bred) used in Sugawara and Sadowsky (2013) when the strain was isolated in e[CO₂] at the SoyFACE facility in Illinois.

Together with physiologic parameters, plant metabolomic can give us valuable information about the status of the soybean-*Bradyrhizobia* symbiosis under current and future [CO₂]. As shown, [CO₂] effect on physiology was dependent on the *B. japonicum* strain and these differential responses are expected to be reflected in the accumulation of specific metabolites. Therefore, metabolomics are a valuable tool to decode the physiologic differences between plants inoculated with different strains and CO₂ levels, helping us understand why the plants symbiosis with the strain that was isolated at e[CO₂] does not perform well at a[CO₂] or why USDA110 is more effective than the other two strains at a[CO₂].

Carbon and Nitrogen Metabolism

Nitrogen fixation in legume nodules is fueled by C fixed through photosynthesis. The current experiment showed that the amount of sucrose in nodules was affected by CO₂ level and by *Bradyrhizobium* strain. Plants inoculated with USDA110 showed the highest sucrose levels (especially at e[CO₂]); on the contrary, SFJ4-24 showed almost no sucrose in both ambient and

elevated [CO₂] when compared with USDA110 (Figure 7). This strain-specific sucrose content was in opposition with the lack of statistically photosynthetic differences observed by Sanz-Sáez et al. (2019) between USDA110 and SFJ4-24. Such results point to the fact that other reasons may exist behind this contrasting sucrose content between plants inoculated with the two strains. More specifically, obtained data would reveal that a greater use of sucrose in the leaf as observed in metabolites implied in glycolysis (glucose, fructose) in leaves of SFJ4-24 at a[CO₂] (Figure 8) which is in accordance of the higher respiration rates observed in leaves of plants inoculated with this strain (Sanz-Sáez et al., 2019) which could limit its export to nodules. On the other hand, nodules of plants inoculated with SFJ4-36 showed greater amount of sucrose under e[CO₂] than under a[CO₂] probably due to a better photosynthetic rate at e[CO₂]. Also, metabolites involved in glycolysis (fructose, glucose, and glucose-6-phosphate) were significant affected by both, *Bradyrhizobium* strain and CO₂ level (Figure 7). These observations suggest a poor supply and/or rapid consumption of C for respiration and C skeletons in nodules of plants inoculated with SFJ4-24 and SFJ4-36 under a[CO₂] (Aranjuelo et al., 2014). Surprisingly, when we observed the content of malate, the main dicarboxylic acid formed after glycolysis (Lodwig and Poole, 2003), and other organic acids involved in Krebs cycle, we saw that they were

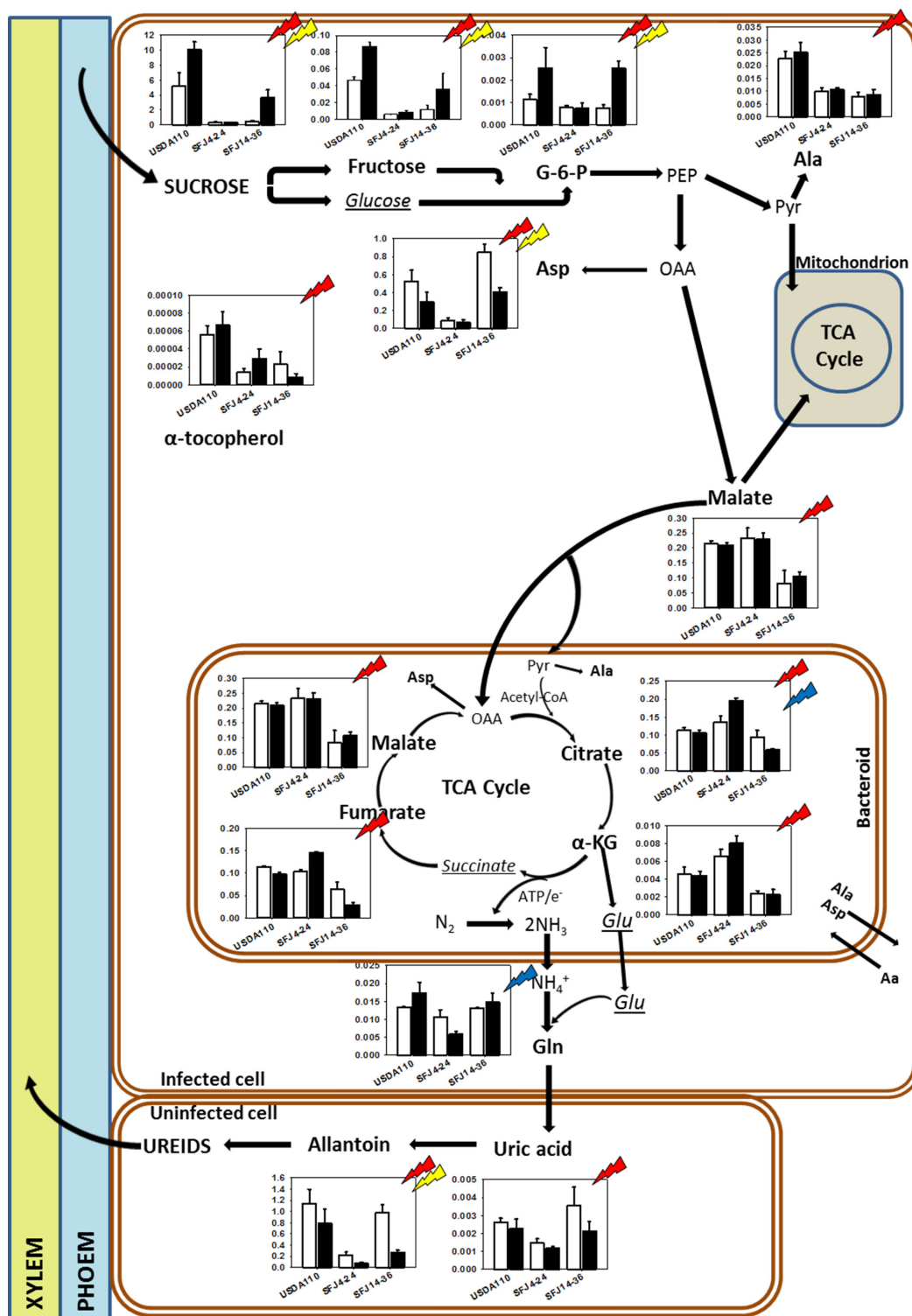


FIGURE 7 | Effects of *Bradyrhizobium japonicum* strain and [CO₂] on soybean nodule metabolism. Bar charts showing the relative abundance of metabolites in ambient [CO₂] (white) and elevated [CO₂] (black) in soybean plants inoculated with three different *Bradyrhizobium japonicum* strains. Red, yellow, and blue lightning signs indicate the significant effect of strain, CO₂, and their interaction, respectively. Metabolites in bold indicate metabolites that were found to have significant effect of strain, CO₂, and/or their interaction; italic metabolites indicate metabolites analyzed that were not found to have significant effect of strain, CO₂, and their interaction, and not bold neither italic indicates metabolites not analyzed.

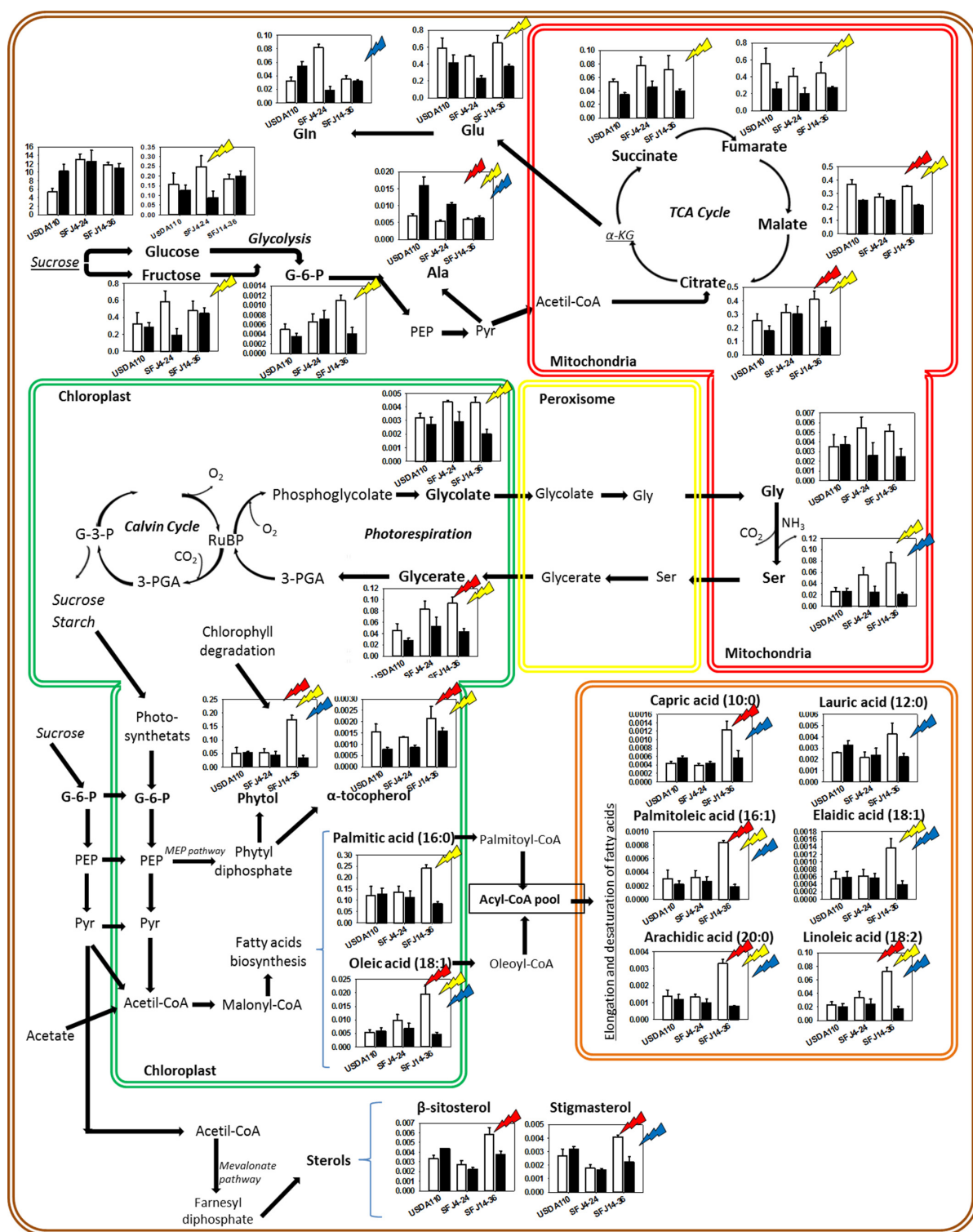


FIGURE 8 | Effects of *Bradyrhizobium japonicum* strain and [CO₂] on soybean leaf metabolism. Bar charts showing the relative abundance of metabolites in ambient [CO₂] (white) and elevated [CO₂] (black) in soybean plants inoculated with three different *Bradyrhizobium japonicum* strains. Red, yellow, and blue lightning signs indicate the significant effect of strain, CO₂, and their interaction, respectively. Bold metabolites indicate metabolites analyzed that were found to have significant effect of strain, CO₂ and/or their interaction; italic metabolites indicate metabolites analyzed that were not found to have significant effect of strain, CO₂, and their interaction, and not bold neither italic indicates metabolites not analyzed.

affected by *Bradyrhizobium* strain but not by CO₂ level content. On the other hand, malate content in SFJ4-24 nodules was similar to USDA110 and higher than in SFJ14-36. These two observations could suggest that in nodules of plants inoculated with SFJ4-24, much of the carbon glycolyzed was derived to maintain nodule energy supply through malate production. Meanwhile in USDA110, although malate content is similar to that observed in SFJ4-24, the preceding substrates (sucrose, fructose, and G-6-P) were at much higher concentrations indicating that part of this C was diverted to the production of other compounds, such as phenolic compounds that were in higher concentration than in the other strains (**Figure 6**), suggesting an active carbon metabolism in soybeans inoculated with USDA110. This depletion in Krebs intermediates could be replenished through the GABA-shunt allowing the synthesis of succinate that can enter the Krebs cycle (Saiz-Fernández et al., 2020) maintaining high its activity and the levels of malate observed with this strain. This role of the GABA-shunt would be confirmed by the increase of the levels of GABA and polyamines (spermidine and spermine) observed in USDA110 under e[CO₂] (**Figure 6**) as it was proposed by Saiz-Fernández et al. (2020) in corn plants. The low malate content in plants inoculated with SFJ14-36 was also showing this C diversion in plants infected by this strain. A significant portion of carbon entering in the Krebs cycle in bacteroids is diverted, *via* anaplerotic reactions, into some amino acids: alanine (Ala), through pyruvate (Igamberdiev and Kleczkowski, 2018); aspartic acid (Asp), through oxaloacetate (OAA) (Melzer and O'Leary, 1991); and glutamic acid (Glu), through α -ketoglutarate (α -KG) (Salminen and Streeter, 1987, 1992; **Figure 7**).

Aspartic acid and Glu were by far the two most abundant amino acids in nodules; whereas by contrast, the content of Ala was low (**Figure 7**). Aspartic acid is a common amino acid and, in nodulated soybean, has been shown to be a form of N-transport, especially under N-stress conditions (Lima and Sodek, 2003). In our work, the highest content of Asp was found in nodules of plants inoculated with SFJ14-36 at a[CO₂], in special when compared with SFJ4-24 strain. However, if a great part of OAA is transaminated to Asp, the Krebs cycle will be shut down because malate and citrate cannot be synthesized (Hayes, 2001), explaining the low content of Krebs cycle metabolites observed in SFJ14-36 strain. Therefore, in nodules, the observed differences in *Bradyrhizobium* strains between glycolysis metabolites and organic acids involved in Krebs cycle may be due to a diversion of C to Asp production *via* anaplerotic reaction; and as a result, lesser organic acids were involved in energy production for N₂ fixation in plants inoculated with SFJ14-36 strain. In the case of USDA 110, the diversion of C from Krebs cycle to phenolic compounds could be replenished through the GABA-shunt.

Although soybean is a ureides exporter, previous studies have revealed that plants with impaired N₂ fixation have shown an enhancement of Asp transport through xylem sap, as a precursor of the products of NH₄⁺ assimilation (Puiatti and Sodek, 1999; Lima and Sodek, 2003). Additionally, when photoassimilates transport to nodules is restricted, as it seems to be the case in plants inoculated with SFJ14-36 strain grown at a[CO₂], plants may use C, N, and energy in a more efficient way, through the

carboxylation of Phosphoenolpyruvate (PEP) into OAA, required for Asp synthesis, instead entering Krebs cycle through malate (Fischinger and Schulze, 2010). This mechanism has been shown mainly in indeterminate nodules (pea); however, Silvente et al. (2003) showed that in ureides exporter nodules (bean), aspartate aminotransferase (AAT) may be acting as an important switching enzyme in driving the metabolic flow of fixed N through amide or ureides synthesis, helping to explain the significant higher concentration of Asp and Krebs cycle metabolites in nodules of plants inoculated with SFJ14-36 strain at a[CO₂] (**Figure 7**). The use of Asp for N assimilation is connected with the carboxylation of PEP, while any use of C through Krebs cycle for N assimilation is connected with a preceding decarboxylation of pyruvate. This means that, while at first the nodule is fixing CO₂, later is losing it. In this sense, nodule CO₂ fixation may represent a C-saving mechanism particularly in occasions of limited C availability (Fischinger and Schulze, 2010; Fischinger et al., 2010), such as the ones infected with SFJ14-36 strain. This Asp produced may be exported through xylem or used for glutamic acid formation and consequently the production of glutamine by glutamine synthetase (GS) and ureides (Lima and Sodek, 2003). The high levels of uric acid and allantoin in nodules of plants inoculated with SFJ14-36 strain at a[CO₂] could support the idea of Asp as an intermediate metabolite in N assimilation under limited C supply to the nodule. However, more studies are necessary to proof this N assimilation route in determinate nodules. These results are in accordance with the ¹⁵N labeled data (**Figures 2, 3A**) which showed a good SFJ14-36 nodule performance (measured as nodule $\delta^{15}\text{N}$) at both CO₂ levels, similar to the one observed in the reference strain (USDA110) at e[CO₂], however, due to a deficient nodulation in SFJ14-36 at a[CO₂] the total amount of N₂ fixed by nodules was very low (**Figure 3A**).

On the other hand, the accumulation of ureides, uric acid and allantoin (Serraj et al., 2001; Ladrera et al., 2007), and Asp (King and Purcell, 2005) has been proposed in soybean nodules to take part in the modulation of the symbiotic activity, acting as an N-feedback mechanism. In our study, allantoin content, similar to Asp, was strongly affected by CO₂ level, especially in plants infected with SFJ14-36 strain, suggesting that the greater content of this N-transporting compound under a[CO₂] could be related with a decline in shoot N demand (Serraj et al., 1999); however, N-feedback effect by these compounds had not been observed since nodule performance (measured as $\delta^{15}\text{N}$) was not reduced at a[CO₂] (**Figure 2**). Additionally, the levels of organic acids implied in Krebs cycle (malate, fumarate, citrate, α -KG) were similar at those observed in plants with this strain grown at e[CO₂], suggesting an active nodule N-fixation but a poor N transport to aerial tissues in SFJ14-36 grown at a[CO₂]. All this data could imply that poor plant fitness observed in SFJ14-36 at a[CO₂] was at the end caused by a N stress. N deficiency was due to a poor nodule implantation, in terms of biomass, to an insufficient C import from leaves (low levels of sucrose), and to a poor N transport of nodule-fixed N that affects N status at whole plant level reducing C fixation through photosynthesis and so total biomass. Nevertheless, these nodules consumed all the sucrose from the aerial part (**Figure 7**) and they try to fix

N in a more efficient way through the carboxylation of PEP to produce Asp; however, more work is needed to confirm the last hypothesis.

Photorespiration Enhance at a[CO₂] Causes a Reorchestration on Krebs Cycle and Glutamic Acid Production in Leaves

Carbon dioxide and O₂ are competitive substrates for ribulose-1,5-bisphosphate carboxylase/oxygenase (Rubisco), and their ratio at the site of catalysis affects the rates of ribulose-1,5-bisphosphate (RuBP) carboxylation and oxygenation (Farquhar et al., 1980; Rachmilevitch et al., 2004). For this reason, increasing atmospheric [CO₂] is expected to promote photosynthesis (C reduction) over photorespiration (C oxidation) as showed in C3 plants (Lin and Wang, 2002; Woodward, 2002) and seen here with the RuBP oxygenation (v_o) estimation (Figure 4A). Our results showed higher concentration of leaf metabolites involved in photorespiratory pathway (glycolate, Gly, Ser, and glycerate) in plants infected with SFJ4-24 and SFJ14-36 grown at a[CO₂], although the difference between CO₂ levels was greater in SFJ14-36. Additionally, all four metabolites showed a similar trend in the combined response to *Bradyrhizobium* strain and CO₂ level as shown in the heat map where they were grouped in the same cluster (Figure 6B) indicating a clear relationship between them. These metabolic results were in accordance with the estimation of v_o , where significant reductions in v_o were found between CO₂ levels for plants inoculated with SFJ4-24 and SFJ14-36 strains but not for USDA110 (Figure 4A), and previous works with soybeans (Booker et al., 1997; Rogers et al., 2006; Ainsworth and Rogers, 2007).

As in the case of photorespiration, some studies had shown that under a[CO₂], respiration and therefore Krebs cycle was up-regulated when compared with plants grown at e[CO₂] (Tcherkez et al., 2008; Soba et al., 2019a,b). Sanz-Sáez et al. (2019) showed that leaves of soybean inoculated with SFJ14-36 showed a significant increase of respiration at a[CO₂] when compared with e[CO₂]; meanwhile, plants inoculated with USDA110 and SFJ14-36 did not show respiratory differences between CO₂ levels. Nevertheless, under a[CO₂], respiration has been suggested to be inhibited by the high levels of mitochondrial NADH, from photorespiration, and ATP/ADP, from photosynthesis (Padan et al., 2005). On the contrary, as commented before, higher Glu quantities are demanded with increased photorespiration rates and, therefore, an increase in 2-oxoglutarate coming from Krebs cycle is expected. Consequently, a complex respiratory homeostasis between two opposing forces: mitochondrial energy requirements and photorespiratory Glu demand is observed in leaves. Our data showed a general increase of dicarboxylic acids involved in Krebs cycle (fumarate, succinate, citrate, and malate) under a[CO₂] that was especially marked in the case of SFJ14-36 strain, which is in accordance with previous respiratory data showed by Sanz-Sáez et al. (2019) and with the enhanced Glu demand by photorespiration in SFJ14-36 under a[CO₂]. Interestingly, α -KG was not significantly

affected by CO₂ treatment in SFJ14-36 and may be due to the fact that is involved in the synthesis of Glu and Gln and, therefore, diverted at a[CO₂] to the production of these two amino acids.

In summary, the observed enhancement of photorespiration in plants inoculated with SFJ14-36 strain grown at a[CO₂] alters the leaf respiratory homeostasis between the downregulation caused by more NADH and the upregulation by more Glu demand. Our data suggest that at a[CO₂], plants inoculated with SFJ14-36 strain showed an upregulation of Krebs cycle to compensate the demand of C skeletons for Glu production. We hypothesize that the imbalance of energy between production (through photosynthesis) and consumption (photorespiration and respiration) observed in SFJ14-36 at a[CO₂] was compensated by a greater fatty acid synthesis as seen in the significant increase of all free fatty acids analyzed (Figures 6B, 8). This could also indirectly reflect a diversion of photoassimilates to the synthesis of organic acids produced by a lower sucrose export to the nodule especially at a[CO₂].

Soybean Inoculated With SFJ14-36 Strain Prematurely Induced Leaf Senescence When Grown at a [CO₂] Probably Caused by N Deficiency

In addition to leaf ontogenic senescence that occurs during the normal aging, prematurely induced senescence can occur when plants are subjected to abiotic/biotic stresses (Troncoso-Ponce et al., 2013). One of the first events that happen is chlorophyll degradation and, as a result, free phytol is produced which can be used as a biomarker of the rapid loss of chlorophyll associated with the degeneration of chloroplast under stress (Lim et al., 2007). In our case, free phytol content in plants inoculated with SFJ14-36 strain at a[CO₂] was five times greater compared with e[CO₂] and with the other inoculation treatments (Figure 8), suggesting an additional stress in this treatment, probably due to a poor supply of N by the nodules.

The resulting free phytol is highly toxic to proteins and membranes, so a large proportion of phytol is incorporated into α -tocopherol, fatty acid phytol esters, and triacylglycerol (Peisker et al., 1989; Ischebeck et al., 2006; Figure 8). α -Tocopherol is the most important lipophilic antioxidant in leaves (Munné-Bosch, 2007), protecting membrane lipids against lipid peroxidation. In our work, α -tocopherol content observed in leaves of SFJ14-36 plants at a[CO₂] was not significantly higher compared with e[CO₂] despite the greater phytol content observed at a[CO₂]. Two possible explanations could be: (1) a poor α -tocopherol synthesis from phytol, in opposition to previous works (Lippold et al., 2012; von Dorp et al., 2015; Mach, 2015) or (2) high tocopherol degradation that provides an excess in their synthesis. The last option could occur when the stress is too severe and consequently lipid peroxidation increases (Munné-Bosch, 2005) and is likely to happen in our case. In addition to α -tocopherol, salicylic acid (SA) increased by 6.3-fold at a[CO₂] in comparison with e[CO₂] in SFJ14-36 plants. Several investigations indicated that SA increases accumulation of phenolics (Kováčik et al., 2008) and enhances the oxidative stress

tolerance (Li et al., 2014) through stimulation of enzymatic and non-enzymatic antioxidant mechanism pathways (El-Esawi et al., 2017). In accordance to this, phenolic acids such as caffeic acid, ferulic acid, and coumaric acid were also upregulated at a[CO₂] in SFJ14-36 plants (**Figure 6B**). Phenolic acids have been reported to be antioxidants that are implied in the scavenging of free radicals (Ghasemzadeh and Ghasemzadeh, 2011). These results indicated that, the antioxidant system was activated due to a severe oxidative stress specifically in leaves of soybeans inoculated with SFJ14-36 strain at a[CO₂] but not in other treatments.

In plants, increased production of Reactive Oxygen Species and the accumulation of lipid peroxidation products have been associated with oxidation of membrane lipids and membrane catabolism during environmental stresses or senescence (Barclay and McKersie, 1994; Berjak and Pammenter, 2008). The amount of all the free fatty acid analyzed (capric, lauric, palmitic, palmitoleic, oleic, elaidic, arachidic, and linoleic acid) showed a significant increase at a[CO₂] when compared with e[CO₂] in plants inoculated with SFJ14-36 strain. However, in the other two strains, the values remained unaltered between CO₂ levels. During maturation, aging, and senescence, the catabolic enzymatic activities become activated, and free fatty acids have been found to be enhanced considerably (Mishra et al., 2006). Together with free fatty acids, sterols (β -sitosterol and stigmasterol) were enhanced at a[CO₂] only in SFJ14-36 plants, remaining unchanged in the other treatments. Sterols have also been found to be enhanced in senescing leaves (Duperon et al., 1984; Bouvier-Navé et al., 2010; Li et al., 2016) where they appear to participate in recycling these fatty acids released from senescing cell membranes to form sterol esters for subsequent transport to other tissues (Holmer et al., 1973; Chen et al., 2007).

All these metabolomic data suggest that in leaves of soybeans inoculated with SFJ14-36 strain grown at a[CO₂], chlorophyll degradation and, therefore, lower photosynthetic ability was likely to occur at the same time as thylakoid membrane degradations. This was previously proposed by Li et al. (2016) during leaf senescence, in our case, probably due to enhancement of oxidative stress and N deficiency. However, this happened only when the plants were grown at current CO₂ conditions not at e[CO₂], showing a soybean-strain specific interaction with atmospheric [CO₂]. As N is essential for synthesis of Rubisco and chlorophylls, in soybean plants inoculated with SFJ14-36 strain, insufficient N-fixation at a[CO₂] (**Figure 3C**) decreased leaf N concentration and, as a consequence, photosynthetic rates were decreased in this *B. japonicum* strains (Sanz-Sáez et al., 2019). In this respect, some studies have shown that a sufficient supply of N through BNF increases photosynthetic rates and delays leaf senescence in soybean (Abu-Shakra et al., 1978; Kaschuk et al., 2010). Therefore, observed induced leaf senescence in SFJ14-36 may be due to an insufficient supply of N from the BNF to the leaves as already observed in soybean (Egli et al., 1978). As stated before, the poor supply of N from nodules to leaves was not likely due to low nodule BNF efficiency (**Figure 2**) but probably to a deficient nodulation as observed by the significant reduction in nodule biomass in SFJ14-36 grown at a[CO₂] when compared with e[CO₂].

CONCLUSION

In this study, with the use of metabolomics and their interaction with physiological measures in soybean nodules and leaves, we revealed alterations in metabolites response to CO₂ fertilization in plants inoculated with different *Bradyrhizobium* strains. This analysis clearly demonstrated that the soybeans inoculated with the strain isolated at e[CO₂] (SFJ14-36) when grown at a[CO₂] suffers changes in metabolic pathways that affect negatively plant growth and development such as a restricted photoassimilate content (sucrose, glucose, fructose). We hypothesize that under these conditions in the nodule, a more efficient use of C and N happened through a carboxylation of PEP to produce Asp instead of decarboxylation of PEP to produce Krebs dicarboxylic acids. In this way, nodule CO₂ fixation may represent a C-saving mechanism and Asp can be used as N-exported compound or to produce ureides. At leaf level, plants inoculated with SFJ14-36 and grown at a[CO₂] showed a complete rearrangement of processes such as photorespiration and Krebs cycle. This metabolic change at a[CO₂] in plants inoculated with SFJ14-36 that were originally isolated at e[CO₂], was probably due to a poor nodulation caused by a change in plant root exudates between elevated and ambient [CO₂], affecting legume-bacteria interaction and, as a result, reducing N₂-fixation and affecting N metabolism. In parallel, induced senescence likely happened as a result of N deprivation and was shown by the enhanced levels of free phytol, free fatty acids, and other compound related with chlorophyll and membrane degradation that may be caused by an oxidative stress due to the poor N status. The metabolism in leaves of the plants inoculated with the SFJ14-36 strain and grown at a[CO₂] seems to swift from C assimilation to catabolism of chlorophyll and macromolecules such as fatty acids caused by N deficiency. However, more research is needed with other strains isolated at e[CO₂] and more soybean cultivars in order to confirm this observations.

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

DS: formal analysis and writing original draft. IA: conceptualization, resource managing, review and editing. UP-L: experimentation, data curation, review, and editing. AM-P: experimentation, data curation, review, and editing. AM-R: resource managing, experimentation, review, and editing. ML: conceptualization, resource managing, experimentation, supervision, review, and editing. AS-S: conceptualization, experimentation, data curation, formal analysis, project administration, supervision, and writing original draft. All authors contributed to the article and approved the submitted version.

FUNDING

This work was financially supported by the following grants: GRUPO Gobierno Vasco IT1022-16 and projects 32-2016-00043, 37-2017-00047, and 000049-IDA2019-38 from the Economic Development and Infrastructures Department of the Basque Country, Spain.

ACKNOWLEDGMENTS

DS was a recipient of a Ph.D. grant supported by the Public University of Navarra. AS-S was partially supported by a postdoctoral fellowship granted by the Education, and Linguistic Policy Department of the Basque Country, Spain and the Alabama Agricultural Experiment Station and the Hatch

Program of the National Institute of Food and Agriculture, United States Department of Agriculture. Technical support by Azucena González, Phytotron Service, SGIker (UPV/EHU) is gratefully acknowledged.

SUPPLEMENTARY MATERIAL

The Supplementary Material for this article can be found online at: <https://www.frontiersin.org/articles/10.3389/fpls.2021.656961/full#supplementary-material>

Supplementary Figure 1 | Classification of metabolites detected by GC-TOF/MS-based analysis in leaves and nodules of plants inoculated with USDA110, SFJ4-24, and SFJ14-36 *B. japonicum* strains under ambient and elevated [CO₂].

REFERENCES

- Abu-Shakra, S. S., Phillips, D. A., and Huffaker, R. A. (1978). Nitrogen Fixation and Delayed Leaf Senescence in Soybeans. *Science* 199, 973–975. doi: 10.1126/science.199.4332.973
- Ainsworth, E. A., and Rogers, A. (2007). The response of photosynthesis and stomatal conductance to rising [CO₂]: mechanisms and environmental interactions. *Plant. Cell Environ.* 30, 258–270. doi: 10.1111/j.1365-3040.2007.01641.x
- Ainsworth, E. A., Davey, P. A., Bernacchi, C. J., Dermody, O. C., Heaton, E. A., Moore, D. J., et al. (2002). A meta-analysis of elevated [CO₂] effects on soybean (*Glycine max*) physiology, growth and yield. *Glob. Chang. Biol.* 8, 695–709. doi: 10.1046/j.1365-2486.2002.00498.x
- Ainsworth, E. A., Rogers, A., Nelson, R., and Long, S. P. (2004). Testing the “source-sink” hypothesis of down-regulation of photosynthesis in elevated [CO₂] in the field with single gene substitutions in *Glycine max*. *Agric. For. Meteorol.* 122, 85–94. doi: 10.1016/j.agrformet.2003.09.002
- Aranjuelo, I., Arrese-Igor, C., and Molero, G. (2014). Nodule performance within a changing environmental context. *J. Plant Physiol.* 171, 1076–1090. doi: 10.1016/j.jplph.2014.04.002
- Aranjuelo, I., Tcherkez, G., Jauregui, I., Gilard, F., Ancín, M., Millán, A. F.-S., et al. (2015). Alteration by thioredoxin f over-expression of primary carbon metabolism and its response to elevated CO₂ in tobacco (*Nicotiana tabacum* L.). *Environ. Exp. Bot.* 118, 40–48. doi: 10.1016/j.envexpbot.2015.05.008
- Barclay, K. D., and McKersie, B. D. (1994). Peroxidation reactions in plant membranes: Effects of free fatty acids. *Lipids* 29, 877–882. doi: 10.1007/BF02536256
- Bei, Q., Liu, G., Tang, H., Cadisch, G., Rasche, F., and Xie, Z. (2013). Heterotrophic and phototrophic ¹⁵N₂ fixation and distribution of fixed ¹⁵N in a flooded rice-soil system. *Soil Biol. Biochem.* 59, 25–31. doi: 10.1016/j.soilbio.2013.01.008
- Berjak, P., and Pammenter, N. W. (2008). From Avicennia to Zizania: Seed Recalcitrance in Perspective. *Ann. Bot.* 101, 213–228. doi: 10.1093/aob/mcm168
- Bertrand, A., Prévost, D., Bigras, F. J., Lalande, R., Tremblay, G. F., Castonguay, Y., et al. (2007). Alfalfa response to elevated atmospheric CO₂ varies with the symbiotic rhizobial strain. *Plant Soil* 301, 173–187. doi: 10.1007/s11104-007-9436-9
- Bertrand, A., Prévost, D., Juge, C., and Chalifour, F. (2011). Impact of elevated CO₂ on carbohydrate and ureide concentrations in soybean inoculated with different strains of *Bradyrhizobium japonicum*. *Botany* 89, 481–490. doi: 10.1139/b11-034
- Bishop, K. A., Betzelberger, A. M. Y. M., Long, S. P., and Ainsworth, E. A. (2015). Is there potential to adapt soybean (*Glycine max* Merr.) to future [CO₂]? An analysis of the yield response of 18 genotypes in free-air CO₂ enrichment. *Plant. Cell Environ.* 38, 1765–1774. doi: 10.1111/pce.12443
- Booker, F. L., Reid, C. D., Brunschön-Harti, S., Fiscus, E. L., and Miller, J. E. (1997). Photosynthesis and photorespiration in soybean [*Glycine max* (L.) Merr.] chronically exposed to elevated carbon dioxide and ozone. *J. Exp. Bot.* 48, 1843–1852. doi: 10.1093/jxb/48.10.1843
- Bouvier-Navé, P., Berna, A., Noiriel, A., Compagnon, V., Carlsson, A., Banas, A., et al. (2010). Involvement of the Phospholipid Sterol Acyltransferase1 in Plant Sterol Homeostasis and Leaf Senescence. *Plant Physiol.* 152, 107–119. doi: 10.1104/pp.109.145672
- Chen, L.-L., Wang, G.-Z., and Zhang, H.-Y. (2007). Sterol biosynthesis and prokaryotes-to-eukaryotes evolution. *Biochem. Biophys. Res. Commun.* 363, 885–888. doi: 10.1016/j.bbrc.2007.09.093
- CO₂.earth (2020). CO₂ Earth: A Pro Oxygen Website. URL: <https://www.co2.earth>
- Das, A., Rushton, P. J., and Rohila, J. S. (2017). Metabolomic Profiling of Soybeans (*Glycine max* L.) Reveals the Importance of Sugar and Nitrogen Metabolism under Drought and Heat Stress. *Plants* 6:21. doi: 10.3390/plants6020021
- Dorp, K., Vom Höhl, G., Plohm, C., Eisenhut, M., Abraham, M., Weber, A. P. M., et al. (2015). Remobilization of phytol from chlorophyll degradation is essential for Tocopherol synthesis and growth of arabidopsis. *Plant Cell* 27, 2846–2859. doi: 10.1105/tpc.15.00395
- Duperon, R., Thiersault, M., and Duperon, P. (1984). High level of glycosylated sterols in species of solanum and sterol changes during the development of the tomato. *Phytochemistry* 23, 743–746. doi: 10.1016/S0031-9422(00)85016-5
- Egli, D. B., Leggett, J. E., and Duncan, W. G. (1978). Influence of N Stress on Leaf Senescence and N Redistribution in Soybeans. *Agron. J.* 70, 43–47. doi: 10.2134/agronj1978.00021962007000010011x
- El-Asawi, M. A., Elansary, H. O., El-Shanhorey, N. A., Abdel-Hamid, A. M. E., Ali, H. M., and Elshikh, M. S. (2017). Salicylic Acid-Regulated Antioxidant Mechanisms and Gene Expression Enhance Rosemary Performance under Saline Conditions. *Front. Physiol.* 8:716. doi: 10.3389/fphys.2017.00716
- FAOSTAT (2020). *ProdStat. Core Production Data Base, Electronic resource*. URL: <http://faostat.fao.org/>.
- Farquhar, G. D., von Caemmerer, S., and Berry, J. A. (1980). A biochemical model of photosynthetic CO₂ assimilation in leaves of C₃ species. *Planta* 149, 78–90. doi: 10.1007/BF00386231
- Fehr, W. R., Caviness, C. E., Burmood, D. T., and Pennington, J. S. (1971). Stage of Development Descriptions for Soybeans, *Glycine Max* (L.) Merrill. *Crop Sci.* 11, 929–931. doi: 10.2135/cropsci1971.0011183X001100060051x
- Fischinger, S. A., Hristozkova, M., Zaman-Allah, M., and Schulze, J. (2010). Elevated CO₂ concentration around alfalfa nodules increases N₂ fixation. *J. Exp. Bot.* 61, 121–130. doi: 10.1093/jxb/erp287
- Fischinger, S., and Schulze, J. (2010). The importance of nodule CO₂ fixation for efficiency of symbiotic nitrogen fixation in pea at vegetative growth and during pod formation. *J. Exp. Bot.* 61, 2281–2291. doi: 10.1093/jxb/erq055
- Ghasemzadeh, A., and Ghasemzadeh, N. (2011). Flavonoids and phenolic acids: Role and biochemical activity in plants and human. *J. Med. Plants Res.* 5, 6697–6703. doi: 10.5897/JMPR11.1404

- Gillon, J. S., and Yakir, D. (2000). Internal conductance to CO₂ diffusion and C¹⁸OO discrimination in C3 leaves. *Plant Physiol.* 123, 201–214. doi: 10.1104/pp.123.1.201
- Gutiérrez, D., Gutiérrez, E., Pérez, P., Morcuende, R., Verdejo, A. L., and Martínez-Carrasco, R. (2009). Acclimation to future atmospheric CO₂ levels increases photochemical efficiency and mitigates photochemistry inhibition by warm temperatures in wheat under field chambers. *Physiol. Plant.* 137, 86–100. doi: 10.1111/j.1399-3054.2009.01256.x
- Hayes, J. M. (2001). Fractionation of Carbon and Hydrogen Isotopes in Biosynthetic Processes. *Rev. Mineral. Geochem.* 43, 225–277. doi: 10.2138/gsrmg.43.1.225
- Holmer, G., Ory, R. L., and Høy, C.-E. (1973). Changes in lipid composition of germinating barley embryo. *Lipids* 8, 277–283. doi: 10.1007/BF02531905
- Igamberdiev, A. U., and Kleczkowski, L. A. (2018). The Glycerate and Phosphorylated Pathways of Serine Synthesis in Plants: The Branches of Plant Glycolysis Linking Carbon and Nitrogen Metabolism. *Front. Plant Sci.* 9:318. doi: 10.3389/fpls.2018.00318
- IPCC (2013). “Summary for policymakers,” in *Climate Change 2013: The Physical Science Basis. Contribution of Working Group I to the Fifth Assessment Report of the Intergovernmental Panel on Climate Change*, eds T. F. Stocker, D. Qin, G.-K. Plattner, M. Tignor, S. K. Allen, J. Boschung, et al. (Cambridge, UK: Cambridge University Press), 3–29.
- Ischebeck, T., Zbierzak, A. M., Kanwischer, M., and Dörmann, P. (2006). A salvage pathway for phytol metabolism in Arabidopsis. *J. Biol. Chem.* 281, 2470–2477. doi: 10.1074/jbc.M50922200
- Kaschuk, G., Hungria, M., Leffelaar, P. A., Giller, K. E., and Kuyper, T. W. (2010). Differences in photosynthetic behaviour and leaf senescence of soybean (*Glycine max* [L.] Merrill) dependent on N₂ fixation or nitrate supply. *Plant Biol.* 12, 60–69. doi: 10.1111/j.1438-8677.2009.00211.x
- Kaschuk, G., Kuyper, T. W., Leffelaar, P. A., Hungria, M., and Giller, K. E. (2009). Are the rates of photosynthesis stimulated by the carbon sink strength of rhizobial and arbuscular mycorrhizal symbioses? *Soil Biol. Biochem.* 41, 1233–1244. doi: 10.1016/j.soilbio.2009.03.005
- Keys, A. J. (1999). “Biochemistry of photorespiration and the consequences for plant performance,” in *Plant carbohydrate biochemistry*, eds J. A. Bryant, M. M. Burrell, and N. J. Kruger (Oxford, UK: BIOS ScientiÆc), 147–161.
- King, C. A., and Purcell, L. C. (2005). Inhibition of N₂ Fixation in Soybean Is Associated with Elevated Ureides and Amino Acids. *Plant Physiol.* 137, 1389–1396. doi: 10.1104/pp.104.056317
- Kováčik, J., Grúz, J., Bačkor, M., Strnad, M., and Repčák, M. (2008). Salicylic acid-induced changes to growth and phenolic metabolism in *Matricaria chamomilla* plants. *Plant Cell Rep.* 28:135. doi: 10.1007/s00299-008-0627-5
- Ladrera, R., Marino, D., Larrainzar, E., González, E. M., and Arrese-Igor, C. (2007). Reduced carbon availability to bacteroids and elevated ureides in nodules, but not in shoots, are involved in the nitrogen fixation response to early drought in soybean. *Plant Physiol.* 145, 539–546. doi: 10.1104/pp.107.102491
- Li, L., Hur, M., Lee, J.-Y., Zhou, W., Song, Z., Ransom, N., et al. (2015). A systems biology approach toward understanding seed composition in soybean. *BMC Genomics* 16:S9. doi: 10.1186/1471-2164-16-S3-S9
- Li, L., Zhao, J., Zhao, Y., Lu, X., Zhou, Z., Zhao, C., et al. (2016). Comprehensive investigation of tobacco leaves during natural early senescence via multi-platform metabolomics analyses. *Sci. Rep.* 6, 2–11. doi: 10.1038/srep37976
- Li, T., Hu, Y., Du, X., Tang, H., Shen, C., and Wu, J. (2014). Salicylic Acid Alleviates the Adverse Effects of Salt Stress in *Torreya grandis* cv. Merrillii Seedlings by Activating Photosynthesis and Enhancing Antioxidant Systems. *PLoS One* 9:e109492. doi: 10.1371/journal.pone.0109492
- 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
- Lima, J. D., and Sodek, L. (2003). N-stress alters aspartate and asparagine levels of xylem sap in soybean. *Plant Sci.* 165, 649–656. doi: 10.1016/S0168-9452(03)00251-6
- Lin, J.-S., and Wang, G.-X. (2002). Doubled CO₂ could improve the drought tolerance better in sensitive cultivars than in tolerant cultivars in spring wheat. *Plant Sci.* 163, 627–637. doi: 10.1016/S0168-9452(02)00173-5
- Lippold, F., vom Dorp, K., Abraham, M., Hölzl, G., Wewer, V., Yilmaz, J. L., et al. (2012). Fatty acid phytol ester synthesis in chloroplasts of Arabidopsis. *Plant Cell* 24, 2001–2014. doi: 10.1105/tpc.112.095588
- Lodwig, E., and Poole, P. (2003). Metabolism of Rhizobium bacteroids. *CRC. Crit. Rev. Plant Sci.* 22, 37–78. doi: 10.1080/713610850
- Mach, J. (2015). Phytol from Degradation of Chlorophyll Feeds Biosynthesis of Tocopherols. *Plant Cell* 27:2676. doi: 10.1105/tpc.15.00860
- Medic, J., Atkinson, C., and Hurburgh, C. R. Jr. (2014). Current Knowledge in Soybean Composition. *J. Am. Oil Chem. Soc.* 91, 363–384. doi: 10.1007/s11746-013-2407-9
- Melzer, E., and O’Leary, M. H. (1991). Aspartic-acid synthesis in C₃ plants. *Planta* 185, 368–371. doi: 10.1007/BF00201058
- Mishra, S., Tyagi, A., Singh, I. V., and Sangwan, R. S. (2006). Changes in lipid profile during growth and senescence of *Catharanthus roseus* leaf. *Brazilian J. Plant Physiol.* 18, 447–454. doi: 10.1590/S1677-04202006000400002
- Molero, G., Tcherkez, G., Roca, R., Mauve, C., Cabrera-Bosquet, L., Araus, J. L., et al. (2019). Do metabolic changes underpin physiological responses to water limitation in alfalfa (*Medicago sativa*) plants during a regrowth period? *Agric. Water Manag.* 212, 1–11. doi: 10.1016/j.agwat.2018.08.021
- Moore, T. C. (1974). *Symbiotic Nitrogen Fixation in Legume Nodules*. In: *Research Experiences in Plant Physiology*. Berlin: Springer, doi: 10.1007/978-3-642-96168-7-26
- Morgan, P., Bollero, G., Nelson, R., Dohleman, F., and Long, S. (2005). Smaller than predicted increase in aboveground net primary production and yield of field-grown soybean under fully open-air [CO₂] elevation. *Glob. Chang. Biol.* 11, 1856–1865. doi: 10.1111/j.1365-2486.2005.001017.x
- Munné-Bosch, S. (2005). The role of α-tocopherol in plant stress tolerance. *J. Plant Physiol.* 162, 743–748. doi: 10.1016/J.JPLPH.2005.04.022
- Munné-Bosch, S. (2007). α-Tocopherol: A Multifaceted Molecule in Plants. *Vitam. Horm.* 76, 375–392. doi: 10.1016/S0083-6729(07)76014-4
- Noctor, G., Veljovic-Jovanovic, S., Driscoll, S., Novitskaya, L., and Foyer, C. (2002). Drought and Oxidative Load in the Leaves of C₃ Plants: a Predominant Role for Photorespiration? 89, 841–850. doi: 10.1093/aob/mcf096
- Obata, T., and Fernie, A. R. (2012). The use of metabolomics to dissect plant responses to abiotic stresses. *Cell. Mol. Life Sci.* 69, 3225–3243. doi: 10.1007/s00018-012-1091-5
- Padan, E., Bibi, E., Ito, M., and Krulwich, T. A. (2005). Alkaline pH homeostasis in bacteria: New insights. *Biochim. Biophys. Acta Biomembr.* 1717, 67–88. doi: 10.1016/J.BBAMEM.2005.09.010
- Peisker, C., Düggelin, T., Rentsch, D., and Matile, P. (1989). Phytol and the Breakdown of Chlorophyll in Senescent Leaves. *J. Plant Physiol.* 135, 428–432. doi: 10.1016/S0176-1617(89)80099-9
- Prévost, D., Bertrand, A., Juge, C., and Chalifour, F. P. (2010). Elevated CO₂ induces differences in nodulation of soybean depending on *bradyrhizobial* strain and method of inoculation. *Plant Soil* 331, 115–127. doi: 10.1007/s11104-009-0238-0
- Puiatti, M., and Sodek, L. (1999). Waterlogging affects nitrogen transport in the xylem of soybean. *Plant Physiol. Biochem.* 37, 767–773. doi: 10.1016/S0981-9428(00)86690-5
- Rabara, R. C., Tripathi, P., and Rushton, P. J. (2017). Comparative Metabolome Profile between Tobacco and Soybean Grown under Water-Stressed Conditions. *Biomed. Res. Int.* 2017:3065251. doi: 10.1155/2017/3065251
- Rachmilevitch, S., Cousins, A. B., and Bloom, A. J. (2004). Nitrate assimilation in plant shoots depends on photorespiration. *Proc. Natl. Acad. Sci. U. S. A.* 101, 11506–11510. doi: 10.1073/pnas.0404388101
- Rogers, A., Gibon, Y., Stitt, M., Morgan, P., Bernacchi, C., Ort, D., et al. (2006). Increased C availability at elevated carbon dioxide concentration improves N assimilation in a legume. *Plant Cell Environ.* 29, 1651–1658. doi: 10.1111/j.1365-3040.2006.01549.x
- Ruuska, S. A., Badger, M. R., Andrews, T. J., and von Caemmerer, S. (2000). Photosynthetic electron sinks in transgenic tobacco with reduced amounts of Rubisco: little evidence for significant Mehler reaction. *J. Exp. Bot.* 51, 357–368. doi: 10.1093/jexbot/51.suppl_1.357
- Saito, K., and Matsuda, F. (2008). Metabolomics for Functional Genomics, Systems Biology, and Biotechnology. *Annu. Rev. Plant Biol.* 61, 463–489. doi: 10.1146/annurev.arplant.043008.092035
- Saiz-Fernández, I., Lacuesta, M., Pérez-López, U., Sampedro, M. C., Barrio, R. J., and De Diego, N. (2020). Interplay between 1-aminocyclopropane-1-carboxylic acid, γ-aminobutyrate and D-glucose in the regulation of high nitrate-induced root growth inhibition in maize. *Plant Sci.* 293:110418. doi: 10.1016/j.plantsci.2020.110418

- Salminen, S. O., and Streeter, J. G. (1987). Involvement of glutamate in the respiratory metabolism of *Bradyrhizobium japonicum* bacteroids. *J. Bacteriol.* 169, 495–499. doi: 10.1128/jb.169.2.495-499.1987
- Salminen, S. O., and Streeter, J. G. (1992). Labeling of Carbon Pools in *Bradyrhizobium japonicum* and *Rhizobium leguminosarum* bv viciae Bacteroids following Incubation of Intact Nodules with ¹⁴CO₂. *Plant Physiol.* 100, 597–604. doi: 10.1104/pp.100.2.597
- Sanz-Sáez, Á., Erice, G., Aguirreola, J., Irigoyen, J. J., and Sánchez-Díaz, M. (2012). Alfalfa yield under elevated CO₂ and temperature depends on the Sinorhizobium strain and growth season. *Environ. Exp. Bot.* 77, 267–273. doi: 10.1016/j.envexpbot.2011.11.017
- Sanz-Sáez, Á., Heath, K. D., Burke, P. V., and Ainsworth, E. A. (2015). Inoculation with an enhanced N₂-fixing *Bradyrhizobium japonicum* strain (USDA110) does not alter soybean (*Glycine max* Merr.) response to elevated [CO₂]. *Plant. Cell Environ.* 38, 2589–2602. doi: 10.1111/pce.12577
- Sanz-Sáez, Á., Koester, R. P., Rosenthal, D. M., Montes, C. M., Ort, D. R., and Ainsworth, E. A. (2017). Leaf and canopy scale drivers of genotypic variation in soybean response to elevated carbon dioxide concentration. *Glob. Chang. Biol.* 23, 3908–3920. doi: 10.1111/gcb.13678
- Sanz-Sáez, Á., Pérez-López, U., del-Canto, A., Ortiz-Barredo, A., Mena-Petite, A., Aranjuelo, I., et al. (2019). Changes in environmental CO₂ concentration can modify *Rhizobium*-soybean specificity and condition plant fitness and productivity. *Environ. Exp. Bot.* 162, 133–143. doi: 10.1016/j.envexpbot.2019.01.013
- Serraj, R., Vadez, V., and Sinclair, T. (2001). Feedback regulation of symbiotic N₂ fixation under drought stress. *Agronomie* 21, 621–626. doi: 10.1051/agro:2001153
- Serraj, R., Vadez, V., Denison, R. F., and Sinclair, T. R. (1999). Involvement of Ureides in Nitrogen Fixation Inhibition in Soybean. *Plant Physiol.* 119, 289–296. doi: 10.1104/pp.119.1.289
- Sharkey, T. D. (1988). Estimating the rate of photorespiration in leaves. *Physiol. Plant.* 73, 147–152. doi: 10.1111/j.1399-3054.1988.tb09205.x
- Silvente, S., Camas, A., and Lara, M. (2003). Molecular cloning of the cDNA encoding aspartate aminotransferase from bean root nodules and determination of its role in nodule nitrogen metabolism. *J. Exp. Bot.* 54, 1545–1551. doi: 10.1093/jxb/erg161
- Soba, D., Ben Mariem, S., Fuertes-Mendizábal, T., Méndez-Espinoza, A. M., Gilard, F., González-Murua, C., et al. (2019a). Metabolic Effects of Elevated CO₂ on Wheat Grain Development and Composition. *J. Agric. Food Chem.* 67, 8441–8451. doi: 10.1021/acs.jafc.9b01594
- Soba, D., Müller, M., Aranjuelo, I., and Munné-Bosch, S. (2020a). Vitamin E in legume nodules: Occurrence and antioxidant function. *Phytochemistry* 172:112261. doi: 10.1016/j.phytochem.2020.112261
- Soba, D., Shu, T., Runion, G. B., Prior, S. A., Fritsch, F. B., Aranjuelo, I., et al. (2020b). Effects of elevated [CO₂] on photosynthesis and seed yield parameters in two soybean genotypes with contrasting water use efficiency. *Environ. Exp. Bot.* 178:104154. doi: 10.1016/j.envexpbot.2020.104154
- Soba, D., Zhou, B., Arrese-Igor, C., Munné-Bosch, S., and Aranjuelo, I. (2019b). Physiological, hormonal and metabolic responses of two alfalfa cultivars with contrasting responses to drought. *Int. J. Mol. Sci.* 20:5099. doi: 10.3390/ijms20205099
- Sugawara, M., and Sadowsky, M. (2013). Influence of Elevated Atmospheric Carbon Dioxide on Transcriptional Responses of *Bradyrhizobium japonicum* in the Soybean Rhizosphere. *Microbes Environ.* 28, 217–227. doi: 10.1264/jsme2.ME12190
- Tcherkez, G., Ben Mariem, S., Larraya, L., García-Mina, J. M., Zamarreño, A. M., Paradelo, A., et al. (2020). Elevated CO₂ has concurrent effects on leaf and grain metabolism but minimal effects on yield in wheat. *J. Exp. Bot.* 71, 5990–6003. doi: 10.1093/jxb/eraa330
- Tcherkez, G., Bligny, R., Gout, E., Mahé, A., Hodges, M., and Cornic, G. (2008). Respiratory metabolism of illuminated leaves depends on CO₂ and O₂ conditions. *Proc. Natl. Acad. Sci. U. S. A.* 105, 797–802. doi: 10.1073/pnas.0708947105
- Troncoso-Ponce, M. A., Cao, X., Yang, Z., and Ohlrogge, J. B. (2013). Lipid turnover during senescence. *Plant Sci.* 20, 13–19. doi: 10.1016/j.plantsci.2013.01.004
- von Caemmerer, S. (2000). *Biochemical models of leaf photosynthesis*. Collingwood, Australia: CSIRO Publishing.
- von Caemmerer, S., Evans, J., Hudson, G., and Andrews, T. (1994). The kinetics of ribulose-1,5-bisphosphate carboxylase/oxygenase in vivo inferred from measurements of photosynthesis in leaves of transgenic tobacco. *Planta* 195, 88–97. doi: 10.1007/BF00206296
- Wang, P., Marsh, E. L., Ainsworth, E. A., Leakey, A. D. B., Sheflin, A. M., and Schachtman, D. P. (2017). Shifts in microbial communities in soil, rhizosphere and roots of two major crop systems under elevated CO₂ and O₃. *Sci. Rep.* 7:15019. doi: 10.1038/s41598-017-14936-2
- Woodward, F. I. (2002). Potential impacts of global elevated CO₂ concentrations on plants. *Curr. Opin. Plant Biol.* 5, 207–211. doi: 10.1016/S1369-5266(02)00253-4
- Zhu, X.-G., Long, S. P., and Ort, D. R. (2008). What is the maximum efficiency with which photosynthesis can convert solar energy into biomass? *Curr. Opin. Biotechnol.* 19, 153–159. doi: 10.1016/j.copbio.2008.02.004

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.

Copyright © 2021 Soba, Aranjuelo, Gakière, Gilard, Pérez-López, Mena-Petite, Muñoz-Rueda, Lacuesta and Sanz-Saez. This is an open-access article distributed under the terms of the Creative Commons Attribution License (CC BY). The use, distribution or reproduction in other forums is permitted, provided the original author(s) and the copyright owner(s) are credited and that the original publication in this journal is cited, in accordance with accepted academic practice. No use, distribution or reproduction is permitted which does not comply with these terms.



Rhizospheric *Bacillus amyloliquefaciens* Protects *Capsicum annuum* cv. Geumsugangsan From Multiple Abiotic Stresses via Multifarious Plant Growth-Promoting Attributes

OPEN ACCESS

Edited by:

Juan Manuel Ruiz-Lozano,
Estación Experimental de Zaidín,
Consejo Superior de Investigaciones
Científicas (CSIC), Spain

Reviewed by:

Susana Redondo-Gómez,
University of Seville, Spain
Gabriela Quiroga,
Estación Experimental de Zaidín,
Consejo Superior de Investigaciones
Científicas (CSIC), Spain

*Correspondence:

Elham Ahmed Kazerooni
elham.ghasemi.k@gmail.com
In-Jung Lee
ijlee@knu.ac.kr

Specialty section:

This article was submitted to
Plant Abiotic Stress,
a section of the journal
Frontiers in Plant Science

Received: 19 February 2021

Accepted: 12 April 2021

Published: 25 May 2021

Citation:

Kazerooni EA,
Maharachchikumbura SSN,
Adhikari A, Al-Sadi AM, Kang S-M,
Kim L-R and Lee I-J (2021)
Rhizospheric *Bacillus*
amyloliquefaciens Protects *Capsicum*
annuum cv. Geumsugangsan From
Multiple Abiotic Stresses via
Multifarious Plant Growth-Promoting
Attributes.
Front. Plant Sci. 12:669693.
doi: 10.3389/fpls.2021.669693

Elham Ahmed Kazerooni^{1*}, Sajeewa S. N. Maharachchikumbura², Arjun Adhikari¹,
Abdullah Mohammed Al-Sadi³, Sang-Mo Kang¹, Lee-Rang Kim¹ and In-Jung Lee^{1*}

¹ Department of Applied Biosciences, Kyungpook National University, Daegu, South Korea, ² School of Life Sciences and Technology, University of Electronic Science and Technology of China, Chengdu, China, ³ Department of Crop Sciences, College of Agricultural and Marine Sciences, Sultan Qaboos University, Al-Khod, Oman

Plant growth-promoting rhizobacteria (PGPR) are beneficial microorganisms that can be utilized to improve plant responses against biotic and abiotic stresses. In this study, we investigated whether PGPR (*Bacillus amyloliquefaciens*) isolated from the endorhizosphere of *Sasamorpha borealis* have the potential to sustain pepper growth under drought, salinity, and heavy metal stresses. The bacterial strain was determined based on 16S rDNA and gyrB gene sequencing and characterized based on the following biochemical traits: nitrogen fixation; 1-aminocyclopropane-1-carboxylate deaminase activity; indole acetic acid production; inorganic phosphate, potassium, zinc, and silicon solubilization; and siderophore production. Various abiotic stresses were applied to 28-day-old pepper seedlings, and the influence of the PGPR strain on pepper seedling growth under these stress conditions was evaluated. The application of PGPR improved survival of the inoculated pepper plants under stress conditions, which was reflected by higher seedling growth rate and improved physiochemical traits. The PGPR-treated plants maintained high chlorophyll, salicylic acid, sugar, amino acid, and proline contents and showed low lipid metabolism, abscisic acid, protein, hydrogen peroxide contents, and antioxidant activities under stress conditions. Gene expression studies confirmed our physiological and biochemical findings. PGPR inoculation led to enhanced expression of XTH genes and reduced expression of *WRKY2*, *BI-1*, *PTI1*, and binding immunoglobulin protein (BiP) genes. We conclude that the PGPR strain described in this study has great potential for use in the phytoremediation of heavy metals and for enhancing pepper plant productivity under stress conditions, particularly those involving salinity and drought.

Keywords: 1-aminocyclopropane-1-carboxylate deaminase, pepper, endophyte, plant growth-promoting rhizobacteria, salinity, drought, phytoremediation

INTRODUCTION

Plants are detrimentally affected by multiple environmental stimuli in the form of various abiotic and biotic stresses. Water deficiency and salinity are the prevalent environmental restraints that affect the morphological, physiological, biochemical, and molecular responses of plants and cause enormous losses in agriculture (Jaleel et al., 2007; Khan et al., 2019). Early responses of plants to these stresses lead to a slowdown of growth and photosynthesis, impair pigment production, increase nutrient deficiency, and reduce enzyme activity (Ali et al., 2014; Glick, 2014). In addition, soil pollution is also prevalent in different places. It is affected by industrial processes, cultivation practices, and human activities. These processes can lead to the spread of, for example, heavy metals, which can create severe ecological problems even at low levels (Wuana and Okieimen, 2011). There are other abiotic stresses that are more location specific, including frost (Keep et al., 2021; Simioniuc et al., 2021), ultraviolet radiation (Böttner et al., 2021; Navarro et al., 2021), and floods (Khan et al., 2020; Liu et al., 2021).

Plant growth-promoting rhizobacteria (PGPR) are occupant microorganisms in the rhizosphere that can enhance the positive results of phytoremediation. Once PGPR are applied in polluted soil, they improve the resistance of plants to heavy metals, expedite the turnover of nutrients, ameliorate the deleterious nature of some metals, boost plant tolerance against pests and pathogen assault, and improve soil structure (Khan and Bano, 2016; Karthik et al., 2017). Plant root exudates contain various metabolites and nutrients that are essential for nourishing rhizobacteria (Han et al., 2005; Babalola, 2010). PGPR provide a plethora of functions by which crop growth and yield can be improved. They have the capacity to fix atmospheric nitrogen, which is associated with the development of numerous growth-promoting plant hormones. Indole-3-acetic acid (IAA) producer strains enhance lateral root formation and improve root length, which assist plant to grow under drought conditions (Gupta and Pandey, 2019). Siderophore production is one of the main PGPR mechanisms to provide iron for plants in stressful situations. Previous studies showed that iron has a role in chlorophyll synthesis and maintain chloroplast structure (Rout and Sahoo, 2015). Additionally, PGPR comprise enzymes that act as mineral solubilizers and help maintain plant growth under stress conditions (Glick et al., 1998; Ma et al., 2009; Oves et al., 2013). The essential mechanisms by which PGPR improve plant growth during stress conditions include an increase in endogenous ethylene (ET) levels *via* 1-aminocyclopropane-1-carboxylate (ACC) deaminase enzyme activity, an increase in photosynthetic pigments, promotion of root development, rhizoremediation, and disease endurance (Belimov et al., 2005; Madhaiyan et al., 2006).

Phytoremediation is a promising technology with great capabilities for ameliorating soil pollution; it comprises the use of plants to “clean-up” environments by diminishing the toxicity, proportion, and diffusion of contaminants in soils (Liao and Chang, 2004). Plants can facilitate the removal of various contaminants, including pesticides and other chemicals, from soil and prevent the dispersion of pollutants

via underground water reservoirs, wind, or rain from one region to another. This technology consists of two critical mutualistic components: plants and plant-associated microbes, which break down toxic substances into nontoxic substances (Saxena et al., 2005).

Pepper (*Capsicum annuum* L.) is one of the most economically important vegetable crops worldwide. It is commonly used as a fresh vegetable or spice owing to its pungent nature. Moreover, its fruit has major nutritional value and anticancer properties (Clark and Lee, 2016). However, this crop is highly susceptible to abiotic and biotic stresses, including salinity, drought, and pathogens; thus, plant yield is affected by such stresses, leading to economic losses (Dagdelen et al., 2004).

Sasamorpha borealis is an indigenous species that is highly prevalent throughout Korea (Lee and Yim, 2002). *S. borealis* is a strong clonal competitor, which successfully restrains other grassy plants and creates dense populations (Doležal et al., 2009). We speculated that PGPR isolated from *S. borealis* could enhance phytoremediation as well as salinity and drought tolerance in pepper plants under adverse environmental conditions. Therefore, this research was conducted to evaluate whether the application of PGPR could improve phytoremediation potential and help maintain the growth of salinity and drought-stressed pepper.

MATERIALS AND METHODS

Collection and Isolation of Rhizospheric and Endophytic Bacteria

A fresh *S. borealis* (Hack.) Nakai plant was collected from Kyungpook National University, Daegu, South Korea. To isolate rhizospheric bacteria, a rhizospheric soil sample was processed based on the methods of Fischer et al. (2007). Endophytic bacteria present in the sample were isolated following a previously described method (Albdaiwi et al., 2019). Briefly, healthy roots were washed thoroughly under running tap water to remove adhering soil particles and then rinsed in double distilled water several times. Root samples were cut into small pieces and then surface sterilized in 70% ethanol for 1 min and 3% sodium hypochlorite solution for 10 min. Subsequently, they were rinsed in sterile distilled water three times. To validate root surface sterilization efficiency, an aliquot (20 μ l) from the third wash solution was dispersed on Luria-Bertani (LB) medium and incubated at $28^{\circ}\text{C} \pm 2^{\circ}\text{C}$ for 1 week. After that, sterilized roots (1 g) were aseptically crushed with a mortar and pestle using 10 ml of 1% NaCl and serial dilutions were prepared. Here, 1 ml of the root extract and the diluents were spread on LB agar medium. The plates were incubated at 28°C and surveilled up to 1 week for bacterial colony emergence. Morphologically dissimilar colonies of bacteria were designated and refined with streaking on LB agar medium. In total, 11 bacterial pure cultures were selected and preserved in corresponding growth medium with 25% glycerol at -20°C until further use. The selected colonies were named B1–B11, respectively.

In vitro Assay for Plant Growth-Promoting Properties

All experiments were repeated three times, and each was conducted following a completely randomized design with three replications for each strain. The uninoculated medium served as a negative control.

Citrate Utilization, Nitrogen Fixation, 1-Aminocyclopropane-1-Carboxylate Deaminase Activity, Indole-3-Acetic Acid, and Siderophore Production

A citrate test of bacterial strains was conducted by adopting the method of Simmons (1926). Citrate utilization ability was confirmed by the change in the color from green to blue. Siderophore production was examined using chrome azurol S (CAS) agar media following a previously described method (Alexander and Zuberer, 1991). The formation of a clear halo zone around the colonies was considered the result of siderophore production. Nitrogen-free bromothymol blue malate (NFB) medium was used for visual detection of nitrogen-fixing activities of bacterial strains (Baldani et al., 2014). A color change from green to blue indicated that the strain probably had nitrogen-fixing activity in the solid culture conditions. IAA production was determined following the methods of Sarwar and Kremer (1995). The appearance of a pink-red color confirmed IAA production by bacterial strains. The ACC deaminase activity of bacterial strains was assessed on sterile minimal Dworkin and Foster (DF) salts media containing 3-mM ACC (Sigma-Aldrich, United States) as a sole nitrogen source (Penrose and Glick, 2003). Bacterial strains multiplying on the ACC-supplemented plates were considered ACC deaminase producers (Ali et al., 2014).

Potassium, Zinc, Phosphate, and Silicon Solubilization Assay

The ability of bacterial strains to solubilize inorganic phosphate was detected using Pikovskaya agar medium supplemented with tricalcium phosphate (Gaur, 1990). For determining potassium solubilization, the bacterial cultures were spot-inoculated onto Aleksandrov medium containing mica powder (Hu et al., 2006). Zinc solubilizing activity was determined in mineral salts agar medium supplemented with 0.1% zinc oxide (Venkatakrishnan et al., 2003). Si solubilization was assessed in the basal media supplemented with potassium alumina silicate (Sharma et al., 2011). The development of a clear zone around the bacterial colonies was considered to indicate solubilizing activity.

In vitro Assay for Stress Tolerance in Response to Abiotic Stresses

The experiment was executed in triplicate with control and multiple test concentrations. The unmodified medium served as a control.

Salinity Stress Tolerance

The intrinsic salt tolerance of bacterial strains was examined by observing their growth on the LB agar medium, which was supplemented with various NaCl concentrations (0.5%, 2.5%, 5%, 7.5%, and 10%). The plates were incubated for 72 h at $28^{\circ}\text{C} \pm 2^{\circ}\text{C}$ (Barra et al., 2016).

Drought Stress Tolerance

The drought endurance of strains was assessed in polyethylene glycol (PEG 6000)-supplemented LB agar medium. The strain growth was surveyed at $28^{\circ}\text{C} \pm 2^{\circ}\text{C}$ for 24 h in LB agar media with different osmotic potentials (-0.05 , -0.15 , -0.3 , -0.49 , and -0.73 MPa) (Ali et al., 2014).

Heavy Metal Stress Tolerance

The heavy metal tolerance of bacterial strains was examined on LB agar medium supplemented with varied cadmium (Cd), chromium (Cr), and nickel (Ni) concentrations ranging from 0.4 to 1.0 g L⁻¹. The significant growth of bacterial strains in the presence of heavy metals during 72 h at $28^{\circ}\text{C} \pm 2^{\circ}\text{C}$ was considered heavy metal tolerance (Cervantes-Vega et al., 1986).

Extraction of Bacterial DNA and Phylogenetic Analysis

The selected bacterial strain (B11) was identified using 16S rDNA and the partial sequence of the gene encoding the subunit B protein of DNA gyrase (*gyrB*) sequencing and phylogenetic analysis. For this purpose, total genomic DNA was isolated as described by Chang et al. (2011). Polymerase chain reaction (PCR) assays were performed for 16S rDNA and *gyrB* regions using primers 27F/1492R and UP1F/UP2R (Supplementary Table 1). The preparation of the PCR reaction mixture and PCR amplification were completed following Ki et al. (2009) and Ahaotu et al. (2013). PCR products were purified and sequenced at SolGent Co., Ltd. (Daejeon, South Korea). The phylogenetic analysis of bacterial strain sequences was performed using RAXML GUI v.1.3 (Silvestro and Michalak, 2012).

Effect of the Selected Bacterial Strain on Pepper Growth Under Drought, Salinity, and Heavy Metal Stresses

Based on the results of *in vitro* assays, the B11 bacterial strain was chosen and used for further assays. Specifically, the growth-promoting and phytoremediation effects of B11 on the growth of pepper under different abiotic stress conditions were assessed.

Preparation of Bacterial Inoculum Suspension

A log phase culture of the B11 bacterial strain at the optical density (600 nm) was used to prepare the bacterial inoculum suspension. The selected bacteria were prepared in LB medium and maintained at 28°C in a shaker at 150 rpm for 24 h until late exponential growth phase. Afterward, the bacterial culture was centrifuged ($8,000 \times g$, 10 min), and the supernatant was

decanted. The obtained pellet was washed three times with sterile distilled water. Afterward, the obtained cell pellets were deferred in sterile distilled water (cell density of 10^7 – 10^8 /ml), vortexed, and then used for plant treatment (50 ml/pot).

Selection of Appropriate Concentration of Salt, Polyethylene Glycol, and Cadmium

Seeds of pepper (*C. annuum* cv. Geumsugangsan) were provided by Takii Korea Ltd. (Seoul, South Korea). They were surface sterilized in 70% ethanol and 1.5% sodium hypochlorite and tested for the efficiency of the sterilization process (Albdaiwi et al., 2019) and viability (Ke et al., 2018). Germinated seeds were planted in plastic pot trays (28 cm × 54 cm) filled with horticultural soil (Shinsung Mineral Co., Ltd., South Korea), with one seed planted per pot. Seeds were then grown in a climate chamber under the following conditions: 250 μ mol photons $m^{-2} s^{-1}$ PAR (photosynthetically active radiation), 65% relative humidity, 25°C $\pm$ 2°C, 16:8 h light:dark cycle, and daily watering of seedlings. Three-week-old seedlings with uniform sizes were transferred to pots (10 cm × 10 cm) and subjected to different treatments. All pepper seedlings were randomly divided into two groups: (i) normal control, grown with distilled water (50 ml/pot), and (ii) salinity treatment, irrigated with NaCl (0.5%, 1%, 1.5%, and 2.5%) (50 ml/pot). Each treatment contained three replicates of 15 plants each. Plants treated with each concentration were measured for various plant growth characteristics. Plant growth parameters such as plant height, root length, stem diameter, leaf length/width, number of leaves, chlorophyll content, plant fresh/dry weight, and root fresh/dry weight were measured after day 8 (8DAT) of treatment. Ultimately, 1% NaCl was found to be the optimal treatment concentration for use in further studies.

The optimal PEG treatment concentration was obtained *via* an experimental method described by Kaya and Doganlar (2019). In addition, we chose to expose pepper plants to 0.1% Cd stress.

Plant Material and Growth Conditions

Experimental work was performed in a growth chamber at the Department of Applied Bioscience, Kyungpook National University. Plants were grown under the aforementioned conditions. In addition, sterilized pepper seeds (one seed/pot) were planted in plastic pot trays containing autoclave-sterilized horticultural soil (Shinsung Mineral Co., Ltd., South Korea). Seedlings of similar size (one seedling/pot) were selected after 3 weeks and then transferred to pots (10 cm × 10 cm). During the experiment, the temperature was maintained constantly at 25°C $\pm$ 2°C (day/night), with a relative humidity of 65% and a light intensity of approximately 200 μ mol $m^{-2} s^{-1}$. Soil moisture (70%), pH ($\sim$ 7), and electrical conductivity (EC) ($\leq$ 1.2) were recorded before the pot experiment; at the end of the experiment, soil samples from individual pots (300 g/pot) in each treatment were examined to again determine moisture, pH, and EC using a humidity tester (Model DM-5; Takemura Electric Works, Ltd., Tokyo, Japan) and conductivity meter (YSI Model 32, United States) (Supplementary Table 1).

Experimental Design and Treatments

A 2 days after transplanting, 3-week-old seedlings were subjected to various treatments. They were divided into two groups: the normal control group, irrigated with sterile distilled water (50 ml/pot), and the bacterized group, irrigated with bacterial inoculum suspension (50 ml/pot). Each group was treated for 7 days, after which the groups of unbacterized and bacterized seedlings were subdivided into two groups containing an equal number of seedlings; this produced eight experimental groups, which are described in Table 1. Each treatment was performed twice in triplicate. The soil moisture level of each pot was monitored daily using a humidity tester (Model DM-5, as above). The pepper seedlings were exposed to selected abiotic stresses for 8 days, and sampling was performed at the end of each treatment. The harvested samples were either immediately used or rapidly deactivated in liquid nitrogen and stored at -80°C .

Physiobiochemical and Molecular Analyses of Plants

Plant Growth Characteristics and Chlorophyll Index Contents

To investigate the effect of each treatment on the seedlings, various plant growth parameters were measured. These parameters included plant height, root length, stem diameter, leaf length/width, plant fresh weight, and the number of leaves, all of which were recorded at 8DAT. A digital Vernier caliper and a ruler were used to measure stem diameter, leaf area (length and width), and plant height. In a preliminary salt screening experiment, chlorophyll content in leaves was examined using a portable CCM-300 Chlorophyll Content Meter (ADC BioScientific Ltd., Herts, United Kingdom).

Photosynthetic Pigments: Chlorophyll a, Chlorophyll b, and Carotenoid Content

Leaf chlorophyll [chlorophyll a (Chla), chlorophyll b (Chlb), and total chlorophyll (total Chl)] and carotenoid content were ascertained by spectrophotometric analysis of chemically extracted pigments (Arnon, 1949). Briefly, freeze-dried ground leaves were extracted in 80% ethanol at room temperature after centrifugation. Pigment absorption was then measured

TABLE 1 | Experimental work plan.

Symbol	Treatment
Cont	Irrigated with sterile distilled water
B11	Irrigated with PGPR B11
S	Irrigated with 1% NaCl
S+B11	Irrigated with 1% NaCl + PGPR B11
Dr	Irrigated with 10% PEG (−0.49 Mpa)
Dr+B11	Irrigated with 10% PEG (−0.49 Mpa) + PGPR B11
Cd	Irrigated with 0.1% Cd
Cd+B11	Irrigated with 0.1% Cd + PGPR B11

PEG, polyethylene glycol; PGPR, plant growth-promoting rhizobacteria.

spectrophotometrically at 663, 645, and 480 nm (Thermo Fisher Scientific, Waltham, MA, United States).

Quantification of Pepper Phytohormones: Absciscic Acid and Salicylic Acid

The extraction and quantification of pepper absciscic acid (ABA) content were performed according to previously described methods (Qi et al., 1998; Kim et al., 2014). The resultant extract was dried with nitrogen gas (N_2), and diazomethane (CH_2N_2) was added for methylation. ABA content was quantified by gas chromatography (GC)–mass spectrometry (MS) (Agilent 6890N Gas Chromatograph, Santa Clara, CA, United States). ThermoQuet software (Manchester, United Kingdom) was used to observe responses to ions (m/e of 162 and 190 for Me-ABA and 166 and 194 for Me- $[^2H_6]$ -ABA).

To measure salicylic acid (SA) level in pepper plants, 0.1 g of freeze-dried sample was used as previously described (Enyedi et al., 1992; Seskar et al., 1998). Briefly, freeze-dried sample was extracted with methanol (90 and 100%) by centrifugation (12,000 rpm for 15 min at 4°C). The combined methanol extracts were vacuum dried, and the dried residue was dissolved in 5% trichloroacetic acid and centrifuged at 10,000 rpm for 10 min. The supernatant was partitioned with ethyl acetate:cyclopentane:isopropanol (49.5:49.5:1.0 ratio, v/v). The top layer of aqueous solution was dried and used for SA quantification using high-performance liquid chromatography (HPLC).

Measurement of Amino Acid Content

Amino acid content was quantified using the method developed by Waqas et al. (2015). About 50 mg of freeze-dried leaves was hydrolyzed in the presence of hydrochloric acid (1 ml of 6-N HCl) for 24 h at 110°C. The extraction was then concentrated and vacuum dried at 80°C for 24 h. Afterward, the residue was diluted with deionized water (2 ml) and evaporated twice. The concentrated residue was then dissolved with hydrochloric acid (1 ml of 0.02-N HCl), and the mixture was passed through a 0.45- μ m filter membrane. Finally, the solution was analyzed using a Hitachi L-8900 Amino Acid analyzer (Hitachi High-Technologies Corporation, Tokyo, Japan).

Estimation of Leaf Protein and Sugar Content

Soluble protein content in the various treatments was quantified based on the methods of Ashraf and Iram (2005). The freeze-dried leaf samples (0.1 g) were ground and then mixed with a 1-ml phosphate buffer (50 mM, pH 7.0). The mixture was centrifuged at 10,000 rpm for 10 min at 4°C. Subsequently, the supernatant was collected and treated with an appropriate reagent, and the absorbance of each sample was assessed at 595 nm. Protein content was estimated in all enzymatic preparations using the Bradford method (Bradford, 1976), with bovine serum albumin as the standard.

Sugar content was estimated according to the defined method of Khan et al. (2018). Briefly, freeze-dried leaves were crushed, extracted using 80% ethanol, and then vacuum dried. The dried residue was redissolved in 1 ml of deionized water and passed

through 0.45- μ m nylon-66 syringe filters. Furthermore, the filtered samples were injected into an HPLC system (Millipore Co., Waters Chromatography, Milford, MA, United States).

Determination of Enzymatic and Nonenzymatic Antioxidant Activity

The activity of antioxidant enzymes such as peroxidase (POD) and polyphenol oxidase (PPO) was analyzed using the method reported by Putter (1974). Superoxide dismutase (SOD) activity was investigated via the method of Sirhindi et al. (2016). To determine flavonoids, 1,1-diphenyl-2-picrylhydrazyl (DPPH) radical scavenging activities, and total polyphenols, samples were processed and analyzed following previously described procedures (Zheng and Wang, 2001; Barka et al., 2006; Wang et al., 2009; Li et al., 2016). The mixture activity and absorbance were measured at selected wavelengths using a Multiskan GO UV/Vis microplate spectrophotometer (Thermo Fisher Scientific, Waltham, MA, United States).

Hydrogen Peroxide and Lipid Peroxidation

Following various treatments, hydrogen peroxide (H_2O_2) levels were determined as previously described (Jana and Choudhuri, 1982; Tsai et al., 2005). Frozen samples were freeze-dried and then ground with a pestle and mortar. The ground sample (0.3 g) was homogenized with 3 ml of ice-cold phosphate buffer [50 mM, 1 mM ethylenediaminetetraacetic acid (EDTA), 1% polyvinylpyrrolidone (PVP), pH 7.0] and centrifuged at 13,000 rpm for 20 min. The supernatant (2 ml) was mixed with 1 ml of 20% (v/v) H_2SO_4 containing 0.1% titanium chloride, and the mixture was centrifuged at 13,000 rpm for 20 min. Supernatant intensity was measured at 410 nm with a T60 UV-Vis Spectrophotometer (PG Instruments Ltd., Wibtoft, United Kingdom).

The level of lipid peroxidation was estimated through the thiobarbituric acid reaction, as described by López-Serrano et al. (2019). Malondialdehyde (MDA) content was calculated using its extinction coefficient. The amount of lipid peroxidation was shown as the level of MDA created per gram of tissue weight.

Quantification of Macronutrients and Sodium and Cadmium Uptake in Bacterial Cells (B11) and Pepper Plants

The potential bacterial strain was grown in LB broth containing 1% NaCl, 10% PEG, and 0.1% Cd. The bacterial pellet was harvested and prepared for analysis as previously explained by Damodaran et al. (2013). For nutrient analysis of pepper plants, samples were first freeze-dried, and processed into a powder. Subsequently, the prepared samples were used to quantify Cd, sodium (Na), and nutrient (potassium, K; phosphorus, P; and calcium, Ca) uptake in pepper plants using inductively coupled plasma mass spectrometry (Optima 7900DV; Perkin-Elmer, United States). Treatments without bacterial inoculation were used to determine the initial concentrations of salt, heavy metals, and macronutrients. The removal efficiency of salt and Cd by the bacterial strain (B11) was calculated as follows: salt/Cd removal

efficiency % = $\frac{C_0 - C_s}{C_0} \times 100$, where C_0 and C_s represent the initial and final concentrations of salt and Cd, respectively.

cDNA Synthesis and Real-Time PCR Analysis

Total RNA was extracted from the pepper leaves (8DAT) and used for cDNA synthesis and quantitative PCR (qPCR) following the procedure of Imran et al. (2018). Specifically, 1 µg of RNA was used to synthesize cDNA with a BioFACT RT-Kit (BIOFACT, Korea) following the manufacturer's standard protocol. The synthesized cDNA was employed as a pattern in a two-step qRT-PCR reaction, which was performed to determine transcript quantity with an Illumina Eco system (Illumina, United States) (Supplementary Table 2).

Statistical Analysis

The experiments were conducted in triplicate consecutively three times. The results were subjected to statistical analysis by ANOVA using R (version 4.0.3). A least significant difference (LSD) test ($p < 0.05$) was used to determine significant differences among treatments. The mean and standard deviation were estimated with Microsoft Excel 2017. Graphs were prepared with GraphPad Prism (version 6.01; San Diego, CA, United States).

RESULTS

Bacterial Isolation and Taxonomic Characterization

A total of 11 rhizobacteria were isolated from the rhizosphere and endosphere of *S. borealis* plants. The 16S rDNA and *gyrB* gene sequencing and phylogenetic analysis disclosed that the selected PGPR strain (B11) had a 100% similarity with the known sequences in GenBank and belonged to *Bacillus amyloliquefaciens*. In addition, phylogenetic analysis of the B11 strain using RAxML GUI v.1.3 software revealed its relevance to other strains of respective species (Figure 1). The 16S rDNA and *gyrB* gene sequences obtained in the present study have been submitted to the NCBI GenBank database and assigned the accession nos. MW599955 and MW602944, respectively.

In vitro Plant Growth-Promoting Assay

The results of the *in vitro* growth-promoting assays in all strains are presented in Table 2 and Supplementary Figure 1. Most bacterial strains exhibited IAA and siderophore production, nitrogen fixation, and ACC deaminase activity (except B1). The strains B3–B8 did not show any citrate utilization activity. Our findings revealed variable solubilization activities: the strains B7, B10, and B11 were able to solubilize potassium, zinc, phosphate, and silicon.

In vitro Stress Endurance in Response to Salt, Drought, and Heavy Metal

The bacterial strains were screened *in vitro* for their potential to tolerate salt, drought, and heavy metal stress conditions (Table 3). Most bacterial strains were able to tolerate salinity levels up to

10%, but some bacterial strains (B1, B2, B7, and B10) could not tolerate such levels. The strains B1 and B2 exhibited growth on LB media supplemented with only 0.5% and 2.5% NaCl. Similarly, the strains B7 and B10 could tolerate up to 5% salinity. The growth of strains B3 and B8 was inhibited on LB media supplemented with various concentrations of NaCl. In terms of their response to PEG-amended media, all bacterial strains were able to survive in drought conditions of “−0.05, −0.15, −0.3” MPa, as well as with “−0.49” and “−0.73” MPa osmotic stress (Table 3). Most bacterial strains showed visible growth on LB media supplemented with the tested Cr and Cd concentrations (Table 3). The specific results revealed different growth responses of the bacterial strains exposed to various Ni concentrations. Exceptionally, the strains B3 and B8 did not exhibit heavy metal resistance. The bacterial strain B11 indicated tolerance in all amended media; therefore, this strain was selected for further study (Table 3 and Supplementary Figures 2, 3).

Effects of Several Salt Concentrations on the Growth of Pepper Seedlings

The effect of salinity on pepper seedlings was investigated at NaCl concentrations of 0.5, 1, 1.5, and 2.5%. Treatment with the lowest NaCl concentration (0.5%) produced no remarkable change compared with the control plants (Supplementary Figure 4 and Supplementary Table 3). However, pepper plants treated with NaCl at 1% showed decreased plant height (33.12%), root length (19.35%), stem diameter (50%), leaf length (45.83%), leaf width (40.90%), plant fresh weight (66.66%), plant dry weight (50%), root fresh weight (55.55%), root dry weight (66.66%), and relative leaf number (27%) ($p < 0.05$) relative to these measures in control plants (Supplementary Figure 4 and Supplementary Table 3). Furthermore, irrigation with the highest level of NaCl (2.5%) caused a drastic reduction in plant growth parameters. Thus, 1% NaCl was the optimal concentration chosen for use in further studies.

Pepper Seedling Response to Plant Growth-Promoting Rhizobacteria (B11) Inoculant Under Salinity, Drought, and Heavy Metal Stresses

Plant Growth Attributes

The influence of the selected bacterial strain (B11) on the growth of pepper seedlings without stress and under salinity, drought, and heavy metal stress conditions was evaluated in pot trials (Supplementary Figure 5). The negative effects of salinity, drought, and heavy metal stresses resulted in reduced growth parameters such as plant height, stem diameter, leaf area (length/width), total plant fresh weight, and number of leaves in relation to corresponding parameters in unstressed and uninoculated pepper plants (Table 4). However, plant height, stem diameter, leaf area (length/width), and fresh weight increased in PGPR-inoculated plants under salinity, drought, and heavy metal stress conditions. Specifically, plant height was improved by 14.77, 18.03, and 31.76% in the salt, drought, and Cd treatments in comparison with the corresponding heights of uninoculated and stressed plants ($p < 0.05$). Similarly, in

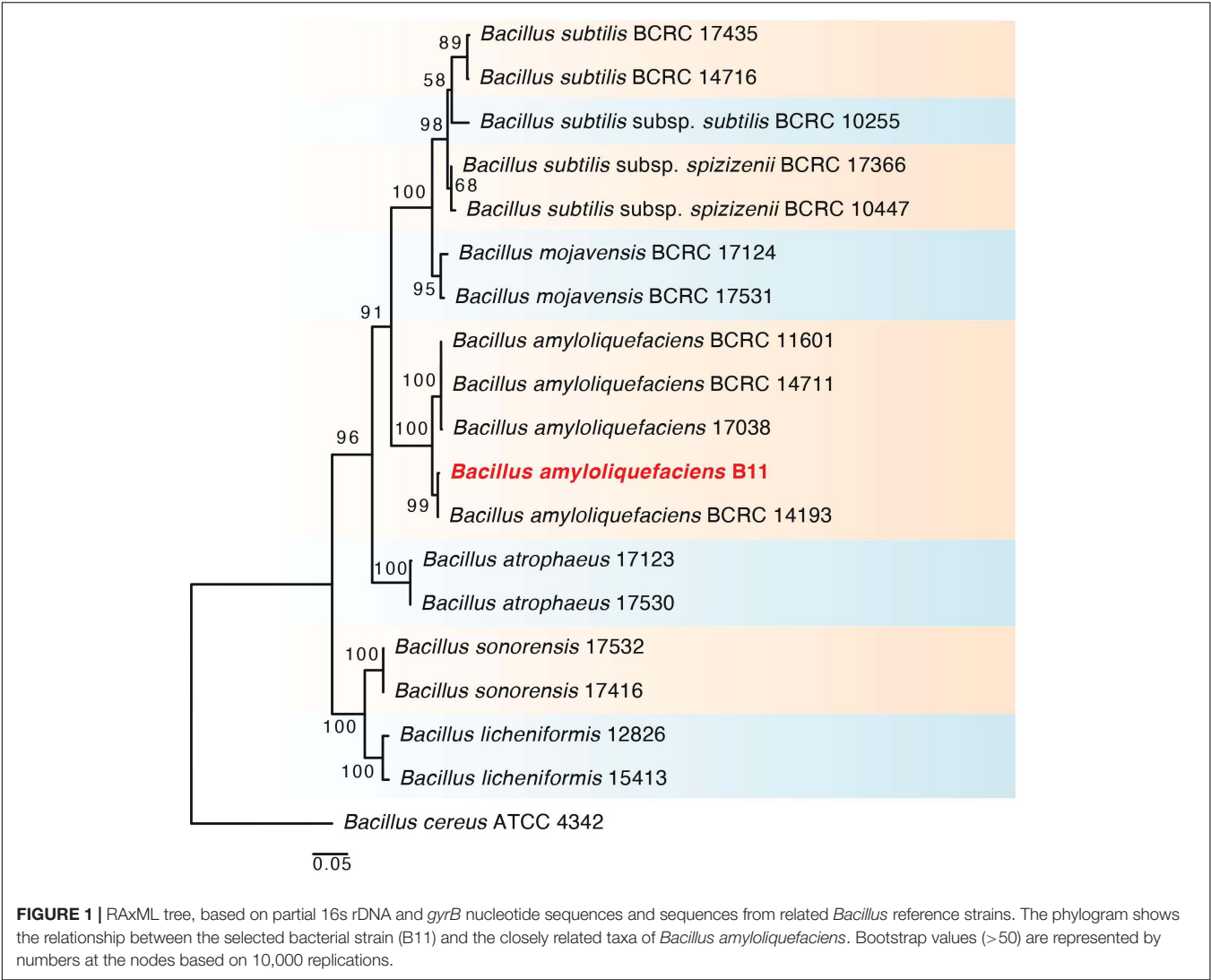


TABLE 2 | Growth-promoting and nutrient solubilization traits of 11 bacterial strains associated with *Sasamorpha* in this study.

Bacterial Isolates	Growth-promoting traits					Nutrient solubilization traits			
	IAA	ACC	Citrate	NF	Siderophore	Halo zone dm (mm)			
						P	Zn	Si	K
B1	+	–	+	+	+	5	4	7	–
B2	+	+	+	+	+	5	4	7	–
B3	+	+	–	+	+	10	–	–	–
B4	+	+	–	+	+	7	7	–	–
B5	+	+	–	+	+	10	5	–	–
B6	+	+	–	+	+	4	–	7	6
B7	+	+	+	+	+	6	10	7	5
B8	+	+	–	+	+	5	–	–	–
B9	+	+	+	+	+	10	5	–	–
B10	+	+	+	+	+	7	5	4	6
B11	+	+	+	+	+	10	10	8	13

+ indicates positive, whereas – indicates negative. ACC, 1-aminocyclopropane-1-carboxylate; IAA, indole-3-acetic acid; NF, nitrogen fixation.

TABLE 3 | Salt, drought, and heavy metal tolerance abilities of bacterial strains associated with pepper in this study.

Bacterial isolates	Growth in NaCl (%)					Growth in PEG 6000 (MPa)					Growth in Heavy metal (g/L)								
	0.5	2.5	5	7.5	10	−0.05	−0.15	−0.3	−0.49	−0.73	Cr			Cd			Ni		
											0.4	0.8	1	0.4	0.8	1	0.4	0.8	1
B1	+	+	−	−	−	+	+	+	+	+	+	+	+	+	+	+	+	−	−
B2	+	+	−	−	−	+	+	+	+	+	+	+	+	−	−	−	−	−	−
B3	−	−	−	−	−	+	+	+	+	+	−	−	−	−	−	−	−	−	−
B4	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	−	−	−
B5	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	−
B6	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	−
B7	+	+	+	−	−	+	+	+	+	+	+	+	+	+	+	+	−	−	−
B8	−	−	−	−	−	+	+	+	+	+	−	−	−	−	−	−	−	−	−
B9	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	−	−	−
B10	+	+	+	−	−	+	+	+	+	+	+	+	+	+	+	+	−	−	−
B11	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+

+ indicates positive, whereas − indicates negative. PEG, polyethylene glycol.

TABLE 4 | Effect of PGPR inoculation on pepper plant growth, chlorophyll a (Chla), chlorophyll b (Chlb), total chlorophyll (total Chl), and carotenoid contents under normal and stress conditions after 8 days of treatment (8DAT).

Treatment	Plant height	Root length	Stem diameter	Leaf length	Leaf width	Total Plant fresh weight	Chla	Chlb	Total Chl	Carotenoid	No. leaf
	(cm)	(cm)	(cm)	(cm)	(cm)	(g)	(μg/g FW)	(μg/g FW)	(μg/g FW)	(μg/g FW)	
8DAT											
Cont	20.6 ± 0.6a	18.0 ± 0.5e	0.3 ± 0.0a	9.4 ± 0.2a	5.5 ± 0.1a	12.11 ± 0.1b	25.9 ± 7.2a	9.9 ± 2.5f	104.4 ± 0.7b	1.3 ± 0.0a	16.0 ± 0.0a
B11	20.1 ± 0.2b	24.0 ± 0.5a	0.3 ± 0.0a	9.2 ± 0.5b	5.5 ± 0.4a	15.38 ± 0.6a	23.8 ± 6.0c	27.6 ± 7.1a	116.7 ± 1.6a	1.0 ± 0.5b	16.0 ± 0.0a
S	15.0 ± 1.0g	14.0 ± 0.5h	0.2 ± 0.0c	7.0 ± 0.5g	3.8 ± 0.4f	4.50 ± 0.7h	13.9 ± 3.0g	17.4 ± 4.0c	69.6 ± 6.3f	0.7 ± 0.9g	12.6 ± 0.5e
S+B11	17.6 ± 0.3d	18.8 ± 0.4d	0.3 ± 0.0a	7.8 ± 0.2e	4.6 ± 0.5d	9.84 ± 0.6e	21.2 ± 4.1d	18.5 ± 3.3b	97.3 ± 1.7c	0.9 ± 1.7d	14.3 ± 0.3b
Dr	15.1 ± 0.4f	14.5 ± 0.2g	0.2 ± 0.0c	7.5 ± 0.1f	4.1 ± 0.2e	6.38 ± 0.1g	13.59 ± 2.6h	12.4 ± 2.1e	68.33 ± 5.9g	0.72 ± 1.2f	13.0 ± 1.1d
Dr+B11	18.3 ± 0.1c	19.8 ± 0.6c	0.3 ± 0.0a	8.6 ± 0.2c	5.1 ± 0.3c	9.96 ± 0.1d	20.96 ± 3.7e	16.0 ± 2.3d	96.04 ± 1.5e	0.93 ± 2.0c	14.0 ± 0.5c
Cd	11.6 ± 2.3h	15.6 ± 0.4f	0.2 ± 0.0c	3.9 ± 2.0h	2.2 ± 1.1g	6.92 ± 0.4f	15.1 ± 2.6f	6.8 ± 0.2h	62.1 ± 1.4h	0.8 ± 1.4e	5.0 ± 2.6f
Cd+B11	17.0 ± 1.1e	22.0 ± 0.5b	0.3 ± 0.0a	8.1 ± 0.3d	5.2 ± 0.4b	10.37 ± 0.3c	23.9 ± 4.4b	9.6 ± 0.4g	97.1 ± 4.7d	1.0 ± 2.2b	13.0 ± 1.0d

Treatments: control + water, control + plant growth-promoting rhizobacteria (PGPR), control + 1%NaCl, PGPR + 1% NaCl, control + 10% polyethylene glycol (PEG), PGPR + 10% PEG, control + 0.1% Cd, and PGPR + 0.1% Cd. Values show the means ± SE (n = 6) and significant differences at $p < 0.05$ [least significant difference (LSD) test].

bacterized plants, the total plant fresh weight was increased by 54.26%, 35.94%, and 33.26% in the salt, drought, and Cd treatments compared with the weights of the control stressed group of plants (Table 4).

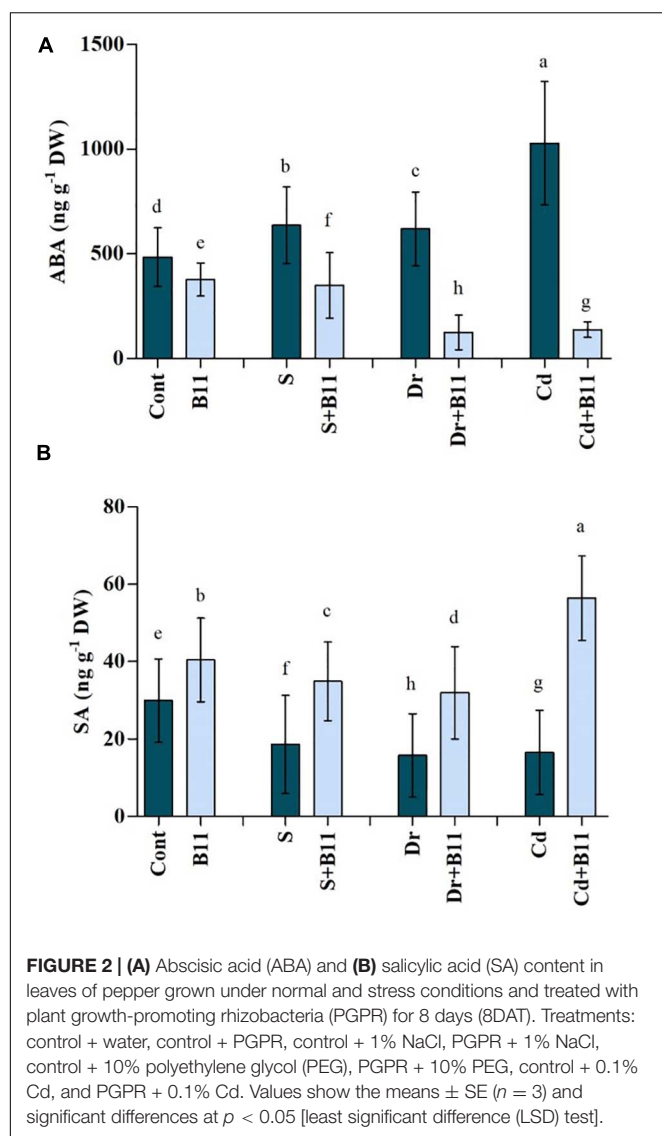
Chlorophyll and Carotenoid Content

Chlorophyll and carotenoid content was determined for pepper plants under both normal and stress conditions. Abiotic stresses are known to negatively affect the photosynthetic pigments of pepper plants. In the present study, plant pigments showed that Chla, Chlb, and carotenoid contents were increased in stressed plants inoculated with the bacterial strain (B11) compared with uninoculated stressed plants. Similarly, all the PGPR-treated stressed plants showed higher values of total chlorophyll content than the control stressed plants (Table 4). A considerable decrease in total chlorophyll content was observed in salt, drought, and heavy metal-stressed plants (33.33, 34.54, and 40.51%, respectively) in comparison with control plants.

However, the PGPR inoculation was effective ($p < 0.05$) and resulted in a 28.46, 28.85, and 36.04% increase in total chlorophyll content under salt, drought, and heavy metal stress conditions, respectively, compared with the control stressed plants (Table 4).

Phytohormone (Abscissic Acid and Salicylic Acid) Regulation

Endogenous ABA and SA contents were investigated over a period of 8 days to determine the influence of PGPR inoculation on pepper growth under control and abiotic stress conditions. Abiotic stresses induced an accumulation of ABA in pepper seedlings. However, PGPR treatment reduced ABA levels in pepper plants relative to the levels in control plants not subjected to abiotic stresses. Upon exposure to salinity, drought, and heavy metal stresses, PGPR-inoculated plants exhibited significantly reduced content of ABA, i.e., 45.15, 79.92, and 86.52%, respectively, compared with the content of uninoculated stressed plants (Figure 2A).



Compared with stressed plants, uninoculated plants showed a decrease in SA concentrations, which were reduced by 37.70% with salinity, 47.42% with drought, and 44.64% with heavy metals relative to the SA concentrations in control plants. Pepper seedlings inoculated with PGPR for 8 days, however, showed remarkable 46.55%, 50.70%, and 70.61% increases in SA content under salt, drought, and heavy metal stresses, respectively, compared with uninoculated stressed plants (**Figure 2B**). Indeed, our results suggested that PGPR inoculation resulted in enhanced SA content in pepper seedlings with or without stress.

Free Amino Acid Content

Eighteen amino acids with different concentrations were detected in pepper seedlings (**Table 5**). Over 8 days, salinity, drought, and heavy metal stresses increased amino acid content in pepper seedlings compared with seedlings under normal conditions. Proline content increased by 71.90%, 64.10%, and 58.83% in salinity-, drought-, and heavy metal-stressed plants, respectively.

Similarly, plants with PGPR showed an increase in proline content. Additionally, 8 days after the application of PGPR in stressed plants, glutamic acid was found at the highest levels, whereas cystine and methionine were found at the lowest level in pepper seedlings subjected to normal and stress conditions (**Table 5**). These findings indicate that PGPR inoculation improved amino acid content under both stress and normal conditions.

Soluble Protein and Sugar Content

Protein content increased in stressed seedlings over 8 days. Under normal conditions, the plant protein level increased by 21.45% upon PGPR inoculation compared with protein levels in uninoculated plants. The protein production rate was increased by 12.58%, 26.80%, and 22.77% under salinity, drought, and heavy metal stresses, respectively, when compared with protein production in unstressed plants ($p < 0.05$) (**Figure 3A**). Conversely, reductions in protein production were detected in PGPR-treated plants when they were exposed to abiotic stresses.

In the leaves of pepper plants, a substantial reduction in sugar content was observed after exposure to stress conditions (**Figure 3B**). Furthermore, sugar content decreased clearly in response to salt (24.31%), drought (17.77%), and heavy metal (41.43%) stresses when compared with the sugar content of plants under normal conditions (**Figure 3B**). The best outcomes were obtained when plants were treated with PGPR, which resulted in an increase in sugar content by 52.10%, 43.98%, and 51.93% under salt, drought, and heavy metal stress conditions relative to the sugar content of stressed plants alone (**Figure 3B**).

H₂O₂ and Malondialdehyde Content

Drought, salinity, and heavy metal stresses caused significant changes in H₂O₂ content in pepper plants (**Figure 3C**). H₂O₂ content was increased by 33.59%, 43.52%, and 47.20% under salinity, drought, and heavy metal stresses, respectively, compared with the H₂O₂ content in control plants. However, the application of PGPR was effective in reducing H₂O₂ content in stressed plants; maximum decreases of 16.05%, 28.23%, and 49.87% in H₂O₂ content were noted in PGPR-inoculated plants under salinity, drought, and heavy metal stresses, respectively ($p < 0.05$).

As shown in **Figure 3D**, stress conditions enhanced MDA production in untreated pepper plants; MDA content increased by 34.32%, 32.27%, and 49.37% under salt, drought, and heavy metal stress conditions, respectively. Compared with uninoculated plants, the reduction in MDA content in PGPR-treated plants was approximately 32.49% under salt, 29.71% under drought, and 31.05% under heavy metal stress conditions ($p < 0.05$).

Antioxidant Content

1,1-diphenyl-2-picrylhydrazyl content in pepper seedlings increased under stress conditions, whereas DPPH content was reduced in PGPR-treated seedlings exposed to all stresses. Specifically, PGPR inoculation contributed to 9.73%, 59.06%, and 37.99% reductions in DPPH content under salinity, drought, and heavy metal stresses relative to the DPPH detected

TABLE 5 | Effect of PGPR inoculation on the amino acid content of pepper plants grown under normal and stress conditions after 8 days of treatment (8DAT).

Amino acid mg/g	Treatment							
	Cont	B11	S	S+B11	Dr	Dr+B11	Cd	Cd+B11
8DAT								
Asp	13.76 ± 0.0h	23.13 ± 0.0b	18.69 ± 0.0f	20.53 ± 0.0c	16.16 ± 0.0g	23.48 ± 0.0a	19.84 ± 0.0e	20.25 ± 0.0d
Thr	6.91 ± 0.0h	12.08 ± 0.0a	10.01 ± 0.0e	10.75 ± 0.0c	8.18 ± 0.0g	11.88 ± 0.0b	8.55 ± 0.0f	10.04 ± 0.0d
Ser	6.30 ± 0.0h	14.22 ± 0.0a	11.03 ± 0.0d	11.85 ± 0.0c	7.0 ± 0.0g	13.19 ± 0.0b	8.53 ± 0.0f	10.84 ± 0.0e
Glu	18.72 ± 0.0h	33.06 ± 0.0d	26.20 ± 0.0f	31.0 ± 0.0e	22.40 ± 0.0g	33.70 ± 0.0c	37.37 ± 0.0b	48.50 ± 0.0a
Gly	8.81 ± 0.0h	14.86 ± 0.0a	12.62 ± 0.0e	13.46 ± 0.0c	10.02 ± 0.0g	14.49 ± 0.0b	11.66 ± 0.0f	13.44 ± 0.0d
Ala	9.35 ± 0.0h	16.09 ± 0.0a	13.28 ± 0.0e	13.94 ± 0.0c	11.46 ± 0.0f	16.02 ± 0.0b	11.10 ± 0.2g	13.82 ± 0.0d
Cys	1.09 ± 0.0h	2.15 ± 0.0e	2.19 ± 0.0d	2.57 ± 0.0b	1.66 ± 0.0g	1.81 ± 0.0f	2.32 ± 0.0c	5.29 ± 0.0a
Val	7.56 ± 0.0h	13.75 ± 0.0a	11.06 ± 0.0e	12.09 ± 0.0c	9.38 ± 0.0f	12.64 ± 0.0b	9.36 ± 0.0g	11.27 ± 0.0d
Met	1.10 ± 0.2h	2.30 ± 0.0b	2.31 ± 0.0a	2.03 ± 0.0d	2.0 ± 0.0e	2.10 ± 0.0c	1.67 ± 0.0g	1.97 ± 0.0f
Ile	6.34 ± 0.0h	11.13 ± 0.0a	8.93 ± 0.0e	9.87 ± 0.0c	7.59 ± 0.0f	10.14 ± 0.0b	7.45 ± 0.0g	9.31 ± 0.0d
Leu	13.91 ± 0.0h	23.92 ± 0.0a	19.59 ± 0.0e	20.80 ± 0.0c	16.77 ± 0.0f	23.17 ± 0.0b	15.76 ± 0.0g	20.06 ± 0.0d
Tyr	4.41 ± 0.0h	7.56 ± 0.0a	6.89 ± 0.0d	7.10 ± 0.2c	5.29 ± 0.0g	7.23 ± 0.0b	5.63 ± 0.0f	6.65 ± 0.0e
Phe	8.03 ± 0.0h	13.82 ± 0.0a	11.32 ± 0.0e	12.22 ± 0.0c	9.68 ± 0.0g	13.47 ± 0.0b	9.97 ± 0.0f	11.89 ± 0.0d
Lys	11.99 ± 0.0h	20.46 ± 0.0a	16.36 ± 0.0e	18.35 ± 0.0c	13.57 ± 0.0g	19.7 ± 0.0b	15.14 ± 0.0f	17.52 ± 0.0d
NH3	1.37 ± 0.0h	3.73 ± 0.0e	2.66 ± 0.0f	4.61 ± 0.0c	1.81 ± 0.0g	4.10 ± 0.2d	4.66 ± 0.0b	4.69 ± 0.0a
His	3.46 ± 0.0h	6.12 ± 0.0a	4.95 ± 0.0f	5.59 ± 0.0c	4.06 ± 0.0g	6.09 ± 0.0b	5.02 ± 0.0e	5.50 ± 0.0d
Arg	7.97 ± 0.0h	14.67 ± 0.0a	12.08 ± 0.0d	13.62 ± 0.0c	9.56 ± 0.0g	13.87 ± 0.0b	9.57 ± 0.0f	11.60 ± 0.0e
Pro	2.74 ± 0.0h	5.78 ± 0.0e	7.10 ± 0.0c	9.74 ± 0.0a	3.32 ± 0.0g	7.63 ± 0.0b	3.67 ± 0.0f	6.65 ± 0.0d

Treatments: control + water, control + plant growth-promoting rhizobacteria (PGPR), control + 1% NaCl, PGPR + 1% NaCl, control + 10% polyethylene glycol (PEG), PGPR + 10% PEG, control + 0.1% Cd, and PGPR + 0.1% Cd. Values show the means ± SE (n = 6) and significant differences at $p < 0.05$ [least significant difference (LSD) test].

in uninoculated stressed plants (**Figure 4A**). Furthermore, total polyphenol content was slightly enhanced under stress conditions, but it decreased under these conditions upon PGPR inoculation (**Figure 4B**).

Enhanced SOD activity was observed in pepper plants under stress conditions; however, SOD activity was lower in PGPR-treated plants subjected to the salinity (15.25%), drought (9.62%), and heavy metal (31.83%) stresses when compared with that of untreated stressed plants (**Figure 4C**).

Similar trends were observed with POD, PPO, and flavonoid activities, which increased distinctly under stress conditions. However, PGPR treatment reduced POD, PPO, and flavonoid content under stress conditions; for instance, their activities decreased (POD, 13.69%; PPO, 12.31%; flavonoid, 6.20%) in PGPR-inoculated seedlings subjected to salt stress ($p < 0.05$) (**Figures 4D–F**).

Macronutrient, Sodium, and Cadmium Content in Plant and Bacterial Cells

To determine the effect of the PGPR inoculant on the macronutrient state of pepper plants and its detoxifying role, five elements, i.e., Ca, K, P, Na, and Cd, were examined (**Table 6**). Under normal conditions, an increase was detected in the concentrations of K and P in plants inoculated with PGPR relative to the respective concentrations in control plants. Furthermore, Ca content was higher in inoculated plants than in control plants under normal conditions. Compared with the stressed plants and the PGPR-inoculated plants, plant macronutrients were regulated in inoculated stressed plants, which showed increases in K and

P concentrations and a decrease in the concentration of Ca under stress conditions. Moreover, PGPR significantly reduced the accumulation of Na and Cd in plants under salinity and heavy metal stresses. Na and Cd uptake by bacterial cells is presented in **Table 6**. These results validate the detoxifying role of PGPR. The removal efficiency of Na and Cd by bacterial strain B11 was 67.08% and 45.24%, respectively.

Effect of Plant Growth-Promoting Rhizobacteria Treatment on the Expression of Salt-, Drought-, and Heavy Metal-Responsive Genes

Pepper seedlings were used to study the expression of abiotic stress-responsive genes. In total, eight genes (**Supplementary Table 2**) were analyzed for their change in expression in pepper plant seedlings under abiotic stresses and PGPR inoculation (B11).

Expression of Genes Related to Binding Immunoglobulin Proteins

This study characterized three binding immunoglobulin protein (BiP) genes (*CaBiP1*, *CaBiP2*, and *CaBiP3*) in pepper plants. These genes showed differential responses in pepper seedlings under abiotic stress and PGPR inoculation. Salt-, drought-, and heavy metal-stressed plants showed an increase in *CaBiP1* expression levels relative to expression in unstressed control plants; however, among salt-, drought-, and heavy metal-stressed plants, the PGPR inoculation reduced expression by

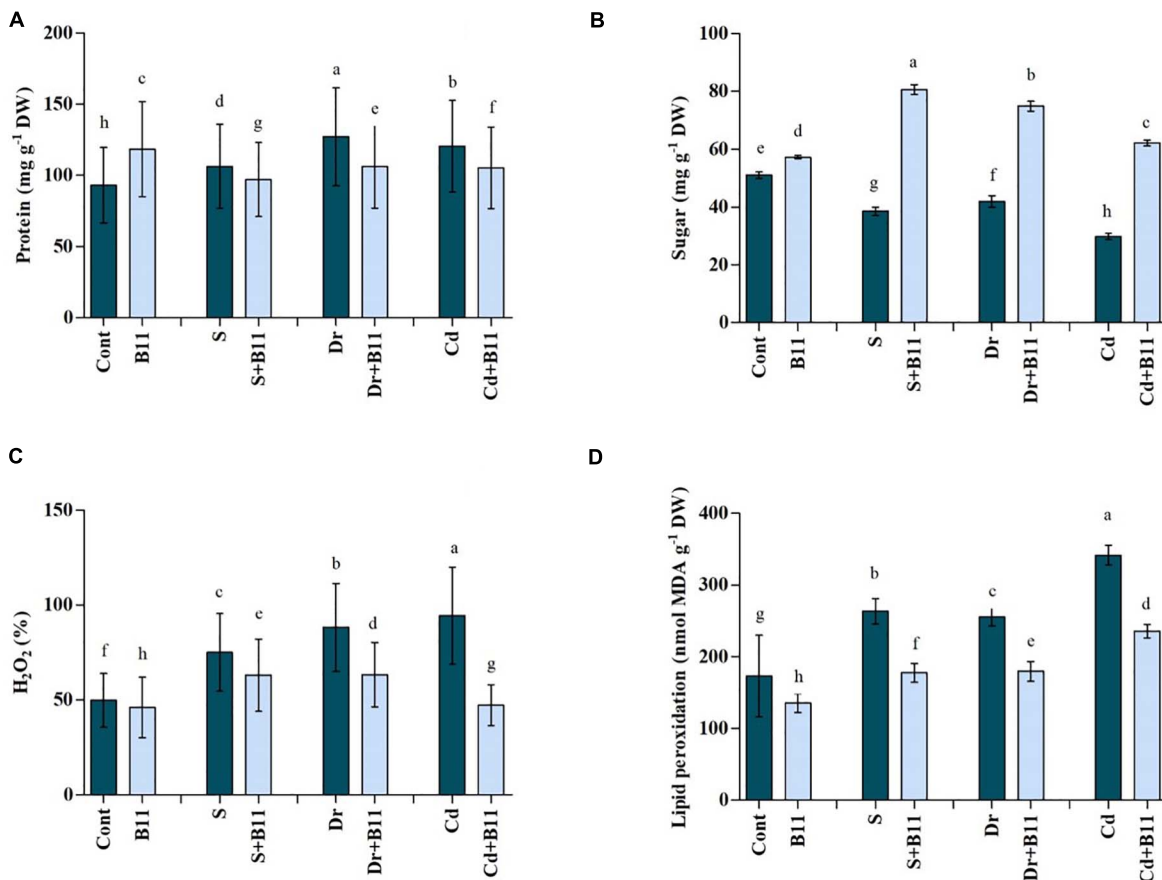


FIGURE 3 | (A) Protein, **(B)** H_2O_2 , **(C)** sugar, and **(D)** malondialdehyde (MDA) content in leaves of pepper grown under normal and stress conditions and treated with plant growth-promoting rhizobacteria (PGPR) for 8 days (8DAT). Treatments: control + water, control + PGPR, control + 1% NaCl, PGPR + 1% NaCl, control + 10% polyethylene glycol (PEG), PGPR + 10% PEG, control + 0.1% Cd, and PGPR + 0.1% Cd. Values show the means $\pm$ SE ($n = 3$) and significant differences at $p < 0.05$ [least significant difference (LSD) test].

76.30, 86.19, and 93.37%, respectively, compared with that in uninoculated plants (Figure 5A). Additionally, abiotic stresses enhanced *CaBiP2* gene expression in uninoculated stressed plants in comparison with inoculated control plants. PGPR-inoculated salt-, drought-, and heavy metal-stressed plants showed lower *CaBiP2* gene expression relative to the expression in uninoculated stressed plants (Figure 5B). *CaBiP3* expression levels were increased significantly in heavy metal-stressed plants in comparison with control plants; however, under heavy metal stress conditions, *CaBiP3* expression reduced remarkably (96.53%) in PGPR-inoculated stressed plants (Figure 5C). Furthermore, PGPR-inoculated salt-stressed plants showed a 60.22% decrease in *CaBiP3* expression compared with expression levels in uninoculated salt-stressed plants.

Expression of Genes Related to Xyloglucan Endotransglucosylase/Hydrolase

The quantitative expressions of *CaXTH1* and *CaXTH2* genes in pepper seedlings under abiotic stresses and PGPR inoculation

are shown in Figures 5D,E. Application of abiotic stresses reduced *CaXTH* gene expression in pepper plants, whereas the expression of these genes increased in PGPR-treated plants. For instance, PGPR exposure enhanced *CaXTH2* gene expression by approximately 75.69%, 66.64%, and 93.26% under salinity, drought, and heavy metal stresses compared with the respective expressions in stressed plants alone.

Expression of Genes Related to BAX Inhibitor 1 (*CaBI-1*)

The effects of salinity, drought, and heavy metal stresses as well as PGPR inoculation on BAX inhibitor 1 (*BI-1*; anti-PCD) was studied in pepper seedlings through the change in the expression of the *BI-1* gene (*CaBI-1*) (Figure 5F). Expression levels of this gene in control and PGPR-inoculated plants displayed minor differences under unstressed conditions; however, higher *CaBI-1* expression was noticed in stressed plants. PGPR-inoculated plants showed a decrease in *CaBI-1* expression compared to uninoculated stressed plants (38.79% under salinity, 97.40% under drought, and 95.79% under heavy metal stress conditions).

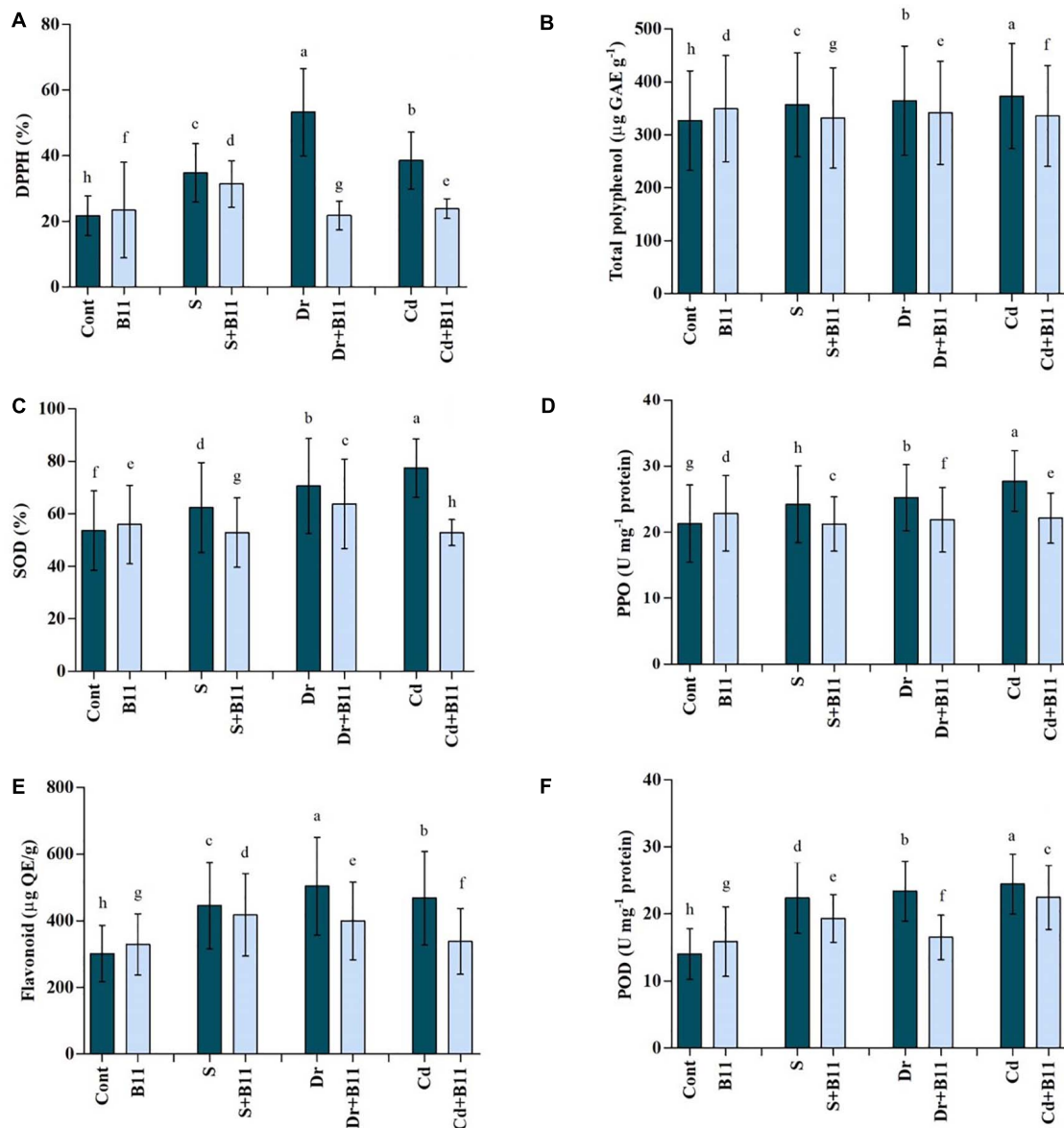


FIGURE 4 | Antioxidant content [DPPH, **A**; total polyphenol, **B**; superoxide dismutase (SOD), **C**; polyphenol oxidase (PPO), **D**; flavonoids, **E**; and peroxidase (POD), **F**] of pepper leaves grown under normal and stress conditions and treated with plant growth-promoting rhizobacteria (PGPR) for 8 days (8DAT). Treatments: control + water, control + PGPR, control + 1% NaCl, PGPR + 1% NaCl, control + 10% polyethylene glycol (PEG), PGPR + 10% PEG, control + 0.1% Cd, and PGPR + 0.1% Cd. Values show the means $\pm$ SE ($n = 3$) and significant differences at $p < 0.05$ [least significant difference (LSD) test].

Expression of Transcription Factor *WRKY2*

The expression of the gene related to the transcription factor *WRKY2* (*CaWRKY2*) was also examined. Salinity-, drought-, and heavy metal-stressed plants exhibited a 66.35%, 65.03%, and 75.03% increase, respectively, in *CaWRKY2* expression levels compared with the expression observed in unstressed plants. However, PGPR-inoculated stressed plants showed a decrease in gene expression levels relative to the expression recorded in uninoculated stressed plants. *CaWRKY2* levels in stressed pepper plants were reduced in PGPR-treated plants

in comparison to uninoculated stressed plants (64.06% under salinity, 93.74% under drought, and 90.61% under heavy metal stresses; **Figure 5G**).

Expression of Genes Related to Ethylene (*CaPTI1*)

The expression level of *CaPTI1* gene was evaluated in pepper seedlings subjected to abiotic stresses and PGPR inoculation. As shown in **Figure 5H**, salinity-, drought-, and heavy metal-stressed plants demonstrated a 94.82%, 93.84%, and 97.19% rise, respectively, in *CaPTI1* expression levels in comparison

TABLE 6 | Macronutrient, Na, and Cd accumulation in pepper plants and bacterial cells grown under stress and control conditions with or without PGPR.

Sample name	Ca ($\mu\text{g/kg}$)	K ($\mu\text{g/kg}$)	P ($\mu\text{g/kg}$)	Na ($\mu\text{g/kg}$)	Cd ($\mu\text{g/kg}$)
8DAT-Plant					
Cont	6.45 $\pm$ 0.05g	41.38 $\pm$ 0.58h	5.01 $\pm$ 0.01h	3.13 $\pm$ 0.03e	ND
B11	8.38 $\pm$ 0.18d	49.64 $\pm$ 0.50g	6.76 $\pm$ 0.23c	4.10 $\pm$ 0.10c	ND
S	12.88 $\pm$ 0.11a	54.11 $\pm$ 1.0e	5.31 $\pm$ 0.11g	13.74 $\pm$ 0.26a	ND
S+B11	8.81 $\pm$ 0.31c	63.35 $\pm$ 2.05a	6.68 $\pm$ 0.22d	8.88 $\pm$ 0.38b	ND
Dr	7.94 $\pm$ 0.05f	50.38 $\pm$ 0.38f	5.40 $\pm$ 0.20f	3.10 $\pm$ 0.10f	ND
Dr+B11	6.28 $\pm$ 0.08h	55.71 $\pm$ 0.99d	6.80 $\pm$ 0.30b	4.10 $\pm$ 0.10c	ND
Cd	11.06 $\pm$ 0.13b	55.81 $\pm$ 0.50c	5.50 $\pm$ 0.20e	3.25 $\pm$ 0.05d	1.65 $\pm$ 0.35a
Cd+B11	8.11 $\pm$ 0.11e	57.63 $\pm$ 1.0b	7.73 $\pm$ 0.26a	4.10 $\pm$ 0.10c	0.52 $\pm$ 0.02b
Sample name	Ca (mg/L)	K (mg/L)	P (mg/L)	Na (mg/L)	Cd (mg/L)
Bacterial cell					
B11	1.69 $\pm$ 0.03c	237.72 $\pm$ 1.07b	70.93 $\pm$ 0.46a	165.53 $\pm$ 0.19b	ND
S+B11	1.94 $\pm$ 0.20a	378.81 $\pm$ 0.27a	67.53 $\pm$ 0.27c	3,462.91 $\pm$ 8.43a	ND
Cd+B11	1.90 $\pm$ 0.02d	234.55 $\pm$ 1.55d	58.02 $\pm$ 0.12b	163.81 $\pm$ 0.90d	631.10 $\pm$ 9.43a
PEG+B11	1.42 $\pm$ 0.04b	214.26 $\pm$ 0.86c	67.57 $\pm$ 0.86d	145.45 $\pm$ 1.33c	ND

Values show the means $\pm$ SE ($n = 6$) and significant differences at $p < 0.05$ (LSD test). LSD, least significant difference; PEG, polyethylene glycol; PGPR, plant growth-promoting rhizobacteria.

with the expression detected in unstressed plants while reduced expression level was observed in PGPR-inoculated stressed plants. PGPR treatment lowered the *CaPT11* expression level by 83.44% under salinity, 94.75% under drought, and 82.07% under heavy metal stresses compared to the uninoculated stressed plants (**Figure 5H**).

DISCUSSION

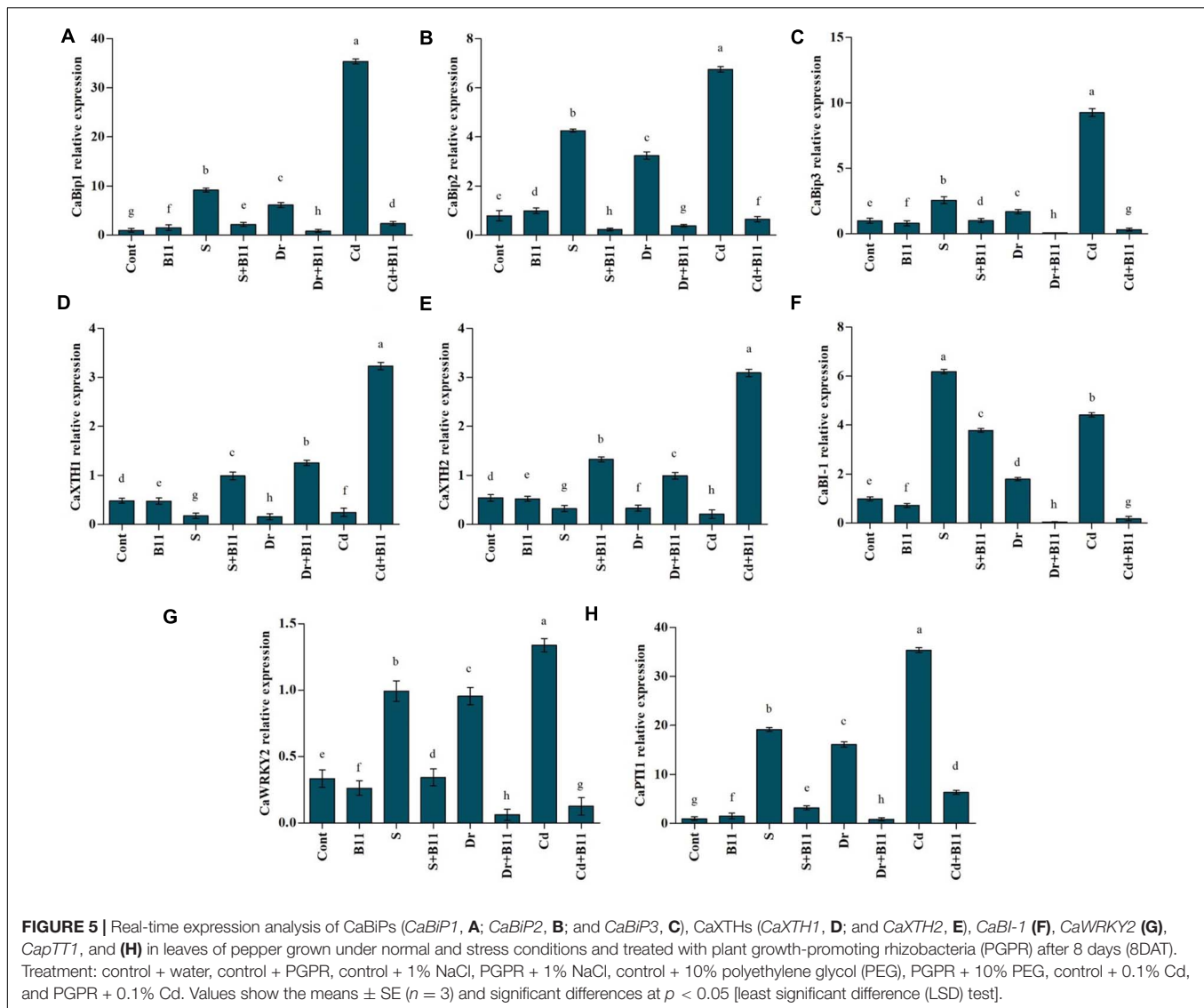
Plants and bacteria have established synergistic interactions to suppress deleterious stresses (Khan et al., 2019; Pandey and Gupta, 2019). The use of PGPR as a bioinoculant is a promising tool for crop improvement (Pan et al., 2019). For example, PGPR is capable of solubilizing phosphate and releasing different chelating agents; hence, it influences the movement and accessibility of various metabolites in the rhizosphere and mediates the process of phytoremediation and nutrient recycling. Additionally, PGPR can also function in plant growth, management of plant diseases, remediation of hazardous substances, and improvement of soil structure and fertility (Jing et al., 2007; Tangahu et al., 2011; Oves et al., 2017). Plant microbe cooperation provides a specific source of carbon to bacteria that allows them to mitigate the toxicity of polluted regions (Khan and Bano, 2016).

Our results demonstrate that stressed seedlings inoculated with PGPR maintain higher growth parameters in contrast to stressed plants without PGPR treatment. We observed increases in chlorophyll and carotenoid contents in the leaves of PGPR-treated plants under stress conditions. Moreover, we found that PGPR treatment increased K and P contents and reduced Ca, Na, and Cd levels relative to the effects observed in uninoculated stressed plants. Previous studies have indicated that PGPR inoculation boosts the photosynthetic pigments in plants under stress conditions (Izanloo et al., 2008;

Bhattacharyya and Jha, 2012). This could be attributed to the higher accessibility and uptake of nutrients from the rhizosphere, which helps maintain plant growth under stress conditions (Çakmakçı et al., 2007; Pii et al., 2015).

Xyloglucan endotransglucosylase/hydrolases (XTHs), as cell growth promoters, play a crucial role in plant development. Moreover, they are involved in plant responses to environmental stimuli including salinity (Cho et al., 2006), water deficit (Choi et al., 2011), heat, cold (Xu et al., 1995), and flood (Saab and Sachs, 1995). In the current study, reduced XTH gene expression was observed in stressed plants, which likely led to decreased cell wall extensibility and retardation in pepper seedlings. In contrast, PGPR-treated stressed plants showed higher XTH gene expression, which led to enhanced plant height and leaf length/width. It has been shown that overexpression of XTH1 and XTH3 enhances salt and drought tolerance in tobacco and pepper plants (Choi et al., 2011; Han et al., 2013). Our results show that PGPR application ameliorates the expression of XTHs (*CaXTH1* and *CaXTH2*) and improves abiotic stress tolerance in pepper seedlings.

Phytohormones regulate the growth and development of plants and help plants acclimate under environmental challenges (Zhang et al., 2006; Yazdanpanah et al., 2011). SA improves photosynthetic and growth parameters in plants and antagonizes oxidative damage in plants in response to abiotic stress (Nazar et al., 2011; Wani et al., 2016). ET is engaged in plant growth, fruit ripening, and leaf senescence along with plant reaction to abiotic and biotic stresses (Gamalero and Glick, 2012). Previous studies have reported the impact of abiotic stress on phytohormone content, including that of SA, ET, and ABA (Yang et al., 2017; Riyazuddin et al., 2020). Yasuda et al. (2008) indicated that antagonistic crosstalk exists between SA and ABA signaling. The present study results indicate that abiotic stresses increase ABA and ET contents but reduce SA levels, which is in accordance with previous reports (Madhaiyan et al., 2006;



Khan et al., 2013; Ryu and Cho, 2015; Wu et al., 2017). Our findings show that treatment with PGPR improves stress tolerance in pepper seedlings by lowering the ABA and ET contents and elevating the SA content.

WRKY, one of the largest transcription factor families, is involved in various developmental and physiological activities including abiotic and biotic stress signaling pathways (Huang and Duman, 2002; Seki et al., 2002; Rizhsky et al., 2004; Oh et al., 2006; Qiu and Yu, 2009; Cheng et al., 2016). Analysis of WRKY expression in the current study revealed its high expression under abiotic stress conditions, which was consistent with a previous report (Pandey and Somssich, 2009). Several WRKY transcription factors have been found to function in ABA and SA signaling pathways (Jiang and Yu, 2009; Jiang and Yu, 2015). ABA and SA also show antagonistic interactions toward each other in response to abiotic stresses, which is supported by our results (Yasuda et al., 2008). One previous study indicated that ABA negatively or positively controls the transcripts of some WRKYs

(Xie et al., 2005). We observed that *WRKY2* transcript levels were higher in pepper plants exposed to abiotic stresses in the presence of ABA. On the other hand, when ABA levels were reduced in PGPR-treated stressed plants, the expression level of *WRKY2* decreased. These observations demonstrate that ABA positively mediates the expression of *WRKY2* in pepper plants. Taken together, our findings related to *WRKY2* (*CaWRKY2*), ABA, and SA levels in PGPR-treated plants allow us to conclude that PGPR helps stressed plants to cope with various abiotic stresses.

Unfavorable environmental conditions have harmful effects on plant growth and development, and they can trigger protein denaturation or misfolding (Wang et al., 2017). Endoplasmic reticulum stress is activated by misfolded proteins that accumulate in the endoplasmic reticulum under adverse environmental conditions and ultimately result in program cell death (Howell, 2013; Mishiba et al., 2013). Accumulation of misfolded proteins leads to upregulation of BiP genes, which prevent protein augmentation and promote plant tolerance to

abiotic and biotic stresses (Mori, 2000). BiPs play key roles in protein quality control by identifying and refolding misfolded proteins (Yang et al., 2016; Wang et al., 2017). In the present study, biochemical/molecular analyses revealed that stressed plants showed increased protein content by the end of the experiment. BiP genes (*CaBiP1*, *CaBiP2*, and *CaBiP3*) were highly expressed due to salinity, drought, and heavy metal stress conditions. This could have been due to an accumulation of unfolded proteins, which may have led to the enhanced induction of BiPs. On the other hand, BiPs were repressed in PGPR-treated plants subjected to stress conditions. Thus, our findings show that PGPR reduces the soluble protein content of stressed plants, which may be due to the stress-relieving effect of PGPR and consequent protein catabolism.

With environmental stresses, plants produce reactive oxygen species (ROS), which at high levels can cause oxidative damage, impair membrane lipid functions, inactivate enzymes, and impede metabolic activities (Huang et al., 2019). In the present study, we observed that H_2O_2 and MDA levels were clearly higher in stressed plants, which agrees with a previous report (Batool et al., 2020). PGPR application, however, obviously mitigated the high H_2O_2 and MDA levels in stressed plants by the end of the experiment. Therefore, PGPR might suppress the production of ROS and thereby prevent oxidative-based cell membrane damage under environmental stress (Han and Lee, 2005; Singh et al., 2015; Banik et al., 2016; Batool et al., 2020).

Reactive oxygen species are known to be key players in programmed cell death (PCD), a cell suicide process that eliminates damaged cells. BCL2-associated x protein (BAX) has been implicated as an important regulator of PCD and is balanced by the activity of an anti-PCD factor, *BI-1* (Hückelhoven, 2004). In pepper plants, *CaBI-1* expression is induced in response to several types of environmental stresses, such as cold, salinity, drought, submergence, and heavy metal stress, and it provides tolerance to plants toward these stresses (Isbat et al., 2009; Jaiswal et al., 2019). Increased expression of *CaBI-1* was detected in the stressed pepper plants studied here, which is in agreement with the findings of a previous study (Isbat et al., 2009). However, we observed reduced *CaBI-1* expression in stressed plants treated with PGPR. This finding confirms the efficiency and ameliorating effect of PGPR in pepper plants subjected to salinity, drought, and heavy metal stresses.

To cope with oxidative damage and reduce excessive ROS accumulation, plants have developed defensive mechanisms that involve antioxidants with enzymatic or nonenzymatic activities (Agarwal and Pandey, 2004). Several studies have demonstrated that PGPR boosts the activity of enzymatic/nonenzymatic antioxidants. In the present study, the activity of antioxidants increased in stressed plants, but this activity was reduced following PGPR treatment in these abiotically stressed plants. This decrease in antioxidant activity indicates that PGPR improves the ability to scavenge excessive ROS and reduces oxidative damage, which contributes to the protection of photosynthetic processes (Banik et al., 2016).

Sugar sustains macromolecule and membrane structure during drastic water loss in plants; environmental stress can lower leaf sugar content and thereby lead to physiological

and biochemical changes (Fenando et al., 2000). It has been demonstrated that an accumulation of soluble sugars mediated by PGPR produces drought resistance in plants because the soluble sugars or sugar by-products function as osmoprotectants under water-deficit conditions (Sánchez et al., 1998). In the current study, a clear increase in leaf sugar content was apparent in pepper seedlings treated with PGPR under normal conditions and stress conditions. Soluble sugars also function as metabolic resources and structural constituents of cells, and they modulate many processes associated with plant development under water stress conditions (Wang and Ruan, 2013). Sugar accumulation in the leaf also triggers the expression of genes connected to photosynthetic activities (Van Oosten and Besford, 1995). In our work, PGPR treatment induced higher sugar accumulation, which potentially acted as an osmoprotectant in photosynthetic organs and helped maintain the photosynthetic efficiency of the plants, resulting in their increased growth under stress conditions.

Amino acids directly or indirectly regulate plant responses to environmental stresses (Ashraf and Harris, 2004). In our experiment, abiotic stresses increased the amino acid content in pepper seedlings. This accumulation suggests that the amino acids play a role in osmotic adjustment as well as in metabolism (Wu et al., 2014). Previous reviews have reported that amino acid content increases in plants under stress (Rare, 1990; Mansour, 2000). The application of PGPR rescued amino acid content in many cases in stressed seedlings during the recovery period. A rapid increase in proline content was observed in stressed seedlings, which is in agreement with the findings of previous studies on other plant species (Khattab, 2007; Bassuony et al., 2008). Accumulation of proline, which functions as osmoprotectant and ROS scavenger, could be one method by which plants endure abiotic stress (Ghars et al., 2008; Dawood and El-Awadi, 2015; Kim et al., 2016; Liu et al., 2018). Indeed, increased proline content has been associated with enhanced plant performance under environmental stresses (Nanjo et al., 1999; Khan et al., 2013). Furthermore, proline may play a role as a reservoir of organic nitrogen that can be consumed during the recovery period to help plants withstand environmental challenges (Rare, 1990; Sairam and Tyagi, 2004). In the present study, application of PGPR led to enhanced proline content in stressed seedlings; this PGPR-induced proline increase could represent an adaptive mechanism that facilitates osmotic adjustment and improves plant survival under stress conditions.

CONCLUSION

Application of rhizospheric *B. amyloliquefaciens* B11 not only augmented pepper growth under salinity, drought, and metal stresses but also undoubtedly urged pepper endurance to these abiotic stresses. The ability of *B. amyloliquefaciens* B11 to solubilize nutrients and secrete IAA, ACC, and siderophore under abiotic induced stress regulated host growth *via* alleviating salt and metal accumulation in pepper plant. Furthermore, *B. amyloliquefaciens* B11 inoculation modified the regulation of host antioxidant system and hormones to minimize the destructive

effects of abiotic stresses. Inoculation of *B. amyloliquefaciens* B11 induced the expression of stress-related genes, namely, CaXTHs, CaWRKY2, CaBI-1, CaPTI1, and CaBiPs. Taken together, our results provided clear evidence to demonstrate the stress-relieving effect of *B. amyloliquefaciens* B11 and propose it as an appropriate candidate for remediation of heavy metal-polluted soils.

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

EK planned the research, conducted the experiments, analyzed the data, and wrote the manuscript. AA and L-RK helped to conduct the experiments. SM analyzed the sequences. SM and AA-S edited the manuscript. S-MK and I-JL provided the resources. All authors approved the manuscript.

FUNDING

This study was supported by the Agenda Program (Project No. PJ015163032021) Rural Development Administration, South Korea.

REFERENCES

- Agarwal, S., and Pandey, V. (2004). Antioxidant enzyme responses to NaCl stress in *Cassia angustifolia*. *Biol. Plant.* 48, 555–560. doi: 10.1023/B:BIOP.0000047152.07878.e7
- Ahaotu, I., Anyogu, A., Njoku, O. H., Odu, N. N., Sutherland, J. P., and Ouoba, L. I. (2013). Molecular identification and safety of *Bacillus* species involved in the fermentation of African oil beans (*Pentaclethra macrophylla* Benth) for production of Ugba. *Int. J. Food Microbiol.* 162, 95–104. doi: 10.1016/j.jfoodmicro.2013.01.001
- Albdaiwi, R. N., Khyami-Horani, H., Ayad, J. Y., Alananbeh, K. M., and Al-Sayaydeh, R. (2019). Isolation and characterization of halotolerant plant growth promoting rhizobacteria from durum wheat (*Triticum turgidum* subsp. durum) cultivated in saline areas of the dead sea region. *Front. Microbiol.* 10:1639. doi: 10.3389/fmicb.2019.01639
- Alexander, D., and Zuberer, D. (1991). Use of chrome azurol S reagents to evaluate siderophore production by rhizosphere bacteria. *Biol. Fertil. Soils* 12, 39–45. doi: 10.1007/bf00369386
- Ali, S. Z., Sandhya, V., and Rao, L. V. (2014). Isolation and characterization of drought-tolerant ACC deaminase and exopolysaccharide-producing fluorescent *Pseudomonas* sp. *Ann. Microbiol.* 64, 493–502. doi: 10.1007/s13213-013-0680-3
- Arnon, D. I. (1949). Copper enzymes in isolated chloroplasts. Polyphenoloxidase in beta vulgaris. *Plant Physiol.* 24, 1–15. doi: 10.1104/pp.24.1.1
- Ashraf, M., and Harris, P. J. C. (2004). Potential biochemical indicators of salinity tolerance in plants. *Plant Sci.* 166, 3–16. doi: 10.1016/j.plantsci.2003.10.024
- Ashraf, M., and Iram, A. (2005). Drought stress induced changes in some organic substances in nodules and other plant parts of two potential legumes differing in salt tolerance. *Flora Morphol. Distrib. Funct. Ecol. Plants* 200, 535–546. doi: 10.1016/j.flora.2005.06.005

ACKNOWLEDGMENTS

We would like to express our deep gratitude to the Kyungpook National University, Department of Applied Biosciences, for providing us with a well-equipped platform with which to undergo our research activities.

SUPPLEMENTARY MATERIAL

The Supplementary Material for this article can be found online at: <https://www.frontiersin.org/articles/10.3389/fpls.2021.669693/full#supplementary-material>

Supplementary Figure 1 | (A) IAA production, **(B)** ACC deaminase activity, **(C)** siderophore production, **(D)** nitrogen fixation, **(E)** citrate utilization, **(F)** potassium solubilization, **(G)** zinc solubilization, **(H)** phosphate solubilization, and **(I)** silicon solubilization.

Supplementary Figure 2 | (A) Salt and **(B)** drought tolerance ability of the selected bacterial strain (B11) associated with *Sasamorpha* in this study.

Supplementary Figure 3 | Heavy metal tolerance ability of the selected bacterial strain (B11) associated with *Sasamorpha* in this study **(A–C)**.

Supplementary Figure 4 | Effects of various salt concentrations on the growth of pepper seedlings following 8 days of treatment (8DAT; **A,B**). Treatments: control + water, 0.5% NaCl, 1% NaCl, 1.5% NaCl, and 2.5% NaCl.

Supplementary Figure 5 | Effects of PGPR inoculation on pepper plant growth under normal and stress conditions after 8 days of treatment (8DAT). Treatments: control + water, control + PGPR, control + 1%NaCl, PGPR + 1%NaCl, control + 10%PEG, PGPR + 10%PEG, control + 0.1%Cd, and PGPR + 0.1%Cd. Values show the means $\pm$ SE ($n = 3$) and significant differences at $p < 0.05$ (LSD test).

- Babalola, O. O. (2010). Beneficial bacteria of agricultural importance. *Biotechnol. Lett.* 32, 1559–1570. doi: 10.1007/s10529-010-0347-0
- Baldani, J. I., Reis, V. M., Videira, S. S., Boddey, L. H., and Baldani, V. L. D. (2014). The art of isolating nitrogen-fixing bacteria from non-leguminous plants using N-free semi-solid media: a practical guide for microbiologists. *Plant Soil* 384, 413–431. doi: 10.1007/s11104-014-2186-6
- Banik, P., Zeng, W., Tai, H., Bizimungu, B., and Tanino, K. (2016). Effects of drought acclimation on drought stress resistance in potato (*Solanum tuberosum* L.) genotypes. *Environ. Exp. Bot.* 126, 76–89. doi: 10.1016/j.envexpbot.2016.01.008
- Barka, E. A., Nowak, J., and Clément, C. (2006). Enhancement of chilling resistance of inoculated grapevine plantlets with a plant growth-promoting rhizobacterium, Burkholderia phytofirmans strain PsJN. *Appl. Environ. Microbiol.* 72, 7246–7252. doi: 10.1128/aem.01047-06
- Barra, P. J., Inostroza, N. G., Acuña, J. J., Mora, M. L., Crowley, D. E., and Jorquera, M. A. (2016). Formulation of bacterial consortia from avocado (*Persea americana* Mill.) and their effect on growth, biomass and superoxide dismutase activity of wheat seedlings under salt stress. *Appl. Soil Ecol.* 102, 80–91. doi: 10.1016/j.apsoil.2016.02.014
- Bassuony, F., Hassanein, R., Baraka, D., and Khalil, R. (2008). Physiological effects of nicotinamide and ascorbic acid on Zea mays plant grown under salinity stress II-Changes in nitrogen constituent, protein profiles, protease enzyme and certain inorganic cations. *Aust. J. Basic Appl. Sci.* 2, 350–359.
- Batool, T., Ali, S., Seleiman, M. F., Naveed, N. H., Ali, A., Ahmed, K., et al. (2020). Plant growth promoting rhizobacteria alleviates drought stress in potato in response to suppressive oxidative stress and antioxidant enzymes activities. *Sci. Rep.* 10:16975. doi: 10.1038/s41598-020-73489-z
- Belimov, A., Hontzas, N., Safronova, V., Demchinskaya, S., Piluzza, G., Bullitta, S., et al. (2005). Cadmium-tolerant plant growth-promoting bacteria associated with the roots of Indian mustard (*Brassica juncea* L. Czern.). *Soil Biol. Biochem.* 37, 241–250. doi: 10.1016/j.soilbio.2004.07.033

- Bhattacharyya, P. N., and Jha, D. K. (2012). Plant growth-promoting rhizobacteria (PGPR): emergence in agriculture. *World J. Microbiol. Biotechnol.* 28, 1327–1350. doi: 10.1007/s11274-011-0979-9
- Böttner, L., Grabe, V., Gablenz, S., Böhme, N., Appenroth, K. J., Gershenzon, J., et al. (2021). Differential localization of flavonoid glucosides in an aquatic plant implicates different functions under abiotic stress. *Plant Cell Environ.* 44, 900–914. doi: 10.1111/pce.13974
- Bradford, M. M. (1976). A rapid and sensitive method for the quantitation of microgram quantities of protein utilizing the principle of protein-dye binding. *Anal. Biochem.* 72, 248–254. doi: 10.1016/0003-2697(76)90527-3
- Çakmakçı, R., Erat, M., Erdoğan, Ü., and Dönmez, M. F. (2007). The influence of plant growth-promoting rhizobacteria on growth and enzyme activities in wheat and spinach plants. *J. Plant Nutr. Soil Sci.* 170, 288–295. doi: 10.1002/jpln.200625105
- Cervantes-Vega, C., Chavez, J., Córdova, N., and Amador, J. V. (1986). Resistance to metals by *Pseudomonas aeruginosa* clinical isolates. *Microbios* 48, 159–163.
- Chang, S.-S., Hsu, H.-L., Cheng, J.-C., and Tseng, C.-P. (2011). An efficient strategy for broad-range detection of low abundance bacteria without DNA decontamination of PCR reagents. *PLoS One* 6:e20303. doi: 10.1371/journal.pone.0020303
- Cheng, Y., JalalAhammed, G., Yu, J., Yao, Z., Ruan, M., Ye, Q., et al. (2016). Putative WRKYs associated with regulation of fruit ripening revealed by detailed expression analysis of the WRKY gene family in pepper. *Sci. Rep.* 6:39000. doi: 10.1038/srep39000
- Cho, S. K., Kim, J. E., Park, J.-A., Eom, T. J., and Kim, W. T. (2006). Constitutive expression of abiotic stress-inducible hot pepper CaXTH3, which encodes a xyloglucan endotransglucosylase/hydrolase homolog, improves drought and salt tolerance in transgenic *Arabidopsis* plants. *FEBS Lett.* 580, 3136–3144. doi: 10.1016/j.febslet.2006.04.062
- Choi, J. Y., Seo, Y. S., Kim, S. J., Kim, W. T., and Shin, J. S. (2011). Constitutive expression of CaXTH3, a hot pepper xyloglucan endotransglucosylase/hydrolase, enhanced tolerance to salt and drought stresses without phenotypic defects in tomato plants (*Solanum lycopersicum* cv. Dotaerang). *Plant Cell Rep.* 30, 867–877. doi: 10.1007/s00299-010-0989-3
- Clark, R., and Lee, S.-H. (2016). Anticancer properties of capsaicin against human cancer. *Anticancer Res.* 36, 837–843.
- Dagdelen, N., Yilmaz, E., Sezgin, F., and Gurbuz, T. (2004). Effects of water stress at different growth stages on processing pepper (*Capsicum annuum* Cv. Kapija) yield water use and quality characteristics. *Pakistan J. Biol. Sci. (Pakistan)* 7, 2167–2172. doi: 10.3923/pjbs.2004.2167.2172
- Damodaran, T., Sah, V., Rai, R., Sharma, D., Mishra, V., Jha, S., et al. (2013). Isolation of salt tolerant endophytic and rhizospheric bacteria by natural selection and screening for promising plant growth-promoting rhizobacteria (PGPR) and growth vigour in tomato under sodic environment. *Afr. J. Microbiol. Res.* 7, 5082–5089.
- Dawood, M. G., and El-Awadi, M. E. (2015). Alleviation of salinity stress on *Vicia faba* L. plants via seed priming with melatonin. *Acta Biol. Colomb.* 20, 223–235.
- Doležal, J., Matsuki, S., and Hara, T. (2009). Effects of dwarf-bamboo understory on tree seedling emergence and survival in a mixed-oak forest in northern Japan: a multi-site experimental study. *Commun. Ecol.* 10, 225–235. doi: 10.1556/comec.10.2009.2.13
- Enyedi, A. J., Yalpani, N., Silverman, P., and Raskin, I. (1992). Localization, conjugation, and function of salicylic acid in tobacco during the hypersensitive reaction to tobacco mosaic virus. *Proc. Natl. Acad. Sci. U.S.A.* 89, 2480–2484. doi: 10.1073/pnas.89.6.2480
- Fenando, E., Boero, C., Gallardo, M., and Gonzalez, J. (2000). Effect of NaCl on germination, growth, and soluble sugar content in *Chenopodium quinona* seeds. *Bot. Bull. Acad. Sin.* 41, 27–34.
- Fischer, S. E., Fischer, S. I., Magris, S., and Mori, G. B. (2007). Isolation and characterization of bacteria from the rhizosphere of wheat. *World J. Microbiol. Biotechnol.* 23, 895–903. doi: 10.1007/s11274-006-9312-4
- Gamalerio, E., and Glick, B. R. (2012). “Ethylene and abiotic stress tolerance in plants,” in *Environmental Adaptations and Stress Tolerance of Plants in The era of Climate Change*, eds P. Ahmad and M. N. V. Prasad (New York, NY: Springer), 395–412. doi: 10.1007/978-1-4614-0815-4_18
- Gaur, A. (1990). “Physiological functions of phosphate solubilizing micro-organisms,” in *Phosphate Solubilizing Micro-Organisms as Biofertilizers*, ed. A. C. Gaur (New Delhi: Omega Scientific Publishers), 16–72.
- Ghars, M. A., Parre, E., Debez, A., Bordenave, M., Richard, L., Lepoint, L., et al. (2008). Comparative salt tolerance analysis between *Arabidopsis thaliana* and *Thellungiella halophila*, with special emphasis on K⁺/Na⁺ selectivity and proline accumulation. *J. Plant Physiol.* 165, 588–599. doi: 10.1016/j.jplph.2007.05.014
- Glick, B. R. (2014). Bacteria with ACC deaminase can promote plant growth and help to feed the world. *Microbiol. Res.* 169, 30–39. doi: 10.1016/j.micres.2013.09.009
- Glick, B. R., Penrose, D. M., and Li, J. (1998). A model for the lowering of plant ethylene concentrations by plant growth-promoting bacteria. *J. Theor. Biol.* 190, 63–68. doi: 10.1006/jtbi.1997.0532
- Gupta, S., and Pandey, S. (2019). Unravelling the biochemistry and genetics of ACC deaminase-An enzyme alleviating the biotic and abiotic stress in plants. *Plant Gene* 18:100175. doi: 10.1016/j.plgene.2019.100175
- Han, H., and Lee, K. (2005). Physiological responses of soybean-inoculation of *Bradyrhizobium japonicum* with PGPR in saline soil conditions. *Res. J. Agric. Biol. Sci.* 1, 216–221.
- Han, J., Sun, L., Dong, X., Cai, Z., Sun, X., Yang, H., et al. (2005). Characterization of a novel plant growth-promoting bacteria strain *Delftia tsuruhatensis* HR4 both as a diazotroph and a potential biocontrol agent against various plant pathogens. *Syst. Appl. Microbiol.* 28, 66–76. doi: 10.1016/j.syapm.2004.09.003
- Han, Y., Wang, W., Sun, J., Ding, M., Zhao, R., Deng, S., et al. (2013). *Populus euphratica* XTH overexpression enhances salinity tolerance by the development of leaf succulence in transgenic tobacco plants. *J. Exp. Bot.* 64, 4225–4238. doi: 10.1093/jxb/ert229
- Howell, S. H. (2013). Endoplasmic reticulum stress responses in plants. *Annu. Rev. Plant Biol.* 64, 477–499. doi: 10.1146/annurev-arplant-050312-120053
- Hu, X., Chen, J., and Guo, J. (2006). Two phosphate-and potassium-solubilizing bacteria isolated from Tianmu Mountain, Zhejiang, China. *World J. Microbiol. Biotechnol.* 22, 983–990. doi: 10.1007/s11274-006-9144-2
- Huang, B., Chen, Y.-E., Zhao, Y.-Q., Ding, C.-B., Liao, J.-Q., Hu, C., et al. (2019). Exogenous melatonin alleviates oxidative damages and protects photosystem II in maize seedlings under drought stress. *Front. Plant Sci.* 10:677. doi: 10.3389/fpls.2019.00677
- Huang, T., and Duman, J. G. (2002). Cloning and characterization of a thermal hysteresis (antifreeze) protein with DNA-binding activity from winter bittersweet nightshade, *Solanum dulcamara*. *Plant Mol. Biol.* 48, 339–350.
- Hückelhoven, R. (2004). BAX Inhibitor-1, an ancient cell death suppressor in animals and plants with prokaryotic relatives. *Apoptosis* 9, 299–307. doi: 10.1023/b:appt.0000025806.71000.1c
- Imran, Q. M., Hussain, A., Mun, B.-G., Lee, S. U., Asaf, S., Ali, M. A., et al. (2018). Transcriptome wide identification and characterization of NO-responsive WRKY transcription factors in *Arabidopsis thaliana* L. *Environ. Exp. Bot.* 148, 128–143. doi: 10.1016/j.envexpbot.2018.01.010
- Isbat, M., Zeba, N., Kim, S. R., and Hong, C. B. (2009). A BAX inhibitor-1 gene in *Capsicum annuum* is induced under various abiotic stresses and endows multi-tolerance in transgenic tobacco. *J. Plant Physiol.* 166, 1685–1693. doi: 10.1016/j.jplph.2009.04.017
- Izanloo, A., Condon, A. G., Langridge, P., Tester, M., and Schnurbusch, T. (2008). Different mechanisms of adaptation to cyclic water stress in two South Australian bread wheat cultivars. *J. Exp. Bot.* 59, 3327–3346. doi: 10.1093/jxb/ern199
- Jaiswal, V., Gahlaut, V., Dubey, M., and Ramchiary, N. (2019). “Genes/quantitative trait loci and associated molecular mechanisms identified in capsicum genome for tolerance to abiotic and biotic stresses,” in *The Capsicum Genome*, eds N. Ramchiary and C. Kole (Cham: Springer), 121–138. doi: 10.1007/978-3-319-97217-6_7
- Jaleel, C. A., Manivannan, P., Sankar, B., Kishorekumar, A., Gopi, R., Somasundaram, R., et al. (2007). Water deficit stress mitigation by calcium chloride in *Catharanthus roseus*: Effects on oxidative stress, proline metabolism and indole alkaloid accumulation. *Colloids Surf. B Biointerfaces* 60, 110–116. doi: 10.1016/j.colsurfb.2007.06.006
- Jana, S., and Choudhuri, M. A. (1982). Glycolate metabolism of three submersed aquatic angiosperms during ageing. *Aqua. Bot.* 12, 345–354. doi: 10.1016/0304-3770(82)90026-2
- Jiang, W., and Yu, D. (2009). *Arabidopsis* WRKY2 transcription factor mediates seed germination and postgermination arrest of development by abscisic acid. *BMC Plant Biol.* 9:96. doi: 10.1186/1471-2229-9-96

- Jiang, Y., and Yu, D. (2015). WRKY transcription factors: links between phytohormones and plant processes. *Sci. China Life Sci.* 58, 501–502. doi: 10.1007/s11427-015-4849-9
- Jing, Y.-D., He, Z.-L., and Yang, X.-E. (2007). Role of soil rhizobacteria in phytoremediation of heavy metal contaminated soils. *J. Zhejiang Univ. Sci. B* 8, 192–207. doi: 10.1631/jzus.2007.B0192
- Karthik, C., Elangovan, N., Kumar, T. S., Govindharaju, S., Barathi, S., Oves, M., et al. (2017). Characterization of multifarious plant growth promoting traits of rhizobacterial strain AR6 under Chromium (VI) stress. *Microbiol. Res.* 204, 65–71. doi: 10.1016/j.micres.2017.07.008
- Kaya, A., and Doganlar, Z. B. (2019). Melatonin improves the multiple stress tolerance in pepper (I). *Sci. Horticult.* 256:108509. doi: 10.1016/j.scienta.2019.05.036
- Ke, Q., Ye, J., Wang, B., Ren, J., Yin, L., Deng, X., et al. (2018). Melatonin mitigates salt stress in wheat seedlings by modulating polyamine metabolism. *Front. Plant Sci.* 9:914. doi: 10.3389/fpls.2018.00914
- Keep, T., Sampoux, J.-P., Barre, P., Blanco-Pastor, J.-L., Dehmer, K. J., Durand, J.-L., et al. (2021). To grow or survive: which are the strategies of a perennial grass to face severe seasonal stress? *Funct. Ecol.* 35, 1145–1158. doi: 10.1111/1365-2435.13770
- Khan, A. L., Al-Harrasi, A., Shahzad, R., Imran, Q. M., Yun, B.-W., Kim, Y.-H., et al. (2018). Regulation of endogenous phytohormones and essential metabolites in frankincense-producing *Boswellia sacra* under wounding stress. *Acta Physiol. Plant.* 40:113.
- Khan, M. I. R., Iqbal, N., Masood, A., Per, T. S., and Khan, N. A. (2013). Salicylic acid alleviates adverse effects of heat stress on photosynthesis through changes in proline production and ethylene formation. *Plant Signal. Behav.* 8:e26374. doi: 10.4161/psb.26374
- Khan, M. I. R., Trivellini, A., Chhillar, H., Chopra, P., Ferrante, A., Khan, N. A., et al. (2020). The significance and functions of ethylene in flooding stress tolerance in plants. *Environ. Exp. Bot.* 179:104188. doi: 10.1016/j.envexpbot.2020.104188
- Khan, N., and Bano, A. (2016). Role of plant growth promoting rhizobacteria and Ag-nano particle in the bioremediation of heavy metals and maize growth under municipal wastewater irrigation. *Int. J. Phytoremed.* 18, 211–221. doi: 10.1080/15226514.2015.1064352
- Khan, N., Bano, A., Rahman, M. A., Rathinasabapathi, B., and Babar, M. A. (2019). UPLC-HRMS-based untargeted metabolic profiling reveals changes in chickpea (*Cicer arietinum*) metabolome following long-term drought stress. *Plant cell Environ.* 42, 115–132. doi: 10.1111/pce.13195
- Khattab, H. (2007). Role of glutathione and polyadenylic acid on the oxidative defense systems of two different cultivars of canola seedlings grown under saline conditions. *Aust. J. Basic Appl. Sci.* 1, 323–334.
- Ki, J.-S., Zhang, W., and Qian, P.-Y. (2009). Discovery of marine Bacillus species by 16S rRNA and rpoB comparisons and their usefulness for species identification. *J. Microbiol. Methods* 77, 48–57. doi: 10.1016/j.mimet.2009.01.003
- Kim, J., Liu, Y., Zhang, X., Zhao, B., and Childs, K. L. (2016). Analysis of salt-induced physiological and proline changes in 46 switchgrass (*Panicum virgatum*) lines indicates multiple response modes. *Plant Physiol. Biochem.* 105, 203–212. doi: 10.1016/j.plaphy.2016.04.020
- Kim, Y.-H., Khan, A. L., Kim, D.-H., Lee, S.-Y., Kim, K.-M., Waqas, M., et al. (2014). Silicon mitigates heavy metal stress by regulating P-type heavy metal ATPases, Oryza sativa low silicon genes, and endogenous phytohormones. *BMC Plant Biol.* 14:13. doi: 10.1186/1471-2229-14-13
- Lee, W., and Yim, Y. (2002). *Plant Geography with Special Reference to Korea*. Chuncheon: Kangwon National University Press, 412.
- Li, M.-W., Muñoz, N. B., Wong, C.-F., Wong, F.-L., Wong, K.-S., Wong, J. W.-H., et al. (2016). QTLs regulating the contents of antioxidants, phenolics, and flavonoids in soybean seeds share a common genomic region. *Front. Plant Sci.* 7:854. doi: 10.3389/fpls.2016.00854
- Liao, S., and Chang, W. (2004). Heavy metal phytoremediation by water hyacinth at constructed wetlands in Taiwan. *Photogramm. Eng. Remote Sens.* 54, 177–185.
- Liu, Y., Ma, M., Ran, Y., Yi, X., Wu, S., and Huang, P. (2021). Disentangling the effects of edaphic and vegetational properties on soil aggregate stability in riparian zones along a gradient of flooding stress. *Geoderma* 385:114883. doi: 10.1016/j.geoderma.2020.114883
- Liu, Z., Cai, J.-S., Li, J.-J., Lu, G.-Y., Li, C.-S., Fu, G.-P., et al. (2018).). Exogenous application of a low concentration of melatonin enhances salt tolerance in rapeseed (*Brassica napus* L.) seedlings. *J. Integr. Agric.* 17, 328–335. doi: 10.1016/s2095-3119(17)61757-x
- López-Serrano, L., Canet-Sanchis, G., Vuletin Selak, G., Penella, C., San Bautista, A., López-Galarza, S., et al. (2019). Pepper rootstock and scion physiological responses under drought stress. *Front. Plant Sci.* 10:38. doi: 10.3389/fpls.2019.00038
- Ma, Y., Rajkumar, M., and Freitas, H. (2009). Inoculation of plant growth promoting bacterium *Achromobacter xylosoxidans* strain Ax10 for the improvement of copper phytoextraction by *Brassica juncea*. *J. Environ. Manag.* 90, 831–837. doi: 10.1016/j.jenvman.2008.01.014
- Madhaiyan, M., Poonguzhali, S., Ryu, J., and Sa, T. (2006). Regulation of ethylene levels in canola (*Brassica campestris*) by 1-aminocyclopropane-1-carboxylate deaminase-containing *Methylobacterium fujisawaense*. *Planta* 224, 268–278. doi: 10.1007/s00425-005-0211-y
- Mansour, M. M. F. (2000). Nitrogen containing compounds and adaptation of plants to salinity stress. *Biol. Plant.* 43, 491–500. doi: 10.1023/A:1002873531707
- Mishiba, K.-I., Nagashima, Y., Suzuki, E., Hayashi, N., Ogata, Y., Shimada, Y., et al. (2013). Defects in IRE1 enhance cell death and fail to degrade mRNAs encoding secretory pathway proteins in the *Arabidopsis* unfolded protein response. *Proc. Natl. Acad. Sci. U.S.A.* 110, 5713–5718. doi: 10.1073/pnas.1219047110
- Mori, K. (2000). Tripartite management of unfolded proteins in the endoplasmic reticulum. *Cell* 101, 451–454. doi: 10.1016/s0092-8674(00)80855-7
- Nanjo, T., Kobayashi, M., Yoshida, Y., Sanada, Y., Wada, K., Tsukaya, H., et al. (1999). Biological functions of proline in morphogenesis and osmotolerance revealed in antisense transgenic *Arabidopsis thaliana*. *Plant J.* 18, 185–193. doi: 10.1046/j.1365-313x.1999.00438.x
- Navarro, N. P., Huovinen, P., Jofre, J., and Gómez, I. (2021). Ultraviolet radiation stress response of haploid and diploid spores of *Mazzaella laminarioides*: do bio-optical traits matter? *Algal Res.* 54:102230. doi: 10.1016/j.algal.2021.102230
- Nazar, R., Iqbal, N., Syeed, S., and Khan, N. A. (2011). Salicylic acid alleviates decreases in photosynthesis under salt stress by enhancing nitrogen and sulfur assimilation and antioxidant metabolism differentially in two mungbean cultivars. *J. Plant Physiol.* 168, 807–815. doi: 10.1016/j.jplph.2010.11.001
- Oh, S.-K., Yi, S. Y., Yu, S. H., Moon, J. S., Park, J. M., and Choi, D. (2006). CaWRKY2, a chili pepper transcription factor, is rapidly induced by incompatible plant pathogens. *Mol. Cells* 22, 58–64.
- Oves, M., Khan, M. S., and Qari, H. A. (2017). Ensifer adhaerens for heavy metal bioaccumulation, biosorption, and phosphate solubilization under metal stress condition. *J. Taiwan Inst. Chem. Eng.* 80, 540–552. doi: 10.1016/j.jtice.2017.08.026
- Oves, M., Khan, M. S., and Zaidi, A. (2013). Biosorption of heavy metals by *Bacillus thuringiensis* strain OSM29 originating from industrial effluent contaminated north Indian soil. *Saudi J. Biol. Sci.* 20, 121–129. doi: 10.1016/j.sjbs.2012.11.006
- Pan, J., Peng, F., Xue, X., You, Q., Zhang, W., Wang, T., et al. (2019). The growth promotion of two salt-tolerant plant groups with PGPR inoculation: a meta-analysis. *Sustainability* 11:378. doi: 10.3390/su11020378
- Pandey, S., and Gupta, S. (2019). ACC deaminase producing bacteria with multifarious plant growth promoting traits alleviates salinity stress in French bean (*Phaseolus vulgaris*) plants. *Front. Microbiol.* 10:1506. doi: 10.3389/fmicb.2019.01506
- Pandey, S. P., and Somssich, I. E. (2009). The role of WRKY transcription factors in plant immunity. *Plant Physiol.* 150, 1648–1655. doi: 10.1104/pp.109.138990
- Penrose, D. M., and Glick, B. R. (2003). Methods for isolating and characterizing ACC deaminase-containing plant growth-promoting rhizobacteria. *Physiol. Plant.* 118, 10–15. doi: 10.1034/j.1399-3054.2003.00086.x
- Pii, Y., Mimmo, T., Tomasi, N., Terzano, R., Cesco, S., and Crecchio, C. (2015). Microbial interactions in the rhizosphere: beneficial influences of plant growth-promoting rhizobacteria on nutrient acquisition process. A review. *Biol. Fertil. Soils* 51, 403–415. doi: 10.1007/s00374-015-0996-1
- Putter, J. (1974). “Peroxidase,” in *Methods of Enzymatic Analysis*, ed. B. Hu (Weinhan: Verlag Chemie), 685–690.
- Qi, Q., Rose, P. A., Abrams, G. D., Taylor, D. C., Abrams, S. R., and Cutler, A. J. (1998). (+)-Absciscic acid metabolism, 3-ketoacyl-coenzyme A synthase gene expression, and very-long-chain monounsaturated fatty acid biosynthesis in *Brassica napus* embryos. *Plant Physiol.* 117, 979–987. doi: 10.1104/pp.117.3.979
- Qiu, Y., and Yu, D. (2009). Over-expression of the stress-induced OsWRKY45 enhances disease resistance and drought tolerance in *Arabidopsis*. *Environ. Exp. Bot.* 65, 35–47. doi: 10.1016/j.envexpbot.2008.07.002

- Rare, E. (1990). Stress physiology: the functional significance of the accumulation of nitrogen-containing compounds. *J. Hortic. Sci.* 65, 231–243. doi: 10.1080/00221589.1990.11516052
- Riyazuddin, R., Verma, R., Singh, K., Nisha, N., Keisham, M., Bhati, K. K., et al. (2020). Ethylene: a master regulator of salinity stress tolerance in plants. *Biomolecules* 10:959. doi: 10.3390/biom10060959
- Rizhsky, L., Davletova, S., Liang, H., and Mittler, R. (2004). The zinc finger protein Zat12 is required for cytosolic ascorbate peroxidase 1 expression during oxidative stress in *Arabidopsis*. *J. Biol. Chem.* 279, 11736–11743. doi: 10.1074/jbc.M313350200
- Rout, G. R., and Sahoo, S. (2015). Role of iron in plant growth and metabolism. *Rev. Agric. Sci.* 3, 1–24. doi: 10.7831/ras.3.1
- Ryu, H., and Cho, Y.-G. (2015). Plant hormones in salt stress tolerance. *J. Plant Biol.* 58, 147–155. doi: 10.1007/s12374-015-0103-z
- Saab, I. N., and Sachs, M. M. (1995). Complete cDNA and genomic sequence encoding a flooding-responsive gene from maize (*Zea mays* L.) homologous to xyloglucan endotransglycosylase. *Plant Physiol.* 108:439. doi: 10.1104/pp.108.1.439
- Sairam, R. K., and Tyagi, A. (2004). Physiology and molecular biology of salinity stress tolerance in plants. *Curr. Sci.* 86, 407–421.
- Sánchez, F. J., Manzanares, M. A., de Andres, E. F., Tenorio, J. L., and Ayerbe, L. (1998). Turgor maintenance, osmotic adjustment and soluble sugar and proline accumulation in 49 pea cultivars in response to water stress. *Field Crops Res.* 59, 225–235. doi: 10.1016/s0378-4290(98)00125-7
- Sarwar, M., and Kremer, R. (1995). Determination of bacterially derived auxins using a microplate method. *Lett. Appl. Microbiol.* 20, 282–285. doi: 10.1111/j.1472-765x.1995.tb00446.x
- Saxena, P., Chauhan, L., and Gupta, S. (2005). Cytogenetic effects of commercial formulation of cypermethrin in root meristem cells of *Allium sativum*: spectroscopic basis of chromosome damage. *Toxicology* 216, 244–252. doi: 10.1016/j.tox.2005.08.008
- Seki, M., Narusaka, M., Ishida, J., Nanjo, T., Fujita, M., Oono, Y., et al. (2002). Monitoring the expression profiles of 7000 *Arabidopsis* genes under drought, cold and high-salinity stresses using a full-length cDNA microarray. *Plant J.* 31, 279–292. doi: 10.1046/j.1365-3113x.2002.01359.x
- Seskar, M., Shulaev, V., and Raskin, I. (1998). Endogenous methyl salicylate in pathogen-inoculated tobacco plants. *Plant Physiol.* 116, 387–392. doi: 10.1104/pp.116.1.387
- Sharma, S. K. M. P., Ramesh, A., and Joshi, O. P. (2011). Characterization of zinc-solubilizing *Bacillus* isolates and their potential to influence zinc assimilation in soybean seeds. *J. Microbiol. Biotechnol.* 22, 352–359. doi: 10.4014/jmb.1106.05063
- Silvestro, D., and Michalak, I. (2012). raxmlGUI: a graphical front-end for RAxML. *Organ. Div. Evol.* 12, 335–337. doi: 10.1007/s13127-011-0056-0
- Simioniuc, D. P., Simioniuc, V., Topa, D., van den Berg, M., Prins, U., Bebeli, P. J., et al. (2021). Assessment of andean lupin (*Lupinus mutabilis*) genotypes for improved frost tolerance. *Agriculture* 11:155. doi: 10.3390/agriculture11020155
- Simmons, J. S. (1926). A culture medium for differentiating organisms of typhoid-colon aerogenes groups and for isolation of certain fungi. *J. Infect. Dis.* 39, 209–214. doi: 10.1093/infdis/39.3.209
- Singh, R. P., Jha, P., and Jha, P. N. (2015). The plant-growth-promoting bacterium *Klebsiella* sp. SBP-8 confers induced systemic tolerance in wheat (*Triticum aestivum*) under salt stress. *J. Plant Physiol.* 184, 57–67. doi: 10.1016/j.jplph.2015.07.002
- Sirihindi, G., Mir, M. A., Abd-Allah, E. F., Ahmad, P., and Gucel, S. (2016). Jasmonic acid modulates the physio-biochemical attributes, antioxidant enzyme activity, and gene expression in glycine max under nickel toxicity. *Front. Plant Sci.* 7:591. doi: 10.3389/fpls.2016.00591
- Tangahu, B. V., Sheikh Abdullah, S. R., Basri, H., Idris, M., Anuar, N., and Mukhlisin, M. (2011). A review on heavy metals (As, Pb, and Hg) uptake by plants through phytoremediation. *Int. J. Chem. Eng.* 2011:939161. doi: 10.1155/2011/939161
- Tsai, Y.-C., Hong, C.-Y., Liu, L.-F., and Kao, C. H. (2005). Expression of ascorbate peroxidase and glutathione reductase in roots of rice seedlings in response to NaCl and H₂O₂. *J. Plant Physiol.* 162, 291–299. doi: 10.1016/j.jplph.2004.06.004
- Van Oosten, J. J., and Besford, R. (1995). Some relationships between the gas exchange, biochemistry and molecular biology of photosynthesis during leaf development of tomato plants after transfer to different carbon dioxide concentrations. *Plant Cell Environ.* 18, 1253–1266. doi: 10.1111/j.1365-3040.1995.tb00185.x
- Venkatakrishnan, S., Sudalayandy, R., and Savariappan, A. (2003). Assessing in vitro solubilization potential of different zinc solubilizing bacterial (ZSB) strains. *Braz. J. Microbiol.* 34, 121–125. doi: 10.1590/s1517-83822004000100020
- Wang, H., Niu, H., Zhai, Y., and Lu, M. (2017). Characterization of BiP genes from pepper (*Capsicum annuum* L.) and the role of CaBiP1 in response to endoplasmic reticulum and multiple abiotic stresses. *Front. Plant Sci.* 8:1122. doi: 10.3389/fpls.2017.01122
- Wang, L., Chen, W., Wang, Q., Eneji, A., Li, Z., and Duan, L. (2009). Coronatine enhances chilling tolerance in cucumber (*Cucumis sativus* L.) seedlings by improving the antioxidative defence system. *J. Agronomy Crop Sci.* 195, 377–383. doi: 10.1111/j.1439-037x.2009.00378.x
- Wang, L., and Ruan, Y.-L. (2013). Regulation of cell division and expansion by sugar and auxin signaling. *Front. Plant Sci.* 4:163. doi: 10.3389/fpls.2013.00163
- Wani, A. B., Chadar, H., Wani, A. H., Singh, S., and Upadhyay, N. (2016). Salicylic acid to decrease plant stress. *Environ. Chem. Lett.* 15, 101–123. doi: 10.1007/s10311-016-0584-0
- Waqas, M., Khan, A. L., Hamayun, M., Shahzad, R., Kim, Y.-H., Choi, K.-S., et al. (2015). Endophytic infection alleviates biotic stress in sunflower through regulation of defence hormones, antioxidants and functional amino acids. *Eur. J. Plant Pathol.* 141, 803–824. doi: 10.1007/s10658-014-0581-8
- Wu, S., Hu, C., Tan, Q., Nie, Z., and Sun, X. (2014). Effects of molybdenum on water utilization, antioxidative defense system and osmotic-adjustment ability in winter wheat (*Triticum aestivum*) under drought stress. *Plant Physiol. Biochem.* 83, 365–374. doi: 10.1016/j.plaphy.2014.08.022
- Wu, W., Zhang, Q., Ervin, E. H., Yang, Z., and Zhang, X. (2017). Physiological mechanism of enhancing salt stress tolerance of perennial ryegrass by 24-epibrassinolide. *Front. Plant Sci.* 8:1017. doi: 10.3389/fpls.2017.01017
- Wuana, R. A., and Okieimen, F. E. (2011). Heavy metals in contaminated soils: a review of sources, chemistry, risks and best available strategies for remediation. *ISRN Ecol.* 2011:402647.
- Xie, Z., Zhang, Z.-L., Zou, X., Huang, J., Ruas, P., Thompson, D., et al. (2005). Annotations and functional analyses of the rice WRKY gene superfamily reveal positive and negative regulators of abscisic acid signaling in aleurone cells. *Plant Physiol.* 137, 176–189. doi: 10.1104/pp.104.054312
- Xu, W., Purugganan, M. M., Polisensky, D. H., Antosiewicz, D. M., Fry, S. C., and Braam, J. (1995). *Arabidopsis* TCH4, regulated by hormones and the environment, encodes a xyloglucan endotransglycosylase. *Plant Cell* 7:1555. doi: 10.1105/tpc.7.10.1555
- Yang, X., Srivastava, R., Howell, S. H., and Bassham, D. C. (2016). Activation of autophagy by unfolded proteins during endoplasmic reticulum stress. *Plant J.* 85, 83–95. doi: 10.1111/tpl.13091
- Yang, Z., Wang, Y., Wei, X., Zhao, X., Wang, B., and Sui, N. (2017). Transcription profiles of genes related to hormonal regulations under salt stress in sweet sorghum. *Plant Mol. Biol. Rep.* 35, 586–599. doi: 10.1007/s11105-017-1047-x
- Yasuda, M., Ishikawa, A., Jikumaru, Y., Seki, M., Umezawa, T., Asami, T., et al. (2008). Antagonistic interaction between systemic acquired resistance and the abscisic acid-mediated abiotic stress response in *Arabidopsis*. *Plant Cell* 20, 1678–1692. doi: 10.1105/tpc.107.054296
- Yazdanpanah, S., Baghizadeh, A., and Abbassi, F. (2011). The interaction between drought stress and salicylic and ascorbic acids on some biochemical characteristics of *Satureja hortensis*. *Afr. J. Agric. Res.* 6, 798–807.
- Zhang, J., Jia, W., Yang, J., and Ismail, A. M. (2006). Role of ABA in integrating plant responses to drought and salt stresses. *Field Crops Res.* 97, 111–119. doi: 10.1016/j.fcr.2005.08.018
- Zheng, W., and Wang, S. Y. (2001). Antioxidant activity and phenolic compounds in selected herbs. *J. Agric. Food Chem.* 49, 5165–5170. doi: 10.1021/jf010697n

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.

Copyright © 2021 Kazerooni, Maharachchikumbura, Adhikari, Al-Sadi, Kang, Kim and Lee. This is an open-access article distributed under the terms of the Creative Commons Attribution License (CC BY). The use, distribution or reproduction in other forums is permitted, provided the original author(s) and the copyright owner(s) are credited and that the original publication in this journal is cited, in accordance with accepted academic practice. No use, distribution or reproduction is permitted which does not comply with these terms.



Plant Growth Promotion and Induction of Systemic Tolerance to Drought and Salt Stress of Plants by Quorum Sensing Auto-Inducers of the *N*-acyl-homoserine Lactone Type: Recent Developments

Anton Hartmann^{1*}, Sophia Klink² and Michael Rothballer²

¹ Microbe-Host Interactions, Faculty of Biology, Ludwig-Maximilians-Universität München, Planegg, Germany, ² Institute of Network Biology, Helmholtz Zentrum München, Neuherberg, Germany

OPEN ACCESS

Edited by:

Mariangela Hungria,
Embrapa Soja, Brazil

Reviewed by:

Francisco Javier Ollero,
University of Seville, Spain
Paula Cerezini,
Brazilian Agricultural Research
Corporation (EMBRAPA), Brazil

*Correspondence:

Anton Hartmann
ahartmannndr@gmail.com

Specialty section:

This article was submitted to
Plant Abiotic Stress,
a section of the journal
Frontiers in Plant Science

Received: 21 March 2021

Accepted: 06 May 2021

Published: 31 May 2021

Citation:

Hartmann A, Klink S and
Rothballer M (2021) Plant Growth
Promotion and Induction of Systemic
Tolerance to Drought and Salt Stress
of Plants by Quorum Sensing
Auto-Inducers of the
N-acyl-homoserine Lactone Type:
Recent Developments.
Front. Plant Sci. 12:683546.
doi: 10.3389/fpls.2021.683546

Keywords: *N*-acyl-homoserine lactones, priming, induced systemic tolerance, ACC deaminase, auxins, osmolytes

INTRODUCTION

During the evolution of land plants, plant-associated microbiomes co-evolved and were integrated into prokaryote-eukaryote holobionts. Functions of these metagenomically organized plants are based on the gene expression of all holobiont participants. The constitution of the holobiont is dependent on specific signaling and perception events between the microbial and plant partners. Microbially produced plant hormones and a diversity of other metabolites have key roles in the process of interkingdom interaction of organisms. In association/symbiosis with plant beneficial bacteria, the ability of plants to cope with abiotic and biotic stresses is enhanced. As example for systemic effects reducing biotic stress, AHL-producing *Serratia liquefaciens* MG1 conferred biocontrol of the phytopathogenic fungus *Alternaria alternata* in tomato plants (Schuhegger et al., 2006). The down-regulation of the stress hormone ethylene by degradation of the precursor 1-aminocyclopropane-1-carboxylate (ACC) with ACC deaminases (Glick, 2014) has a key role in the beneficial interaction of bacteria with plants under abiotic stress conditions. The ability of degrading the ethylene precursor ACC is frequently found in bacteria isolated from the rhizosphere of salt-tolerant plants (Jha et al., 2012; Suarez et al., 2015; Hryniewicz et al., 2019). Other mechanisms for the mitigation of salinity and drought stress in plants, like the involvement of IAA by rhizobacteria (Egamberdieva et al., 2017), antioxidants, extracellular polymeric substances (EPS) or volatile organics were recently reviewed by Kumar et al. (2020). However, the induction of systemic tolerance to abiotic stress conditions by the quorum sensing auto-inducers of *N*-acyl-homoserine lactone-type (AHL) or AHL-producing rhizobacteria was not mentioned in this recent review and is generally not much recognized yet.

Since more than two decades, a very sensitive and effective communication system within bacterial populations is known, based on auto-inducers. In Gram-negative bacteria, molecules of the *N*-acyl-homoserine lactone type with hydrophilic (short) and hydrophobic (long) CH-chain are produced by *luxI*-type genes (Hartmann and Schikora, 2012; Rothballer et al., 2018). These small molecules are synthesized by the bacteria themselves but also by others in their vicinity depending on environmental conditions in the bacterial habitat. Their perception and subsequent

concentration dependent transcriptional regulation mediated by *luxR*-type receptors allow to efficiently adapt gene expression to environmental changes. This makes the auto-inducer signaling an essential and evolutionary relevant tool for the efficiency of bacterial behavior (Hense and Schuster, 2015). Interkingdom signaling of bacteria with host plants based on AHL auto-inducers was demonstrated in many Gram-negative rhizobacteria as reviewed by Schikora et al. (2016). The most recently observed role of AHLs in the improvement of abiotic stress tolerance of some plants deserves to be widened for more different crops to be finally applied in practical agriculture.

ROLES OF PHYTOHORMONES AND OSMOLYTES IN SALT AND DROUGHT TOLERANCE OF PLANTS

Plant hormones play an essential role in the regulation of responses at environmental stress situations such as drought or high salinity. It has been reviewed in detail, which different mechanisms are active in a wide number of diverse plant growth promoting rhizobacteria (PGPR) conferring drought and salt tolerance to crop plants (Vurukonda et al., 2016). In all living organisms the key physiological basis of salt and drought tolerance is the accumulation of so-called osmolytes. Important osmolytes are certain amino acids (especially proline), amines (glycine betaine or ectoine) and sugars, which have specific physicochemical properties to protect cellular protein structures and balance high osmotic pressure. Oxygen stress is also accompanying abiotic and biotic stress situations. Therefore, the oxygen radical scavenging enzymes are also very important in these situations. For example, the oxygen radical scavenging enzyme superoxide dismutase of *Gluconacetobacter diazotrophicus* PAL5 is obligatory for endophytic colonization of rice (Alquerres et al., 2013). Concerning the hormonal drought-stress responses in plants, abscisic acid (ABA) and other phytohormones, such as jasmonates and ethylene are involved. In addition, the auxin indole-3-acetic acid (IAA) in an optimal ratio to other phytohormones plays an integral part in plant adaptation to water stress. It is therefore of particular importance under abiotic stress conditions that root associated bacteria themselves produce and excrete auxins and other phytohormones or influence the phytohormone balance supporting plants which have too low auxin levels due to salt inhibition. The use of salt-tolerant phytohormone producing bacteria in combination with high osmolyte containing seaweeds is widely distributed and successfully applied to support crop growth in salt affected soils as reviewed by Nabti et al. (2015). Another approach to solve the lack of IAA in plants under salt stress is the inoculation with IAA-overproducing *Ensifer meliloti* conferring drought tolerance to *Medicago sativa* (Defez et al., 2017b). Inoculation with IAA-overproducing strains of endophytic *Burkholderia cepacia* strain RRE25 caused stimulated root development and improved nutrient use efficiency (especially of phosphate uptake) leading to increased plant growth (Singh et al., 2013). In endophytic diazotrophic bacteria *Enterobacter cloacae* RCA25 and *Klebsiella variicola* RCA26 overproduction

of IAA upregulated nitrogen fixation in bacterial cultures and inoculated rice plants (Defez et al., 2017a). *Azospirillum brasilense* Cd mutants which overproduce IAA could be selected using the L-tryptophan toxic antimetabolite 5-fluor-tryptophan (Hartmann et al., 1983), leading to plants with increased root system at low nitrogen supply. Furthermore, using the proline antimetabolite 3-dehydropoline (DHP), *A. brasilense* Sp7 mutants with increased osmotolerance have been isolated (Hartmann et al., 1992). Concerning proline-overproducing strains, many salt-tolerant bacteria are already DHP-resistant. The screening for DHP-resistance was not yet systematically applied to yield strains with improved salt and drought tolerance.

QUORUM SENSING AUTOINDUCER N-ACYL-HOMOSERINE LACTONES AND AHL-PRODUCING RHIZOBACTERIA STIMULATE PLANT GROWTH UNDER NORMAL AND ABIOTIC STRESS CONDITIONS

The influence of AHLs on plant performance is dependent on the molecular structure of the AHL-signal. Treatment with water-soluble C4- and C6-homoserine lactones (HSL) changes in the phytohormone balance occurred in roots and shoots of *Arabidopsis thaliana* (von Rad et al., 2008). It was found that the presence of GCR1/GPA1 genes in the plant host mediates the stimulation of root growth (Liu et al., 2012). In barley plants, AHL-molecules applied in axenic model experiments with seedlings initiated a nitrous oxide (NO)-burst and Ca^{2+} -fluxes in root cells as well as morphological changes in roots (Rankl et al., 2016). As shown in *A. thaliana*, calmodulin receptors were involved in primary root elongation stimulated by 3-oxo-C6 HSL (Zhao et al., 2015). Furthermore, it was shown that ATMYB44 was involved in the enhanced elongation of primary roots (Zhao et al., 2016). Using several independent experimental approaches, it could be clearly proven that water-soluble AHLs are taken up by plant roots through the vascular system into the shoot by an energy-dependent process. However, this is only possible in plants, like *Arabidopsis thaliana*, wheat, or barley, which are devoid of AHL-degrading enzymes, like lactonases. In plant shoots, hydrophilic C4-, C6-, and C8-HSLs influence the activities of enzymes with e.g., anti-oxidative capacity or xenobiotic phase II detoxifying enzymes improving stress tolerance (Goetz-Roesch et al., 2015).

Water-insoluble AHLs with long CH-chains (e.g., C12- and C14-HSL) are also effective in modifying plant performance (Schenk and Schikora, 2015). Several lines of evidences point to the involvement of a specific receptor for water-insoluble AHLs in plant cells and the activation of a specific signaling cascade. In barley it was demonstrated that the ability of AHL-priming of systemic resistance is a genetically determined property (Wehner et al., 2019). Salicylic acid (SA) and oxylipin 12-oxo-phytodienoic acid (*cis*-OPDA) are involved in the systemic response and priming (Schenk and Schikora, 2015; Shrestha et al., 2020). In addition, the

activation of MAP-kinases MPK3 and MPK6 were enhanced and prolonged, along with the upregulation of defense-related transcription factors WRKY22 and WRKY29, glutathione-S-transferase GST6-gene and heat shock protein Hsp60-gene in *Arabidopsis* by a mixture of short- and long CH-chain AHLs (Shrestha et al., 2019). AHL-producing *Burkholderia graminis* M12 and M14 strains could induce tolerance to salt stress in wild type and genetically engineered tomato plants (Barriuso et al., 2008). It was recently shown by Zhao et al. (2020) that AHL-enhanced tolerance to salt stress in *Arabidopsis thaliana* and wheat was linked to ABA-dependent and ABA-independent signaling pathways and also the Salt Overly Sensitive (SOS) pathway. 3-oxo-C6-HSL treated plants had an increased proline and chlorophyll content, while lipid peroxidation, as measured by tissue content of malondialdehyde, and the Na^+/K^+ ratio was decreased. It was clearly demonstrated that inoculation with AHL-producing plant beneficial Gram-negative bacteria like *Gluconacetobacter diazotrophicus* PAL5 supports plant growth under adverse conditions. For example, *G. diazotrophicus* mitigates drought stress in *Oryza sativa* (Filgueiras et al., 2020), resulting in higher biomass production, higher levels of gas exchange and osmoprotecting solutes (proline and glycine betaine) in shoots and roots under drought conditions. Under reduced water availability malondialdehyde accumulation, a product of lipid peroxidation, increased drastically as result of cell damages, but in PAL5-inoculated plants the accumulation of malondialdehyde was much reduced. In addition, the anti-oxidant enzymes superoxide dismutase (*sodA*), glutathione reductase (*gor*),

catalase (*katE*), pyrrolinbe-5-carboxylate reductase (*P5CR*) and betaine aldehyde dehydrogenase (*BADH*) were stimulated by inoculation with *G. diazotrophicus* PAL5 under increased water stress. Interestingly, this “induced systemic tolerance” at reduced water availabilities was accompanied by an increased transcription of both pathogenesis related PR-1 and PR-10 genes, which are known to function in the salicylic acid (SA) and jasmonic acid (JA) pathways. Furthermore, in *G. diazotrophicus* PAL5 exposed to reduced water availability, AHL synthase gene expression increased dramatically, which is linked to a stimulation of N-acyl-homoserine lactone production. Thus, AHLs are probably the key bacterial signals behind the induced systemic tolerance response in red rice, reported by Filgueiras et al. (2020).

AHL-DEVOID MUTANTS ARE IMPAIRED IN BENEFICIAL PLANT EFFECTS

The central role of AHLs in the plant beneficial effects was proven several times with mutants lacking the *luxI*- or *luxR* gene homologue or in AHL-depleted transconjugants of PGPR. *Rhizobium radiobacter* F4 has been first demonstrated as endofungal bacterium of the plant growth promoting fungus *Serendipita indica* (syn. *Piriformospora indica*) (Sharma et al., 2008; Varma et al., 2012). However, even without the fungus, *R. radiobacter* F4 (RrF4) proved to have very similar plant beneficial effects as *S. indica*, like improved abiotic and biotic stress tolerance (Glaeser et al., 2015). Recently, it was

The presence of AHLs or AHL-producing bacteria initiate modifications on:

Plant cell level:

- nitrous oxide (NO)-burst and Ca^{2+} influx
- increased levels of osmolytes (proline, glycine betaine, trehalose)
- reduced Na^+/K^+ -ratio
- activation of MPK3 and MPK6-genes and priming
- low content on malondialdehyd (reduced lipid peroxidation)
- increased chlorophyll content
- activation of Salt Overly Sensitive (SOS) pathway

Whole plant (systemic) level:

- enhanced elongation of roots
- uptake of C4- to C8-HSL through vascular system into shoots
- increase of anti-oxygen stress (e.g. superoxide dismutase) enzymes
- C12-HSL initiate priming through SA- and cis-OPDA pathway (*lox2* and *NPR1* genes)
- activation of ABA-dependent and -independent pathways
- transcription of PR-1 and PR-10 genes
- higher levels of gas exchange
- higher biomass production

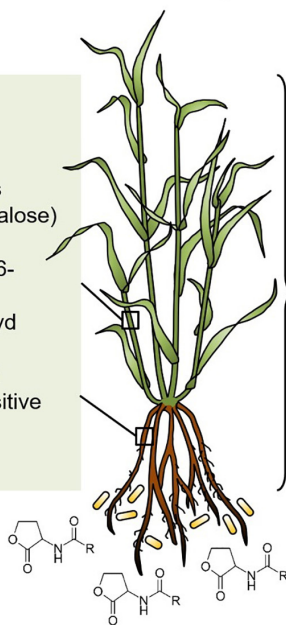


FIGURE 1 | Induced systemic tolerance against drought and salt stress in N-acyl-homoserine lactone-treated plants. MPK, Mitogen-associated protein kinase; SA, Salicylic acid; cis-OPDA, 12-oxo-phytodienoic acid; PR-1 and PR-10, Pathogenesis related proteins; ABA, Absciscic acid; lox, Lipoxigenase; NPR1, Nonexpressor of pathogenesis-related genes 1.

demonstrated that *R. radiobacter* F4 produced a spectrum of short and medium CH-chain AHL molecules (Alabid et al., 2020). *R. radiobacter* transconjugants F4NM13 with a lactonase-producing plasmid, which are devoid of AHLs, showed only reduced colonization abilities of roots of *Arabidopsis thaliana* and barley and only marginal plant beneficial effects. In the PGPR *Acidovorax radialis* N35 (Li, 2010), producing 3-OH-C10-HSL, *araI*-deleted mutants were less effective in root colonization in competition with the wild type. While the AHL-producing wild type induced a plant expression profile of stimulation and some priming, the AHL-deleted mutant caused increased expression of defense responses like flavonoid biosynthesis (Han et al., 2016). Thus, in *A. radialis*, AHL-production could influence the perception by a host plant. This was also indicated by *Arabidopsis thaliana* and barley inoculation with the AHL-negative transconjugant *Rhizobium radiobacter* F4NM13 (Alabid et al., 2020), but more studies are necessary to further document this important issue.

POSSIBLE ROLES OF *LuxR*-SOLOS IN PLANT BENEFICIAL EFFECTS

In the frequently applied *Azospirillum brasilense* strains Ab-V5, Sp245, Sp7, or Az39 and also in other *Azospirillum* species strains (Gualpa et al., 2019) *luxR*-solo or *luxR*-orphan genes were found, i.e., *luxR*-homologous receptors without the presence of a *luxI*-type AHL-synthase. It has been shown by Fukami et al. (2018) that the addition of 3-oxo-C6-HSL to *luxR*-solo *A. brasilense* Ab-V5 caused a significant stimulation of plant colonization traits, demonstrating that external available AHLs were perceived and resulted in a positive response of stimulated bacteria-plant interaction. It has been proposed by Patel et al. (2013) that bacterial *luxR*-solos have evolved to respond to AHL-related or even rather unrelated signals from plants. This evolution of the *luxI*-/*luxR*-type quorum sensing regulon may be regarded as the basis of a novel signal/receptor couple for plant-bacteria communication (Schaefer et al., 2013, 2016). According to Gualpa et al. (2019), quorum quenching activities were prevalent in *A. brasilense* Az39, which also lacks a *luxI*-type AHL-biosynthesis gene. It may be possible, that *A. brasilense* Az39 has already gone steps forward in the evolution of the QS system and specialized on the degradation of AHLs. This may provide the bacterium an advantage to neutralize competing AHL-producing bacteria in the rhizosphere and in parallel facilitates receiving communication signals from the plant through *luxR*-solo receptors.

FUTURE PERSPECTIVES

A high diversity of rhizobacteria support plants in environmental stress conditions, especially under salt and drought stress using different mechanisms (Kumar et al., 2020). In addition, QS signals of the N-acyl-homoserine lactone-type from Gram-negative bacteria stimulate plant development and prime systemic tolerance to environmental stress in crop plants. This

stimulation occurs at cellular as well as whole plant level, as summarized in **Figure 1**. Thus, it should be a desirable goal to use AHL-producing bacteria or their AHL-signals as well as AHL quorum sensing related activities to stimulate plant tolerance to abiotic stress. In this context, synthetic biology would be a helpful approach (Stephens and Bentley, 2020). The entire network of interactions of the plant microbiome with host plants under different biotic and abiotic stress situations needs to be further developed also in a systems biology approach (Rodriguez et al., 2019). Besides the stimulatory interaction of AHL-producing bacteria with plants, C4- and C6-HSL molecules as foliar spray or seed treatment should be applied to improve plant performance, yields and resistance to biotic and abiotic stresses as was successfully demonstrated by Moshynets et al. (2019). Furthermore, a nanocomposite fertilizer using magnetic carbon nanofibers coupled with C4-HSL stimulated seed germination and growth of crops with enhanced resistance to oxidative and high salinity stresses (Gupta et al., 2019). Thus, the application of a synthetic AHL concentrate for bacteria-plant stimulation started already quite promising and AHL-QS research should be emphasized in the future involving more different crop plants. However, it should be kept in mind that not all cultivars are susceptible for AHL-perception (Shrestha et al., 2019). More basic understanding of the underlying mechanisms should further support successful field applications to modify and improve crop growth in abiotic and biotic challenging conditions.

AUTHOR CONTRIBUTIONS

AH designed the structure and content of the manuscript. SK wrote part of the manuscript, corrected the final manuscript, and contributed the figure. MR wrote part of the manuscript and corrected the final manuscript. All authors contributed to the article and approved the submitted version.

FUNDING

Deutsche Forschungsgemeinschaft (DFG) Grant No. RO2340/4-1 supported SK in the ongoing project. We thank Helmholtz Zentrum München for institutional funding of the former Research Unit Microbe-Plant Interactions, headed by AH, the special inhouse funding of the interdisciplinary research project “Molecular signaling in the Rhizosphere” and the current Institute of Network biology (MR). The DFG-project RO2340/4-1 covers the fees for open access publication.

ACKNOWLEDGEMENTS

The support of Helmholtz Center Munich, German Research Center for Health and Environment, München/Neuherberg, during the interdisciplinary in house funding topic “Molecular Interactions in the Rhizosphere” is greatly appreciated. The contribution of Sophia Klink to this manuscript was supported by Deutsche Forschungsgemeinschaft (DFG), grant RO 2340/4-1.

REFERENCES

- Alabid, I., Hardt, M., Imani, J., Hartmann, A., Rothballer, M., Li, D., et al. (2020). The N-acyl-homoserine lactone depleted *Rhizobium radiobacter* RrF4NM13 shows reduced growth-promoting and resistance-inducing activities in mono- and dicotyledonous plants. *J. Plant Dis. Prot.* 8:127. doi: 10.1007/s41348-020-00360-8
- Alqueres, S., Meneses, C., Rouws, L. M., Rothballer, M., Baldani, I., Schmid, M., et al. (2013). The bacterial superoxide dismutase and glutathione reductase are crucial for endophytic colonization of rice roots by *Gluconacetobacter diazotrophicus* PAL5. *Mol. Plant Microbe Interact.* 26, 937–945. doi: 10.1094/mpmi-12-12-0286-r
- Barriuso, J., Solano, B. R., Fray, R. G., Cámara, M., Hartmann, A., and Gutierrez Manero, F. J. (2008). Transgenic tomato plants alter quorum sensing in plant growth-promoting rhizobacteria. *Plant Biotech. J.* 6, 442–452. doi: 10.1111/j.1467-7652.2008.00331.x
- Defez, R., Andreozzi, A., and Bianco, C. (2017a). Overproduction of IAA in endophytes upregulate nitrogen fixation in both bacterial cultures and inoculated rice plants. *Microb. Ecol.* 74, 441–452. doi: 10.1007/s00248-017-0948-4
- Defez, R., Andreozzi, A., Dickinson, M., Charlton, A., Tadini, L., Pesaresi, P., et al. (2017b). Improved drought stress response in Alfalfa plants nodulated by an IAA- overproducing *Rhizobium* strain. *Front. Microbiol.* 8:e2466. doi: 10.3389/fmicb.2017.02466
- Egamberdieva, D., Wirth, S. J., Alqarawi, A. A., Abd Allah, E. F., and Hashem, A. (2017). Phytohormones and beneficial microbes: essential components for plants to balance stress and fitness. *Front. Microbiol.* 8:2104. doi: 10.3389/fmicb.2017.02104
- Filgueiras, L., Silva, R., Almeida, I., Vidal, M., Baldani, J. I., and Meneses, C. H. S. (2020). *Gluconacetobacter diazotrophicus* mitigates drought stress in *Oryza sativa* L. *Plant Soil* 451, 57–73. doi: 10.1007/s11104-019-04163-1
- Fukami, J., Abrantes, J. L. F., del Cerro, P., Nogueira, M. A., Ollero, F. J., Megias, M., et al. (2018). Revealing strategies of quorum sensing in *Azospirillum brasilense* strains Ab-V5 and Ab-V6. *Arch. Microbiol.* 200, 47–56. doi: 10.1007/s00203-017-1422-x
- Glaeser, S. P., Imani, J., Alabid, I., Guo, H., Kämpfer, P., Rothballer, M., et al. (2015). The endofungal bacterium *Rhizobium radiobacter* RrF4 colonizes plant roots and exhibits beneficial activity independently of its fungal host *Piriformospora indica*. *ISME J.* 10, 871–884. doi: 10.1038/ismej.2015.163
- Glick, B. R. (2014). Bacteria with ACC deaminase can promote plant growth and help to feed the world. *Microbiol. Res.* 169, 30–39. doi: 10.1017/j.micres.2013.09.009
- Goetz-Roesch, C., Riedel, T., Schmitt-Kopplin, P., Hartmann, A., and Schroeder, P. (2015). Influence of bacterial N-acyl-homoserine lactones on growth parameters, pigments, anti-oxidative capacities and the xenobiotic phase II detoxification enzymes in barley and yam bean. *Front. Plant Sci.* 6:205. doi: 10.3389/fpls.2015.00205
- Gualpa, J., Lopez, G., Nieves, S., Coniglio, A., Halliday, N., Cámara, M., et al. (2019). *Azospirillum brasilense* Az39, a model rhizobacterium with AHL quorum-quenching capacity. *J. Appl. Microbiol.* 14:269. doi: 10.1111/jam.14269
- Gupta, G. S., Kumar, A., and Verma, N. (2019). Bacterial homoserine lactones as a nanocomposited fertilizer and defense regulator for chickpeas. *Environ. Sci. Nano.* 6, 1246–1258. doi: 10.1039/c9en00199a
- Han, S., Li, D., Trost, E., Mayer, K. F., Vlot, C., et al. (2016). Systemic response of barley to the 3-hydroxy-decanoyl-homoserine lactone producing plant beneficial endophyte *Acidovorax radialis* N35. *Front. Plant Sci.* 7:e1868. doi: 10.3389/fpls.2016.01868
- Hartmann, A., Gündisch, C., and Bode, W. (1992). *Azospirillum* mutants improved in iron acquisition and osmotolerance as tools for the investigation of environmental fitness traits. *Symbiosis* 13, 271–279.
- Hartmann, A., and Schikora, A. (2012). Quorum sensing of bacteria and trans-kingdom Interactions of N-acyl homoserine lactones with eukaryotes. *J. Chem. Ecol.* 38, 704–713. doi: 10.1007/s10886-012-0141-7
- Hartmann, A., Singh, M., and Klingmüller, W. (1983). Isolation and characterization of *Azospirillum* mutants excreting high amounts of indole acetic acid. *Can. J. Microbiol.* 29, 916–923. doi: 10.1139/m83-147
- Hense, B. A., and Schuster, M. (2015). Core principles of bacterial auto-inducer systems. *Microbiol. Mol. Biol. Rev.* 79, 153–169. doi: 10.1128/MMBR.00024-14
- Hryniewicz, K., Patz, S., and Ruppel, S. (2019). *Salicornia europaea* L. as an underutilized saline-tolerant plant inhabited by endophytic diazotrophs. *J. Adv. Res.* 19, 49–56. doi: 10.1016/j.jare.2019.05.002
- Jha, B., Gontia, I., and Hartmann, A. (2012). The roots of the halophyte *Salicornia brachiata* are source of new halotolerant diazotrophic bacteria with plant growth promoting potential. *Plant Soil* 356, 265–277. doi: 10.1007/s11104-011-0877-9
- Kumar, A., Singh, S., Gaurav, A. K., Srivastava, S., and Verma, J. P. (2020). Plant growth-promoting bacteria: biological tools for the mitigation of salinity stress in plants. *Front. Microbiol.* 11:e1216. doi: 10.3389/fmicb.2020.01216
- Li, D. (2010). Phenotypic variation and molecular signalling in the interactions of the rhizosphere bacteria *Acidovorax radialis* N35 and *Rhizobium radiobacter* F4 with roots. Ph D thesis, Germany: Ludwig-Maximilians-Universität München.
- Liu, F., Bian, Z., Jia, Z., Zhao, Q., and Song, S. (2012). The GCR1 and GPA1 participate in promotion of *A. thaliana* primary root elongation induced by N-acyl-homoserine lactones, the bacterial QS-signals. *Mol. Plant Microbe Interact.* 25, 677–683. doi: 10.1094/MPMI-10-11-0274
- Moshynets, O. V., Babenko, L. M., Rogalsky, S. P., Iungin, O. S., Foster, J., Kosakivska, I. V., et al. (2019). Priming winter wheat seeds with the bacterial quorum sensing N-hexanoyl-L-homoserine lactone (C6-HSL) shows potential to improve plant growth and seed yield. *PLoS One* 14:e0209460. doi: 10.1371/journal.pone.0209460
- Nabti, E., Schmid, M., and Hartmann, A. (2015). *Application Of Halotolerant Bacteria To Restore Plant Growth Under Salt Stress*. Halophiles: Springer-Verlag, 235–259.
- Patel, H. K., Suárez-Moreno, Z. R., Degraess, G., Subramoni, S., González, J. F., and Venturi, V. (2013). Bacterial *LuxR* solos have evolved to respond to different molecules including signals from plants. *Front. Plant Sci.* 4:e447. doi: 10.3389/fpls.2013.00447.03
- Rankl, S., Gunsé, B., Sieper, T., Schmid, C., Poschenrieder, C., and Schröder, P. (2016). Microbial homoserine lactones (AHLs) are effectors of root morphological changes in barley. *Plant Sci.* 253, 130–140. doi: 10.1016/j.plantsci.2016.09.014
- Rodriguez, P. A., Rothballer, M., Chowdhury, S. P., Nussbaumer, T., Gutjahr, C., and Falter-Braun, P. (2019). Systems Biology of Plant-Microbiome Interactions. *Molec. Plant* 12, 804–821. doi: 10.1016/j.molp.2019.05.006
- Rothballer, M., Uhl, J., Kunze, J., Schmitt-Kopplin, P., and Hartmann, A. (2018). “Detection of bacterial quorum sensing signaling molecules N-acyl-homoserine lactones and N-acyl homoserine with an enzyme-linked immunosorbent assay (ELISA) and via ultrahigh performance liquid chromatography coupled to mass spectrometry (UPLC-MS),” in *Quorum Sensing: Methods and Protocols. Springer series “Methods in Molecular Biology”*, eds L. Leoni and G. Rampioni (Netherlands: Springer Science), 61–72. doi: 10.1007/978-1-4939-7309-5_5
- Schaefer, A. L., Lappala, C. R., Morlen, R. P., Pelletier, D. A., Lu, T. S., Lankford, P. K., et al. (2013). *LuxR*- and *LuxI*-type quorum sensing circuits are prevalent in members of the *Populus deltoides* microbiome. *Appl. Environ. Microbiol.* 79, 5745–5752. doi: 10.1128/AEM.01417-13
- Schaefer, A. L., Oda, Y., Coutinho, B. G., Pelletier, D. A., Weiburg, J., Venturi, V., et al. (2016). A *luxR* homolog in a cottonwood tree endophyte that activates gene expression in response to a plant signal or specific peptides. *mBio* 7, 1101–1116. doi: 10.1128/mBio.01101-16
- Schenk, S. T., and Schikora, A. (2015). AHL-priming functions via oxylipin and salicylic acid. *Front. Plant Sci.* 5:e784.
- 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
- Schuhegger, R., Ihring, A., Gantner, S., Bahnweg, G., Knappe, C., Vogt, G., et al. (2006). Induction of systemic resistance in tomato plants by N-acyl-homoserine lactone-producing rhizosphere bacteria. *Plant Cell Environ.* 29, 909–918. doi: 10.1111/j.1365-3040.2005.01471.x
- Sharma, M., Schmid, M., Rothballer, M., Hause, G., Zuccaro, A., Imani, J., et al. (2008). Detection and identification of mycorrhizal helper bacteria intimately associated with representatives of the order *Securinales*. *Cell. Microbiol.* 10, 2235–2246. doi: 10.1111/j.1462-5822.2008.01202.x

- Shrestha, A., Elhady, A., Adss, S., Wehner, G., Böttcher, C., Heuer, H., et al. (2019). Genetic differences in barley govern the responsiveness to N-acyl homoserine lactone. *Phytobiomes J.* 3, 191–202. doi: 10.1094/phytobiomes-03-19-0015-r
- Shrestha, A., Grimm, M., Ojio, I., Krumweide, J., and Schikora, A. (2020). Impact of quorum sensing molecules on plant growth and immune system. *Front. Microbiol.* 11:e1545.
- Singh, R. K., Malik, N., and Singh, S. (2013). Improved nutrient use efficiency increases plant growth of rice with the use of IAA-overproducing strains of endophytic *Burkholderia cepacia* strain RRE25. *Microb. Ecol.* 66, 375–384. doi: 10.1007/s00248-013-0231-2
- Stephens, K., and Bentley, W. E. (2020). Synthetic biology for manipulating quorum sensing in microbial consortia. *Trends Microbiol.* 2, 633–643. doi: 10.1016/j.tim.2020.03.009
- Suarez, C., Cardinale, M., Ratering, S., Steffens, D., Jung, S., Montoya, A. M. Z., et al. (2015). Plant growth promoting effects of *Hartmannibacter diazotrophicus* on summer barley (*Hordeum vulgare*, L.) under salt stress. *Appl. Soil. Ecol.* 95, 23–30. doi: 10.1016/j.apsoil.2015.04.017
- Varma, A., Bakshi, M., Lou, B., Hartmann, A., and Oelmueller, R. (2012). *Piriformospora indica*: A novel plant growth-promoting mycorrhizal fungus. *Agric. Res.* 1, 117–131. doi: 10.1007/s40003-012-0019-5
- von Rad, U., Klein, I., Dobrev, P. I., Kottova, J., Zazimalova, E., Fekete, A., et al. (2008). The response of *Arabidopsis thaliana* to N-hexanoyl-DL-homoserine-lactone, a bacterial quorum sensing molecule produced in the rhizosphere. *Planta* 229, 73–85. doi: 10.1007/s00425-008-0811-4
- Vurukonda, S. S. K. P., Vardharajula, S., Shrivastava, M., and Skz, A. (2016). Enhancement of drought stress tolerance in crops by plant growth promoting rhizobacteria. *Microbiol. Res.* 184, 13–24. doi: 10.1016/j.micres.2015.12.003
- Wehner, G., Kopalmke, D., Richter, K., Kecke, S., Schikora, A., and Ordon, F. (2019). Priming is a suitable strategy to enhance resistance towards leaf rust in barley. *Phytobiome J.* 18:41. doi: 10.1094/PBIOMES-09-18-0041-R
- Zhao, Q., Li, M., Jia, Z., Liu, F., Ma, H., Huang, Y., et al. (2016). AtMYB44 positively regulates the enhanced elongation of primary roots induced by N-oxo-hexanoyl homoserine lactone in *Arabidopsis thaliana*. *Mol. Plant Microbe Interact.* 29, 774–785. doi: 10.1094/mpmi-03-16-0063-r
- Zhao, Q., Yang, X. Y., Li, Y., Liu, F., Cao, X. Y., Jia, Z. H., et al. (2020). N-3-oxo-hexanoyl-homoserine lactone, a bacterial quorum sensing signal, enhances salt tolerance in *Arabidopsis* and wheat. *Bot. Stud.* 61:8. doi: 10.1186/s40529-020-00283-5
- Zhao, Q., Zhang, C., Jia, Z., Huang, Y., Li, H., Song, S., et al. (2015). Involvement of calmodulin in regulation of primary root elongation by N-3-oxo-hexanoyl homoserine lactone in *Arabidopsis thaliana*. *Front. Plant Sci.* 5:807. doi: 10.3389/fpls.2014.00807

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.

Copyright © 2021 Hartmann, Klink and Rothballer. This is an open-access article distributed under the terms of the Creative Commons Attribution License (CC BY). The use, distribution or reproduction in other forums is permitted, provided the original author(s) and the copyright owner(s) are credited and that the original publication in this journal is cited, in accordance with accepted academic practice. No use, distribution or reproduction is permitted which does not comply with these terms.



Arbuscular Mycorrhiza-Mediated Regulation of Polyamines and Aquaporins During Abiotic Stress: Deep Insights on the Recondite Players

Karuna Sharma, Samta Gupta, Sarda Devi Thokchom, Pooja Jangir and Rupam Kapoor*

Department of Botany, University of Delhi, New Delhi, India

OPEN ACCESS

Edited by:

Alvaro Sanz-Saez,
Auburn University, United States

Reviewed by:

Milan Borisev,
University of Novi Sad, Serbia
Fernando Torralbo,
University of Missouri, United States
Nieves Goicoechea,
University of Navarra, Spain

*Correspondence:

Rupam Kapoor
kapoor_rupam@yahoo.com

Specialty section:

This article was submitted to
Plant Abiotic Stress,
a section of the journal
Frontiers in Plant Science

Received: 15 December 2020

Accepted: 11 May 2021

Published: 17 June 2021

Citation:

Sharma K, Gupta S, Thokchom SD,
Jangir P and Kapoor R (2021)
Arbuscular Mycorrhiza-Mediated
Regulation of Polyamines and
Aquaporins During Abiotic Stress:
Deep Insights on the Recondite
Players. *Front. Plant Sci.* 12:642101.
doi: 10.3389/fpls.2021.642101

Environmental stresses of (a)biotic origin induce the production of multitudinous compounds (metabolites and proteins) as protective defense mechanisms in plants. On account of the regulation of some of these compounds, arbuscular mycorrhizal fungi (AMF) reinforce the inherent tolerance of plants toward the stress of different origins and kind. This article reviews two specific fundamental mechanisms that are categorically associated with mycorrhiza in alleviating major abiotic stresses, salt, drought, and heavy metal (HM) toxicity. It puts emphasis on aquaporins (AQPs), the conduits of water and stress signals; and polyamines (PAs), the primordial stress molecules, which are regulated by AMF to assure water, nutrient, ion, and redox homeostasis. Under stressful conditions, AMF-mediated host AQP responses register distinct patterns: an upregulation to encourage water and nutrient uptake; a downregulation to restrict water loss and HM uptake; or no alterations. The patterns thereof are apparently an integrative outcome of the duration, intensity, and type of stress, AMF species, the interaction of fungal AQPs with that of plants, and the host type. However, the cellular and molecular bases of mycorrhizal influence on host AQPs are largely unexplored. The roles of PAs in augmenting the antioxidant defense system and improving the tolerance against oxidative stress are well-evident. However, the precise mechanism by which mycorrhiza accords stress tolerance by influencing the PA metabolism *per se* is abstruse and broadly variable under different stresses and plant species. This review comprehensively analyzes the current state-of-art of the involvement of AMF in “PA and AQP modulation” under abiotic stress and identifies the lesser-explored landscapes, gaps in understanding, and the accompanying challenges. Finally, this review outlines the prospects of AMF in realizing sustainable agriculture and provides insights into potential thrust areas of research on AMF and abiotic stress.

Keywords: abiotic stress, arbuscular mycorrhizal fungi, aquaporins, polyamines, salt stress, drought stress, heavy metal toxicity

INTRODUCTION

Environmental factors like light, temperature, water, and soil status are important abiotic components that regulate the plant life cycle, and any abnormality in these factors predisposes the plants to stress, a state of altered physiology (Sade et al., 2013). Abiotic stresses, such as salinity, drought, and heavy metals (HMs), result in a 70% reduction in global crop yield worsening the dwindling equilibrium between crop production and exponential population growth (Singh et al., 2016). In addition to this, abiotic stresses, particularly salinity and HMs, depress the nutrient availability in soil and lead to nutritional disorders in plants (Juniper and Abbott, 1993; Evelin et al., 2012; Gusman et al., 2013; Gupta et al., 2021). Soil salinity and HM contamination have been demonstrated to limit the accumulation of minerals, such as Ca, Fe, Mg, and Zn in edible parts of crop plants (Gusman et al., 2013; Chakraborty et al., 2016; Alam et al., 2019; Liu et al., 2020), consequently reducing the nutritive value of the crops. Since these minerals are essential for the human diet, any decrease in their concentrations will have a major impact on nutritional security of food and can give rise to hidden hunger, which is a grave issue especially in a scenario where the global burden of crop production and micronutrient deficiency remains alarming (FAO, 2017).

High soil salinity (>4 dS/m; Juniper and Abbott, 1993) stems from rapid uptake and translocation of Na^+ and Cl^- ions by the plants at the expense of important nutrients such as K, P, and Ca and sequential translocation of these ions into the shoot tissues, thereby increasing the toxic load. Drought stress results from a moderate water loss, which, when gets extensive, results in cellular desiccation (Jaleel et al., 2009) that can cause potential loss of enzyme activity, gross disturbance of plant metabolism, and handicapped cell structure and function. Moreover, essential (Cu, Mn, Zn, Fe, Mo, Co, and Ni) and non-essential (As, Pb, Sn, Cd, Hg, Al, and Cr) HMs also generate toxic/lethal effects on plant growth and metabolism by the inhibition of nutrient assimilation and biomass accumulation, degradation of chlorophyll, passivation of enzymes, disturbed water balance, and senescence. These stresses exacerbate the risk to food security of the world with a teeming population that is projected to mushroom to 11.2 billion by the end of the century (Roser et al., 2013).

Most of the mitigation strategies that are employed to cope with adverse impacts of abiotic stress are either long term, such as breeding stress-tolerant varieties, or are inaccessible or costly to farmers (Bharti et al., 2017). We endeavor to usher sustainable crop production in a way that does not cause economical brunt on farmers. However, noteworthy is the fact that, despite considerable research into the effects of salt and drought on crops, the release of abiotic-tolerant/resistant cultivars has not been commensurate. The reason is most likely due to the complex nature of the effect of stressors on crop plant; as in a multigenic trait, like tolerance to stress, the hierarchy of different aspects of tolerance may differ among and within species (Flowers and Yeo, 1995; Flowers et al., 1997). Abiotic tolerance is way too complex to be easily amenable to refinement through the selection of a trait *per se*. On that account, arbuscular mycorrhizal fungi (AMF)

emerge to the occasion as a group of potential stress mitigators in plants owing to their tolerance to extremities, diversity in genetic makeup, ubiquity, and promiscuous interaction with plants and soil (Bharti et al., 2017). In view of that, the deployment of AMF to alleviate abiotic stress in plants is a better, an economical, and an effective agronomic option.

Intrinsic protective mechanisms aside, plants can counteract environmental stress by associating with AMF that form a promiscuous mutualistic relationship with the majority of terrestrial plants ($>100,000$ species) (Wang and Qiu, 2006). AMF orchestrate various biochemical and physiological pathways that coordinate to impart tolerance to the host plant. They maintain nutrients, ions, and redox homeostasis; enhance water acquisition by serving as extended plant roots, ensure photosynthetic efficiency and osmoregulation, and reinforce antioxidant metabolism (Evelin et al., 2019). Much of these protective roles conferred by mycorrhizal associations during stress can be ascribed to multiple factors, of which AMF-mediated modulation of polyamines (PAs) and aquaporins (AQPs), the recondite players in abiotic stress mitigation, are discussed at length in this review.

Due to their positively charged characteristics, PAs interact with anionic functional groups of membranes and proteins (Slocum et al., 1984). They influence the stability and permeability of cell membranes by the formation of electrostatic bonds with phospholipid head groups (Besford et al., 1993). They have also been reported to prevent loss of chlorophyll from thylakoid membranes by stabilizing the photosystem complexes (Popovic et al., 1979). PAs enhance osmolyte and antioxidant production and interact with other important metabolic routes that are of the essence to stress tolerance in plants. As highlighted in the review, there exists coordination between AMF and the PA metabolism (Sannazzaro et al., 2007; Cicatelli et al., 2010; Wu et al., 2012a; Abdel-Fattah et al., 2013; Talaat and Shawky, 2013; Zhang et al., 2020). However, the understanding of an exact mechanism underlying AMF-mediated modulation of PA pool in relation to salt/drought/HM tolerance is an uncharted area; ergo, merits more research for deeper insights and practical implications. AQPs are intrinsic membrane proteins that facilitate transcellular passive conduction of water and neutral molecules, a significant role that is implicated in the alleviation of water stress. Studies demonstrating the differential regulation of AQP genes in AMF-inoculated plants under water-deficit conditions (salinity and drought) consolidate the role of AMF in helping plants combat water scarcity, but the mechanisms underlying these responses at cellular and molecular level still remain elusive. However, the role of AQPs in combating metal stress in plants is limited to few records and demands extensive exploration. Information on the direct relationship among the two biomolecules (PA and AQP) and AMF *per se* is sketchy. However, it can be speculated that AMF-mediated PA homeostasis could be involved in regulating AQP protein abundance, thereby contributing to the maintenance of plant–water relations; although no concrete evidence exists to reinforce this postulation. Future research should venture into such investigations to expand the dimensions and depth of our understanding on this subject.

Along this review, we explore and analyze the contribution of various studies on the role of AMF in the modulation of the PA metabolism and AQP gene expression as significant mechanisms to impart tolerance to abiotic stresses in plants. As devising strategies that are oriented to improve stress recovery and stress resistance in plants become an enormous challenge, this review will outline the prospects of AMF in realizing sustainable agriculture.

POLYAMINE METABOLISM AND THEIR CROSS TALK WITH OTHER METABOLIC ROUTES INVOLVED IN STRESS TOLERANCE

Polyamines are ubiquitous biostimulants, with low-molecular-weight, that participate in an array of plant growth, developmental and defense processes under stressful conditions (Chen et al., 2019). Triamine spermidine (Spd), tetramine spermine (Spm), and their precursor, diamine putrescine (Put) are the principal free PAs found in plants (Galston and Sawhney, 1990).

PA metabolism: Putrescine occupies a central position in the common PA biosynthetic pathway whose biosynthesis transpires *via* two parallel routes: (1) arginine-derived pathway involving arginine decarboxylase (ADC) and (2) ornithine-derived pathway involving ornithine decarboxylase (ODC) (Hanfrey et al., 2001; Kakkar and Sawhney, 2002). The aminopropyl residues that are successively incorporated into the Put skeleton to yield Spd and Spm are furnished from decarboxylated S-adenosyl methionine (dcSAM). The addition of the aminopropyl moieties to the Put precursor is effectuated by the enzymes Spd synthase (SPDS) and Spm synthase (SPMS), respectively. The catabolism of PA is impelled by the action of two major classes of amine oxidases: Cu-containing diamine oxidase (DAO) and flavoprotein-containing PA oxidase (PAO). Stringent regulation of cellular PA titers is imperative for evoking responses to environmental cues (such as stress) through an intricate crosstalk with other metabolic pathways that are involved in the synthesis of stress signals and metabolites [ethylene, proline, gamma-aminobutyric acid (GABA), nitric oxide (NO), and abscisic acid (ABA)] involved in amplifying the defense response of plants against encountered stress (**Figure 1**).

Cross talk of PA metabolism with stress-defensive metabolites: Ethylene and PAs are interlinked by a common precursor, S-adenosyl methionine (SAM). The deviation of metabolic flux from ethylene synthesis toward PA synthesis confers tolerance as ethylene temporarily and reversibly hinders cell cycle and speeds up senescence in plants that are under the grip of environmental stress (Even-Chen et al., 1982; Dubois et al., 2018). The synthesis of proline is also in strong correlation with the PA metabolic route by virtue of the common precursor, glutamate (Sannazzaro et al., 2007; Mohapatra et al., 2009). A significant contribution of Put degradation by DAO to proline accumulation has also been reported. The ensuing accumulation of proline by the PA oxidation mediates osmoprotection and serves as a nitrogen reserve that can be exploited by the plant during

recovery. Stress-triggered changes in the level of PAs translate into the alteration in cellular content of a non-protein amino acid, GABA, which further boosts the antioxidant capacity and synthesis of protective osmolytes, proline and soluble sugars like trehalose, that are essential for cellular osmotic adjustments (Wang et al., 2017; Priya et al., 2019). PAs also induce the production of a signaling molecule, NO (Tun et al., 2006), that may bridge the PA-mediated stress response with other mediators of stress. NO also, in turn, increases the PA titers under stress (Tailor et al., 2019). PAs, in alliance with their catabolic products, H₂O₂ and NO, formed *via* different metabolic routes, appear to act cooperatively to fine-tune ABA responses in stomatal closure during physiological drought conditions (Yamasaki and Cohen, 2006). The presence of ABA-responsive elements, ABRE (namely, ABRE-related motifs) in the promoter sequence of PA biosynthetic genes has reinforced the notion that the participation of PAs in stress tolerance is ABA-dependent. PAs, precisely Put, and ABA are invested in a positive feedback circuit within which they mutually foster production of each other to ameliorate acclimation of plant to abiotic stress (Alcázar et al., 2010). Put activates the *NCED* gene that prompts the ABA accumulation *via* zeaxanthin. This further activates the ABRE elements that stimulate AREB/ABF transcription factors (TFs). These TFs, in turn, regulate the *ADC* gene and direct the synthesis of principal PA, Put (Espasandin et al., 2014) (**Figure 1**). Additionally, ABA can encourage methionine to produce a generous amount of higher PAs *via* dcSAM (Li et al., 2020).

Interaction among PAs, phytohormones, and other metabolites: There exists a highly conserved pathway that is expressed in plants subjected to stress, integrating different phytohormones that regulate plant processes *via* changes in glutamate metabolism (Podlešáková et al., 2018); and therefore, in the PA metabolism, regulating the reactive oxygen species (ROS) responses and NO production, which successively also regulate phytohormone signaling in stressed plants (Freschi, 2013). In general, PAs and plant hormone biosynthesis and signaling are closely related (reviewed by Anwar et al., 2015). Their extensive scrutiny of published data revealed that generally Put is positively correlated with the expression of genes regulating ABA biosynthesis (Alcazar et al., 2005; Singh et al., 2018) but have an inverse effect on hormones, such as ethylene, jasmonic acid, and gibberellic acid (GA) (Alcazar et al., 2005; Cuevas et al., 2008) while the action of Spd is totally antipodal to that (Radhakrishnan and Lee, 2013a; Li Z. et al., 2016). Conversely, Spm promotes the expression of genes involved in ethylene and jasmonate biosynthesis (Ozawa et al., 2009; Gonzalez et al., 2011) while downregulating those for gibberellins (Gonzalez et al., 2011) and ABA biosynthesis (Radhakrishnan and Lee, 2013b). Spd is found to positively regulate salicylic acid (SA) signaling (Lazzarato et al., 2009), and those of cytokinins and auxins are linked to Spm action (Anwar et al., 2015; Sharma et al., 2019). Overall, the nature of these cross-talks between PAs and phytohormones varies with the set of PAs and the hormone involved, and also with the developmental stage of the plant and abiotic conditions to which it is subjected. Mycorrhizal plants perceive the stress cues that trigger the modification in the endogenous levels of phytohormones and/or PA (phyto-regulators). This influences

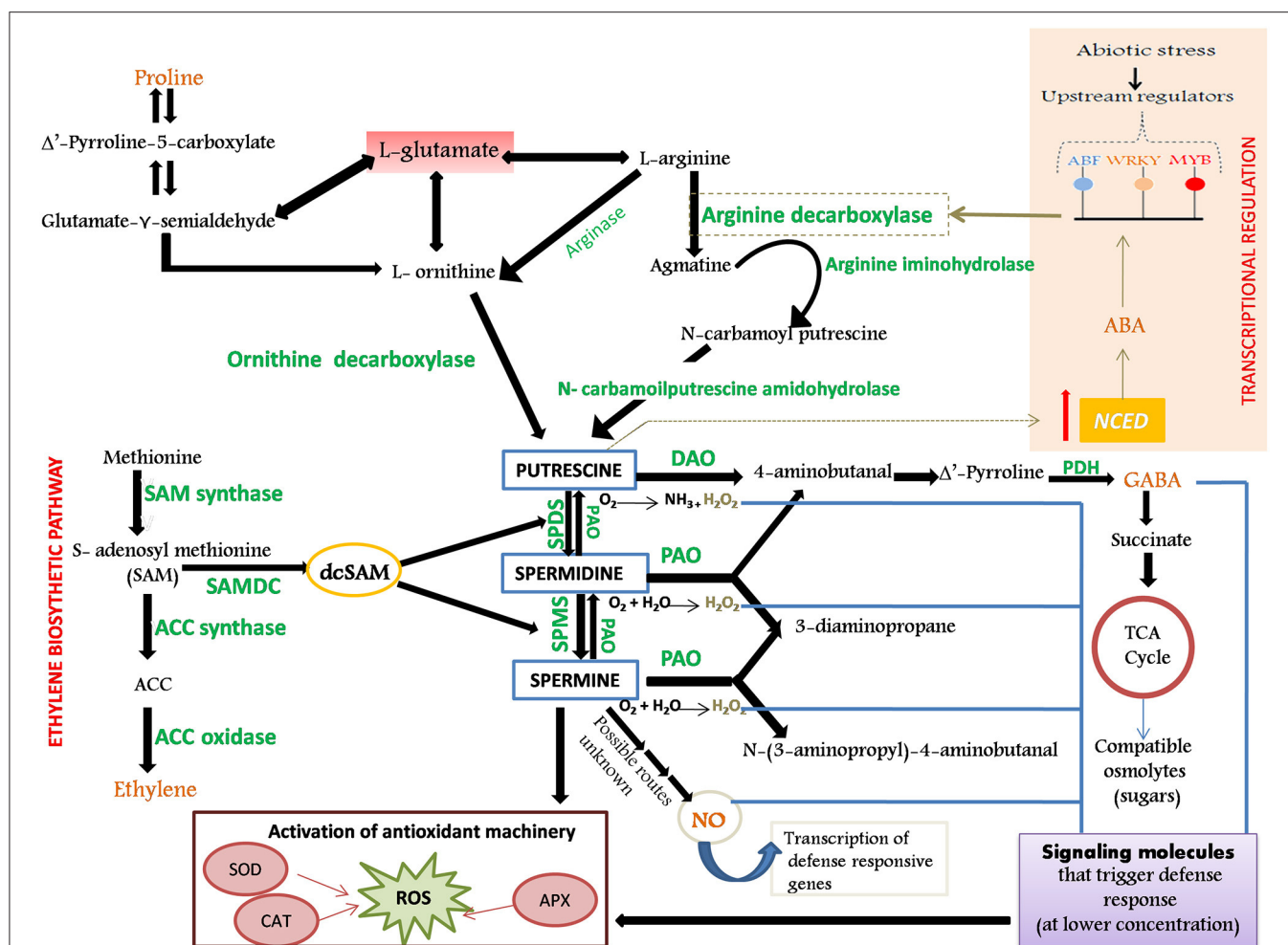


FIGURE 1 | Schematic representation of biosynthesis and catabolism of polyamines (PAs), their role in the activation of defense response in plants and cross-talk with stress-defensive metabolites. The enzymes involved in the metabolic processes are shown in green color. Putrescine (Put), the central molecule of PA biosynthesis, can be synthesized either by arginine (by ADC) or by ornithine (by ODC) derived pathway. Synthesis of diamine spermidine (Spd) and tetramine spermine (Spm) is brought about by the subsequent addition of aminopropyl groups (furnished from the decarboxylated penultimate precursor of ethylene, dcSAM) to the Put skeleton, and is catalyzed by SPDS and SPMS, respectively. Degradation of Put is catalyzed by DAO and that of Spd and Spm by PAO. PAs, along with their catabolism by-products (GABA, H_2O_2 , and NO), serve as stress messengers and participate in the reinforcement of antioxidant machinery. Production of GABA from PA further boosts the production of compatible osmolytes (such as sugars) under stress. Put and Pro are linked by the common precursor glutamate and share precursor-product relationship regulated by DAO and GABA. Through possibly unknown mechanisms, both PA and NO are found to increase production of each other during stress. Spd possibly intensifies NOS activity that boosts NO production. Put and abscisic acid (ABA) are invested in a positive feedback circuit; Put activates *NCED* gene that triggers ABA accumulation, which further activates TFs that in turn regulate the ADC gene. ODC, Ornithine Decarboxylase; ADC, Arginine Decarboxylase; DAO, Diamine Oxidase; PAO, Polyamine Oxidase; PDH, Pyrroline Dehydrogenase; SPMS, Spermine Synthase; SPDS, Spermidine Synthase; ACC, 1-aminocyclopropane-1-carboxylic acid; NO , Nitric Oxide; GABA, Gamma-Aminobutyric Acid; H_2O_2 , Hydrogen Peroxide; dcSAM, decarboxylated S-Adenosyl Methionine; Pro, Proline; NOS, Nitric Oxide Synthase; *NCED*, 9-Cis-Epoxyxycarotenoid Dioxygenase.

the expression of genes involved in primary metabolism and those relating to amino acid metabolism (proline, GABA, and glycine betaine), carbohydrate metabolism (trehalose and sucrose), and antioxidant metabolism [ascorbate-glutathione (AsA-GSH) cycle and enzymatic antioxidant defense]. AMF-protected plants exhibit reinforced PA production (Sannazzaro et al., 2007; Evelin et al., 2013), upregulated osmolyte production (Evelin et al., 2013; Garg and Saroy, 2020), and regulated synthesis of phytohormones (Shaul-Keinan et al., 2002; Khalloufi et al., 2017; Ren et al., 2018), all of which might potentially interconnect at various levels and ameliorate the tolerance mechanism for cumulative stress response (Hashem et al.,

2018). Different phytohormones influence overlapping processes such that the result of phytohormone action relies on a certain hormone combination rather than on their discrete actions (Iqbal et al., 2014).

AMF-MEDIATED MODULATION OF POLYAMINES AND THEIR RESPONSES UNDER STRESS

The close association of PAs with the defense response of plants is ascribed to multiple reasons: (1) transcriptional activity of

PA biosynthetic genes [*ADC*, *ODC*, *SPDS*, *SPMS*, and *SAM decarboxylase (SAMDC)*] and catalytic ability of the enzymes that participate in the PA metabolism are reinforced in the presence of stress, (2) induced suppression of PA synthesis by inhibitors such as difluoromethyl ornithine (DFMO), difluoromethyl arginine (DFMA), and cyclohexylamine (CHA) or by knocking down/out PA synthesizing genes, corresponds to a compromised defense response (Urano et al., 2003), (3) abundance of free and conjugated PAs in stress-tolerant cultivars under stressful conditions (Zapata et al., 2004), and (4) overexpression of PA biosynthetic genes results in a concomitant increase in the ability of plants to counter and acclimate to stress (Wen et al., 2008). Most studies till now have reiterated the positive impact of exogenous application of PAs on root/shoot architecture, development, and stress response in plants (for instance, *Panax ginseng*; Parvin et al., 2014, *Zoysia japonica* Steud; Li S. et al., 2016; *Bakraii citrus* seedlings; Khoshbakht et al., 2018, and *Cucumis sativus*; Wu et al., 2018). However, it is increasingly becoming a realm of scientific interest to strategize techniques by which endogenous PA production can be boosted so as to improve the overall development of plant as well as to magnify stress adaptiveness. Transgenic approaches oriented toward overexpressing PA biosynthetic genes and manipulating the intertwined metabolic web in a way that directs the metabolic flux toward the synthesis of stress messengers and scavengers are deemed as efficient techniques. Albeit they are successful, these high-throughput approaches are beyond the financial frontiers of many developing and low-middle-income nations. In this respect, use of AMF to optimize the PA metabolism emerges as an economically effective agronomic option. As aforementioned, AMF ensure the PA homeostasis that further helps in stress mitigation by the maintenance of pH and ion homeostasis, production of osmolytes and improvement of plant water status, ROS scavenging, stability of membrane and photosystem framework, modified expression of stress-responsive genes, regulation of root plasticity (Talaat and Shawky, 2013) (**Figure 2**), and the regulation of ribosomes, amino acids, and energy metabolism (Li et al., 2018). The mediation of stress tolerance using AMF by reinforcing the PA metabolism as a strategy is discussed in the following sections.

Salt Stress

High soil salinity directly results in various downstream stresses, viz., hyperionic stress, physiological drought, hyperosmotic stress, and nutrient imbalance (Evelin et al., 2009; Porcel et al., 2012). Several studies have dealt with the association of AMF and the PA metabolism in plants to improve tolerance to salt stress (Sannazzaro et al., 2007; Echeverria et al., 2013; Evelin et al., 2013; Talaat and Shawky, 2013; Abeer et al., 2014).

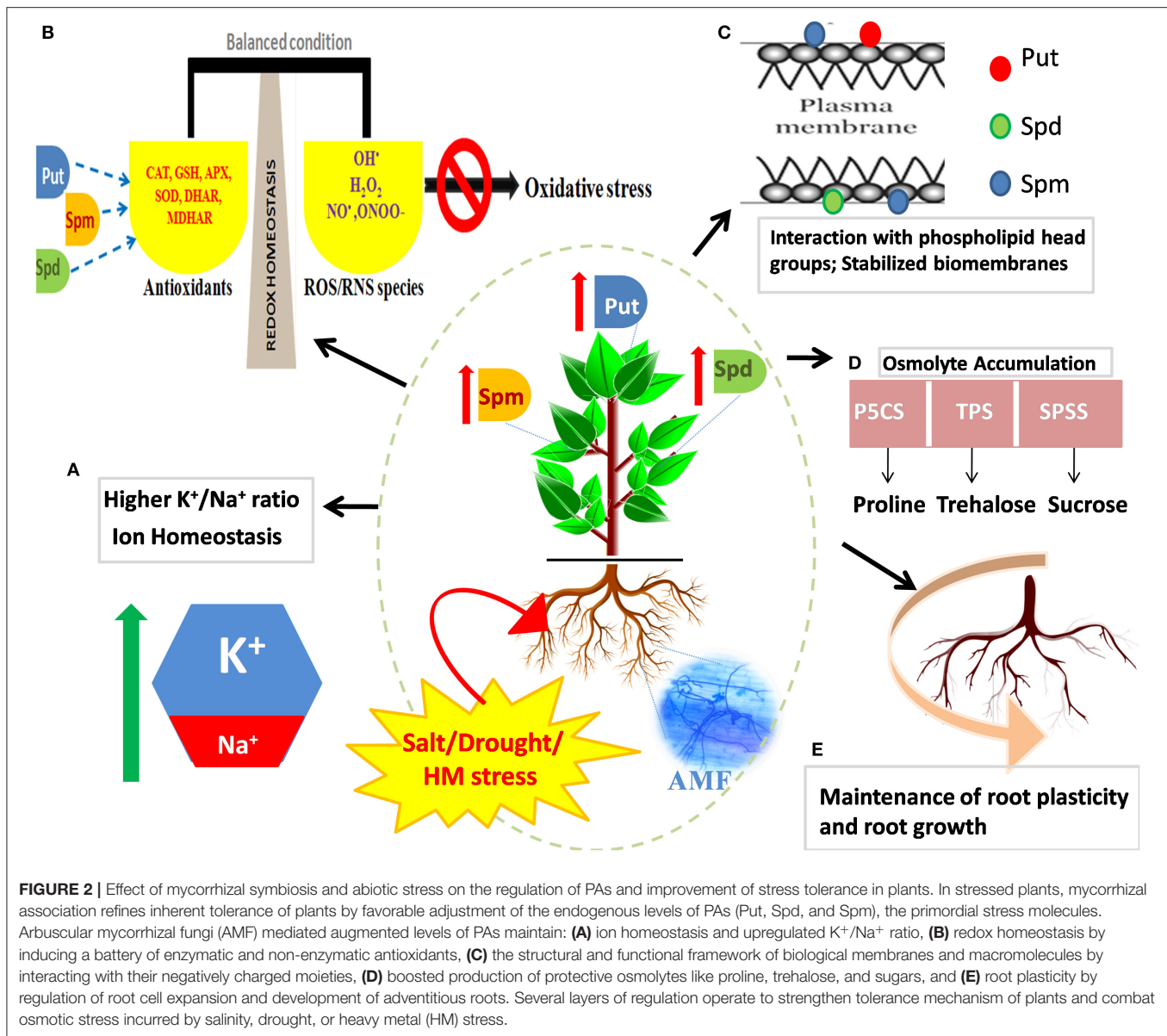
Sannazzaro et al. (2007) reported an increase in the Spd+Spm/Put ratio in salinity-stressed roots of *Lotus glabra* and ascribed it as a mechanism deployed by AMF to mediate the defense response of plants under unfavorable conditions. Lesser free Put in the leaves could be postulated as a deviation of the metabolic pool either toward the synthesis of higher PAs, Spd, and Spm (that have better protective capacity than Put by virtue of additional NH₂ groups) or toward proline

synthesis (as glutamate is the common precursor of proline and PA, **Figure 1**). However, the validation of this hypothesis would require exhaustive investigation to elucidate the relationship among mycorrhization, root architecture, and the balance between primary and secondary metabolism. Additionally, they concluded that both mycorrhizal and non-mycorrhizal plants would be equally stressed since they accumulate similar proline contents. Moreover, no correlation was observed between an increased Spd+Spm/Put ratio and the proline accumulation (Sannazzaro et al., 2007). Contrastingly, the AMF inoculation leads to a significant reduction in DAO and PAO activity in salt-stressed wheat plants that accounted for increased content of free Put, Spd, and Spm, and technically highest Put level, which boosted the fitness of plants to salt stress and influenced the ROS-scavenging antioxidant mechanism in inoculated plants (Talaat and Shawky, 2013). Likewise results were obtained in inoculated *Trigonella foenum-graecum* (Evelin et al., 2013), *Lotus tenuis* (Echeverria et al., 2013), and *Vicia faba* (Abeer et al., 2014) plants, wherein AMF application augmented the free PA titers along with other favorable influences on plant metabolism (**Table 1**). Premised on a tight correlation of Put synthetases and root architecture, it can be argued that AMF-triggered root Put (and not Spm and Spd) refines root traits, hence fostering better nutrient acquisition and improved drought acclimation, as confirmed by Wu et al. (2012b) in *Citrus tangerine* plants.

The synergistic role of PAs and AMF in helping the plant combat stress is also substantiated by mounting evidence that demonstrates how PA and AMF mutually foster each other under stress and concomitantly exert their beneficial effects to make plants more resilient to stress (**Figure 3**), as the dual administration of AMF and PA is found to be more efficient in maintaining/improving phenotype of the plant under stress than AMF inoculation or PA administration alone (Niemi et al., 2006; Ibrahim et al., 2011; Abdel-Fattah et al., 2013). A comprehensive conclusion can be squeezed out from all the investigations carried out hitherto; there exists a tightly regulated coordination between AMF and the PA metabolism that is committed to refine physiological plasticity of plants and resilience to assure its survival under unfavorable environments. These implications may be of practical importance in realizing the economically feasible, yet efficient, development of salt-tolerant crops.

Drought Stress

Notwithstanding the precise role of PAs in abiotic stress tolerance and the intimate mechanisms of such an effect are uncertain, there has been mounting evidence evincing the role of PAs in drought-stressed plants. Several studies corroborate the fact that the PA reservoir of plants corresponds to drought stress tolerance. For instance, Nahar et al. (2016) reported in *Vigna radiata* the Spm-mediated upregulation of antioxidant genes and decreased methylglyoxal toxicity by the activity of refined glyoxalase system. This system plays a critical role in counteracting oxidative stress by recycling reduced GSH, which is entrapped by methylglyoxal, consequently maintaining GSH homeostasis. Higher PA levels were recorded in water-stressed wheat, as postulated to be responsible for drought



tolerance and grain filling (Liu et al., 2016). PAs moderate the evolution rate of ethylene (antagonistic behavior), which might have accounted to better grain filling in wheat plants. In a pioneering study by Hura et al. (2015), drought stress tolerance in triticale during late developmental stages (from tillering to anthesis) was associated with specific accumulation of cell membrane-bound PAs and decreased content of free PAs. This could be accredited to the role of PAs in the reinforcement of plant cell wall under stress. Besides, PAs are also capable of regulating stomatal movements under drought stress conditions by encumbering inward rectifying K^+ channels (KIRC) (Liu et al., 2000) (Figure 2). The binding of Put ions to the KIRC site propels the outward displacement of K^+ ions and further retards K^+ movement across the channel (Chen et al., 2020). PAs are significant targets in AMF-mediated drought stress

regulation. However, inconsistency in PA accrual patterns has been observed in AM plants in response to water deficit. The first pioneering study in reference to this was conducted by Goicoechea et al. (1998) wherein the inoculation of water-stressed alfalfa plants with *Glomus fasciculatum* resulted in the following: (1) an increment of free Spm and Spd levels, which might serve as a nitrogen reserve that can be used to fulfill immediate energy demands of the plants soon after it recuperates from stress and (2) a higher enhancement of PA concentration in leaves than that in roots, which might be due to a high transpiration rate observed in drought-stressed plants that fostered a long-distance transport of PAs from root to shoot *via* transpiration stream. Also, the free Spd contents of stressed AM plants were closely correlated with proline levels. In contrast to that, Zhang et al. (2020) observed that besides boosting

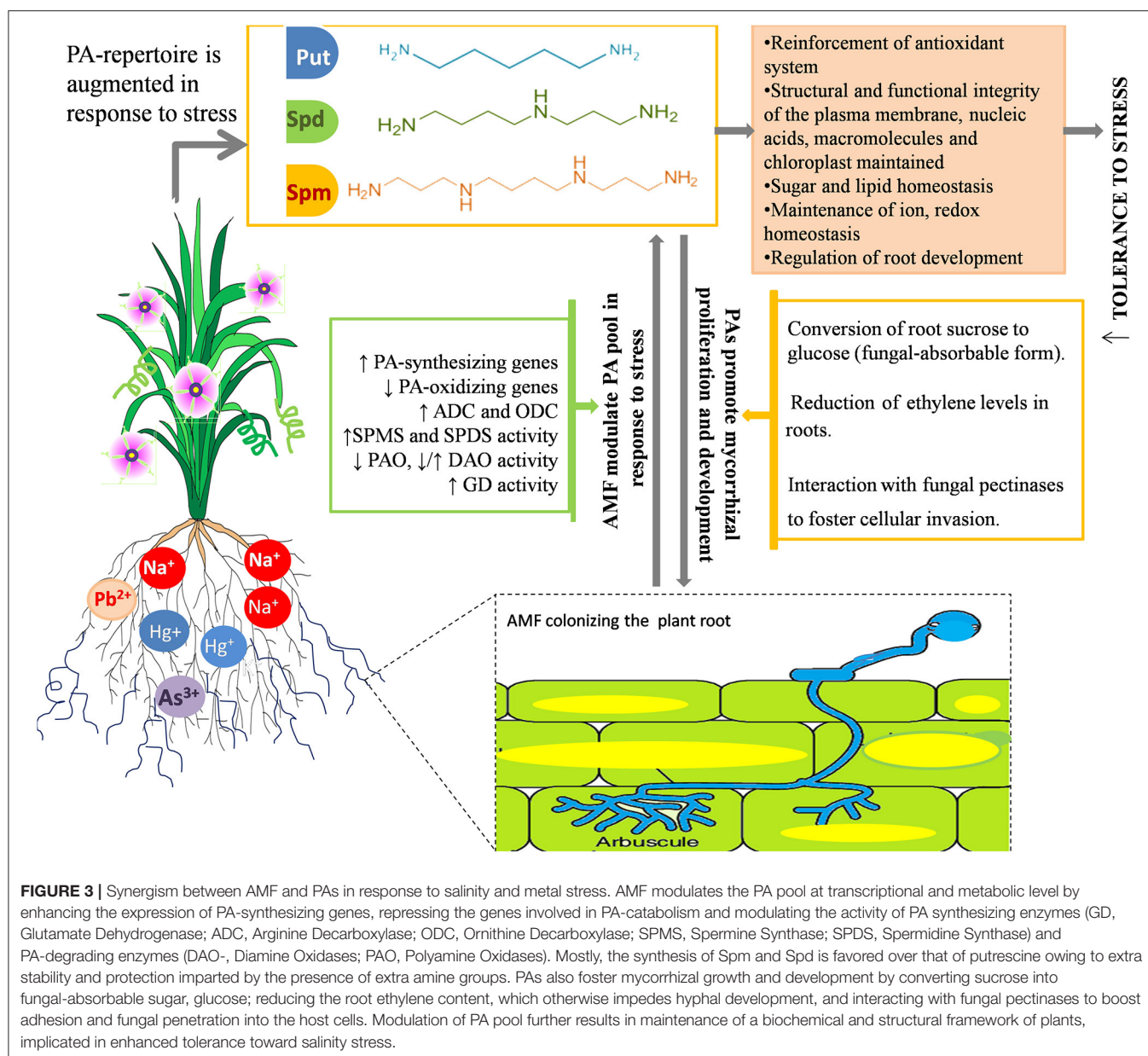
TABLE 1 | Effects of arbuscular mycorrhizal fungi (AMF) inoculation on modulation of polyamine ratio under abiotic stresses.

Abiotic stress	Plant species	AMF Species	Effect on polyamine ratio	References
SALT STRESS (0 and 200 mM)	<i>Lotus glaber</i>	<i>Glomus intraradices</i>	↑(Spd + Spm)/Put ratio; ↑Put in shoots of tolerant plants, ↑Spd and Spm in both tolerant and sensitive plants	Sannazzaro et al., 2007
SALT STRESS (0, 50, 100, and 200 mM)	<i>Trigonella foenum-graecum</i>	<i>G. intraradices</i>	↑Put, ↑Spd, ↑Spm, Increased content of other osmolytes (total soluble sugars, proline, glycine betaine)	Evelin et al., 2013
SALT STRESS (0 and 150 mM)	<i>Lotus tenuis</i>	<i>G. intraradices</i>	AM salt-stressed plants showed a higher root PA (Spm, Spd, Put) level than their corresponding non-AM controls; no significant effect of AM under salt stress on total, root, stem, and leaf free PA contents in comparison to control plants	Echeverria et al., 2013
SALT STRESS (4.7 and 9.4 dS m ⁻¹)	<i>Triticum aestivum</i> (Giza 168, Sids 1)	Mixture of <i>Glomus</i> spp.	Significant reduction in DAO and PAO activities under salt stressed plants; ↑Put, ↓Spm and Spd in Giza 168, ↓Put, ↑Spm and ↑Spd in Sids 1	Talaat and Shawky, 2013
SALT STRESS (0, 50, and 100 mM)	<i>Vicia faba</i>	<i>Funneliformis mosseae</i> , <i>Rhizophagus intraradices</i> , and <i>Claroideoglomus etunicatum</i>	Significant increase in Put, Spd, Spm levels under all conditions of salinity, with maximum increase in Put concentration (under 100 mM treatment)	Abeer et al., 2014
DROUGHT STRESS (2 cycles of moisture stress)	<i>Medicago sativa</i>	<i>G. fasciculatum</i>	Higher free polyamine (Spd and Spm) content in symbiotic water-stressed plants; No significant enhancement of polyamine concentration in roots	Goicoechea et al., 1998
DROUGHT STRESS (45–50% max field water capacity)	<i>Poncirus trifoliata</i>	<i>G. mosseae</i>	↓Put and Spd, ↑Spm content, ↑SPMS activity in drought stressed AM plants	Luo, 2009
DROUGHT STRESS (50% of max. water holding capacity)	<i>P. trifoliata</i>	<i>F. mosseae</i>	↑Put and Cad, ↓Spd and Spm concentrations, ↑PA catabolic enzyme activity (CuAO; PAO) and Put-synthases (ODC and ADC)	Zhang et al., 2020
DROUGHT STRESS (mild T1 and moderate T2 drought)	<i>Zea mays</i>	<i>R. irregularis</i>	Significantly, ↓Put content in water stressed (T1 and T2) AM plants, ↑DAO and ↑GABAT activity, ↑GABA accumulation	Hu and Chen, 2020
DROUGHT STRESS (soil WW status (18.08%))	<i>P. trifoliata</i>	<i>F. mosseae</i>	↑ADC, ↑ODC, ↑SPMS, ↑SPDS, ↑DAO, ↑PAO activity. ↑precursor of PA (agmatine, L-ornithine and SAM), ↑Put, ↑Cad, ↓Spd	Zou et al., 2021
HEAVY METAL STRESS (Cd)	<i>Plantago lanceolata</i>	<i>G. fasciculatum</i>	No significant difference in leaf PA ratio between mycorrhizal and non-mycorrhizal plants. Mycorrhizal roots registered lower (Put/Spd+Spm) ratio	Parádi, 2003
HEAVY METAL STRESS (Cu and Zn)	<i>Populus alba</i>	<i>G. mosseae</i> and <i>G. intraradices</i>	Induction of <i>PaSPDS1</i> and <i>PaSPDS2</i> and <i>PaADC</i> ; ↑free and conjugated PA titers in stressed AM plants; ↑stabilization of heavy metals in soil	Cicatelli et al., 2010
HEAVY METAL STRESS (Pb)	<i>Calopogonium mucunoides</i>	<i>G. etunicatum</i>	Mycorrhization influenced free amino acid profile in leaves; resulted in depleted arginine content, prioritizing PA synthesis over protein metabolism	Souza et al., 2014

growth traits and AQP expression in *Poncirus trifoliata* plants, *Funneliformis mosseae* augmented the cellular concentration of specifically two diamines, Put and cadaverine (Cad), and diminished the concentrations of root Spd and Spm under water-deficit conditions. This is ascribed to a concomitant increase in the activity of Put-synthases (ADC and ODC) and Put-oxidases (PtPAO1, PtPAO2, and PtPAO3) that convert Spd back to Spm and further to Put, by successive deamination (Mo et al., 2015). The upregulation of PAO expression is important to stimulate stress responsiveness in plants probably because it results in the production of H₂O₂, which, when present in moderate amounts,

serves as a ligand in stress signaling (Sequera-Mutiozabal et al., 2017).

Another study on *P. trifoliata* inoculated with *Glomus mosseae* and subjected to drought stress exhibited substantially lowered concentrations of Put and Spd and significantly elevated levels of Spm in inoculated (vs. non-inoculated) plants, thereby advocating the essential role of Spm in trifoliolate orange (Luo, 2009). The increased titers of free Spm could be explained by the higher activity of the SPMS gene and drought-spurred reduction of its metabolic precursors, Put and Spd. Better protective role of higher PAs (owing to the additional amine



groups) could also offer a good explanation for the higher Spd concentration obtained. The results of an experiment on maize seedlings exposed to water-deficit conditions suggested that Put oxidation into GABA [by the activity of copper amine oxidase (CuAO)] was the target pathway in AM-facilitated regulation of drought tolerance (Hu and Chen, 2020). GABA shunt is vital for C/N homeostasis in plants (Shelp et al., 2012) and drought acclimation (Mekonnen et al., 2016). Moreover, the activity of GABA transaminase (GABAT) was significantly high in AM roots under drought. GABAT converts GABA into a subsequent substrate, succinate, which is consumed in the tricarboxylic acid cycle (TCA) cycle to synthesize carbohydrates. In a recent study by Zou et al. (2021), AMF-regulated PA homeostasis in the roots of *P. trifoliata* plants was found to

be associated with enhanced tolerance against soil moisture deficit stress (SMDS). Mycorrhizal plants exhibited a significantly higher concentration of PA precursors, L-ornithine, agmatine, and SAM, increased Put and Cad, and reduced Spd content. In response to SMDS, AMF colonization augmented the activities of PA-synthetic (ADC, ODC, SPMS, and SPDS) and catabolizing (PAO and DAO) enzymes with much less degree of damage to the membrane and lower generation of ROS *vis-à-vis* non-mycorrhizal plants (Table 1). It can be speculated from the studies so far that the accrual pattern of PAs may totally vary depending on the type of host plant, the interacting AMF partner, the degree, duration, and nature of stress, and most importantly, the developmental stage and tissue of the plant under investigation; all these factors might account for

the variation in free and/or conjugated PA pool observed in different plants.

The inherent and AMF-facilitated modulation of PA titers may behave either as an indicator or a harbinger (to communicate the perceived environmental cues) to modify spatio-temporal response of plants to stress (Rangan et al., 2014). That being said, further studies should be dedicated to unraveling the molecular signaling pathways of AM-induced regulation of these metabolic hubs. With the advent of forward and reverse genetic approaches, the functional essence of PAs in stress acclimation has been elucidated to some extent. However, a straight cause-and-effect relationship between the accumulation of PAs and drought stress is not firmly established yet (Pál et al., 2015). The knowledge acquired so far about the AMF-mediated modulation of PAs has constructed a strong case for future studies earmarked for vigilant analyses of fungal genes contributing to the modulation of PA pool upon stress exposure. High-throughput investigations involving transcriptomics, metabolomics, and microarray approaches along with a careful analysis of coordinating partners of PA metabolic enzymes will be a great deal of help in better understanding the mechanism of stress tolerance/resistance and whether it involves PA-induced epigenetic changes in the plant cells.

Heavy Metal Stress

In the process of phytoremediation, many plant-associated microbes reinforce the metal detoxification capacity of the plants (microbe-assisted phytoremediation). HM-contaminated soils can be redeemed and revegetated by using AMF that can potentially expedite the phytostabilization and phytoextraction process (Khalid et al., 2021). The potential of AMF as an ameliorator of HM toxicity has been well-proved and promoted (Christie et al., 2004; Bai et al., 2008; Merlos et al., 2016; Sharma et al., 2017; Gupta et al., 2021). Fungus-assisted phytoremediation is deemed as the most befitting and eco-friendly technology for the redemption of HM polluted soil (Khalid et al., 2021). Among various recognized mechanisms deployed by AMF for the prevention of HM uptake by plants (metal immobilization, modulation of metal transporter activity, and production of organic acids and glomalin), the regulation of the PA metabolism under metal stress is inconsiderably explored (Ezawa et al., 2002; Smith and Read, 2008; Chen et al., 2012; Tajti et al., 2018). PAs can stimulate metal chelation, boost antioxidant defense, maintain hormone homeostasis, and ensure membrane stability (Tajti et al., 2018). The stress mitigatory roles of PAs explain their inherent regulation during metal stress.

In cadmium-treated (Cd) and copper-treated (Cu) wheat leaf segments, a respective increase and decrease in Put and Spm concentration were obtained (Groppa et al., 2007). Such results might be attributed to the following: (1) accelerated PA (Spd and Spm) degradation into Put by higher PAO activity or (2) higher SAM flux toward ethylene metabolism, formation of which is detrimental to higher PAs. Resultantly, the reduction of Put to Spd and Spm was hampered in spite of Put availability. Similar fluctuations in PA titers have been observed in Cu-stressed rice (Lin and Kao, 1999), Cd-stressed *Potamogeton crispus* L. (Yang

et al., 2010), and chromium- (Cr) stressed *P. trifoliata* (Shahid et al., 2018), etc. Besides, exogenous feeding of PAs also refines the resistance of plants to HM stress (Rady and Hemida, 2015; Nahar et al., 2016; Taie et al., 2019). Garg and Saroy (2020) demonstrated that the combined application of AMF (*Rhizoglyphus intraradices*) with PAs furnished a remarkable improvement in biomass (root and shoot) yield of plants, nutrient acquisition, ureids and trehalose accumulation, and nodulation potential in *Cajanus cajan* under nickel (Ni) stress.

A plethora of reports on both AMF-mediated and PA-mediated increase in oxidative defense strategy, such as the reinforced activity of enzymes participating in AsA-GSH cycle, contributing to stress tolerance in plants, exist in the literature (to cite a few, Garg and Bhandari, 2016; Tseng et al., 2013; Liu et al., 2017; Hashem et al., 2018). In a recent study, Saroy and Garg (2021) analyzed the effectiveness of AMF and PAs in modulating the AsA-GSH cycle in two genotypes of *C. cajan* varying in their Ni tolerance. They found out that exogenous co-supplementation of PAs (mainly Put) and *R. intraradices* contributed the most in upsurge in the activity of enzymes of AsA and GSH pool [ascorbate peroxidase (APOX), monodehydroascorbate reductase (MDHAR), dehydroascorbate reductase (DHAR), glutathione reductase (GR)] and also improved GSH/glutathione oxidized (GSSH) ratio, consequently providing tolerance against the HM-induced oxidative damage in pigeon pea genotypes. Beyond the repressive mechanisms of PAs on HM uptake, the latter also boost the production of phytochelatins (PCs), which bind, transport, and sequester the metal ions into the vacuole (Pál et al., 2017); possibly because they contribute to GSH pool, which is the building block of PCs. Higher PAs behave as stronger chelators or amplify chelation mechanisms (Nahar et al., 2016). Furthermore, PAs can also block cation channels in the sequence, $\text{Spm}^{4+} > \text{Spm}^{3+} > \text{Put}^{2+}$, hence, they succor the sequestration of surplus metal ions during stress (Liu et al., 2014).

To what extent the innate stress resistance mechanisms of the host are persuaded by mycorrhizal symbiosis still remains ambiguous. Although mycorrhizal mediation of metallothionein (*MT*), *PC* and HM tolerance (*HMT*) genes under HM stress have been unequivocally established, the “buffer effect” of AMF in improving the HM resistance of AM plants by orchestrating the PA metabolism has been very modestly studied and reported. Parádi (2003) studied the influence of *Glomus intraradices* on free PA content and ratios in Cd-exposed *Daucus carota* L. plants. AM plants did not register any significant change in the PA ratio unlike non-AM counterparts that showed reduced Spd and Put content. This could be due to the “equalizing effect” of AMF upon Cd exposure against the alteration in PA titers widely reported in various stress conditions. In poplar plants (clone AL35) grown in Cu and Zn polluted soil, inoculation with *G. mosseae* (*Gm*) and *G. intraradices* (*Gi*) provoked an overall increment in free and conjugated PAs by the induction of *PaSPDS2* and *PaADC* expression (Cicatelli et al., 2010, 2014). Contrary to the first sampling stage (S1), *PaADC* expression was downregulated at second sampling stage (S2) in mycorrhizal plants under stress. In the presence of metal stress, both *PaSPDS1* and *PaSPDS2* were upregulated in *Gi*-inoculated poplar plants. At S2, both SPDS transcripts were induced by the inoculation with AMF

on both non-stressed and stressed soil. At S1, on non-polluted soil, free PA levels were strongly reduced in the presence of AMF, while those of conjugated PAs were significantly higher in AM plants. On polluted soil, free Spd titers were significantly higher in the presence of AMF, especially *Gi*; conjugated Spd and Spm titers were also dramatically enhanced (up to 5-fold) relative to uninoculated controls, but only in *Gi*-inoculated plants (Cicatelli et al., 2010, 2014). Amelioration of lead (Pb) toxicity in mycorrhized *Calopogonium mucunoides* was related to the alteration in amino acid profile, deviating metabolic flux toward PA synthesis from protein synthesis (Souza et al., 2014) (**Table 1**). Taken together, these results advocate that stress amelioration is driven from AMF-mediated regulation of these protective molecules under the combined effect of toxic metals and AMF.

Research on AMF-regulated PA metabolism and function during metal stress is in its infancy; hence, countless efforts are required to unravel the molecular dialog of AMF and PA and their protective role in HM stress tolerance. Employment of “-omics” approaches is needed to amplify AMF-mediated PA actions toward HM toxicity. Also, the effect of co-inoculation of AMF with growth-promoting bacteria in reversing metal toxicity should be subjected to thorough scrutiny to fathom the potential of synergistic interactions of plant-associated microbes in the mediation of crop abiotic stress tolerance.

Impact of Polyamines on Mycorrhizal Colonization

The extent of mycorrhizal colonization, spore germination, and hyphal growth and propagation are also affected by various stressors (Wu et al., 2010; Shekoofeh et al., 2012). In that respect, PAs have been reported to, in turn, enhance the AM symbiosis by actively participating in signaling episodes of plant–fungus interaction (El Ghachtouli et al., 1995). This positive correlation between mycorrhizal colonization and PAs is validated by several studies. Based on their type and method of administration (soil drench or foliar application), PAs exerted their effectiveness on mycorrhizal (*G. intraradices*) development and concomitantly improved the quantity and quality of corm, and enhanced flower parameters in *Freesia hybrida* (Rezvanypour et al., 2015). The application of PAs in grape plants yielded beneficial effects on the infection rate, spore number, and hyphal growth of *Gigaspora margarita*, which are significantly reduced by the application of a PA biosynthetic inhibitor, methylglyoxal bis-guanyldrazone (Yong et al., 2003). These results have made the stimulatory effects of PAs on the symbiotic efficiency of AMF manifestable and apparently directed toward the role of PAs as potential “regulatory factors” in the mycorrhizal association. Wu et al. (2012b) reported that 14 weeks of exogenous PA application on *C. tangerine* inoculated with *Paraglomus occultum* resulted in accelerated mycorrhizal colonization, a greater number of infectious propagules (vesicles and arbuscules), an enhanced mycorrhizal status, and a higher root glucose concentration, suggesting that PAs probably facilitate the transformation of root sucrose to glucose (usable form of carbohydrate for the fungus) to promote mycorrhizal proliferation and development. The PA-mediated stimulation of root colonization can be attributed to

various reasons: (1) PAs directly interact with fungal pectinases and boost adhesion, fungal penetration, and growth into the cell wall of plants (Nogales et al., 2009), (2) PAs reduce the ethylene levels in roots, which otherwise inhibit root colonization (Foo et al., 2016; Mattoo and White, 2018), and (3) AMF proliferation and development begins with chromatin decondensation of the host nucleus, corresponding to higher transcriptional activity (Berta et al., 1990), essential to which are PAs as they stabilize RNA against RNase activity (Serafini-Fracassini et al., 1984). Thus, PAs function as regulatory molecules in plant–AMF interactions (El Ghachtouli et al., 1995) (**Figure 3**).

AQUAPORINS: TYPES AND BACKGROUND

One of the crucial parameters that govern the development and stages of growth in plants is the stringent regulation of transcellular water movement, conduction, and mobilization. Diffusion of water across cells is facilitated by AQPs, the members of major intrinsic proteins (MIPs) family, that serve as a nexus between and within the cells, passively conducting water, gases, and solutes. Multiple isoforms of AQPs are found in plants, the fact that is reflective of a high degree of compartmentalization of cells, selectivity and size specificity of transport, and regulation properties that fine-tune water transport (Kapilan et al., 2018). As per the amino acid sequence homology, MIPs can be categorized into four major subgroups: (1) plasma membrane intrinsic proteins (PIPs) constitute the largest subfamily of MIPs and sit predominantly in the plasma membrane of the cells localized in the plant organs marked by high water fluxes, such as guard cells and root cells; (2) tonoplast intrinsic proteins (TIPs) chiefly reside in the vacuolar membrane serving as conduits for the transportation of water, small solutes, metabolites, and gases, thereby contributing to rapid osmotic homeostasis of the cytosol and maintenance of the cellular hydrostatic pressure; (3) nodulin-26 like intrinsic proteins (NIPs) are the aqua-glyceroporins found localized in the peribacterial membranes of the root nodules, hence being supposed to be presumably involved in the exchange of metabolites (glycerol and water, chiefly) betwixt the host plant and the N₂-fixing bacterial symbionts; and (4) small basic intrinsic proteins (SIPs) are localized in the membrane of endoplasmic reticulum (ER); however, their precise physiological functionality is indeterminate (Ishikawa et al., 2005).

Expression and activity of AQPs are regulated by several processes, such as gating, which deals with the phosphorylation and dephosphorylation of AQPs, heterotetramerization, divalent cations, hormones, and even ROS generated during abiotic stresses (Kapilan et al., 2018). Tight regulation of these molecular gears can enable efficient regulation of water conduction under stress. Owing to the isoform multiplicity of AQPs and distinct expression patterns under stress (it may reduce, increase, or may remain unchanged), the discrete and integrated functionality of AQPs under diverse physiological conditions under stress remains elusive. The regulation of water conduction by AQPs under a variety of abiotic stresses depends on multiple parameters: the nature, duration and intensity of stress,

conditions of plant growth, developmental stage, the type of tissue expressing AQP genes, and the type/isoform of AQP being expressed (Siemens and Zwiazek, 2004). There is a considerable dearth of such studies in the literature that directly integrate the relationship among the two biomolecules (PA and AQP) and AMF *per se*. However, it can be speculated that PA homeostasis has some roles to play in regulating the distribution and abundance of AQP proteins by virtue of which plant–water relations are maintained (Tailor and Bhatla, 2021). The authors studied the effect of potent PA inhibitors (DFMA and DFMO) on the expression of two major AQP families: PIP2 and TIP1 in salt-stressed *Helianthus annuus* L. seedlings. The seedling that was raised in 500 μ M of DFMO and DFMA exhibited a significant root extension, irrespective of the salt-stress imposed, and resulted in further exacerbating the decrease in relative water content (RWC) in roots and cotyledons. This was found to be correlated with augmented levels of PIP2 and TIP1 proteins in the roots but not in the cotyledons, of the seedling (Tailor and Bhatla, 2021). This arena of study integrated with the role of AMF colonization in further modulating the two biomolecules can offer a good research question to investigate. On this account, it can be deduced, *a priori*, that AMF-regulated PA homeostasis that accounts for improved adaptation of plants to water-deficit conditions (Hu and Chen, 2020; Hu et al., 2020; Zhang et al., 2020) could be correlated to an extent as a function of modulation of the abundance of AQP subfamilies. However, no direct correlation between PAs and AQPs has been established hitherto.

AMF-MEDIATED REGULATION OF AQUAPORINS UNDER STRESS

Complete establishment of AM symbiosis in plants needs extensive morphological and molecular reprogramming with most of the morphological alterations concerned with changes in membrane system of vacuole and cytoplasm. Since AM symbiosis potentially alters root hydraulic conductivity in plants, the fact that it regulates AQP gene expression and protein abundance seems more convincing. The first wave of an attempt to characterize AMF-mediated changes in the AQP profile rose by Roussel et al. (1997), who reported mycorrhiza-induced TIP expressions in *Petroselinum crispum*, followed by that of Krajinski et al. (2000), who conducted the experiment along similar lines in *Medicago truncatula* and completely credited the changes of *MtAQP1* expression profile to AMF colonization. They proposed that mycorrhiza-induced heightened expression of *MtTIP* is crucial to optimize water conduction after the changes that commence in plants on symbiosis establishment. Arbuscule-containing root cortical cells may register an altered ratio of cytoplasmic content and vacuolar space, due to which, changes in the tonoplast may be required to buffer the osmotic instability in the cytoplasm. Higher *MtTIP* expression could offer a way of redressing the reduced tonoplast water permeability of the highly compartmented vacuoles in the cells harboring fungal symbionts. Analogously, *Medicago* plants inoculated with *G. mosseae* showed an apparently enhanced expression of *MtPIP2;1* and *MtNIP1* while transcript abundance of other isoforms

were uninfluenced by mycorrhization (Uehlein et al., 2007) (Table 2). The peri-arbuscular membrane (PAM) surrounding the arbuscules is a site of transmembrane water and solute transport across the plant–fungus interface. The strong induction of AQP expression during mycorrhization might be indicative of the physiological alterations, i.e., the optimization of water and solute transport system in the roots (the site of plant–fungus interaction), as symbiotic exchange occurs through AQPs localized in the PAM. AQPs furnish a very low-resistance transcellular water conduction pathway through the membrane, which seems to be predominantly managed by TIPs and PIPs that are the central regulators of the conduction pathway (Maurel, 2007). Many AMF-regulated AQPs conduct solutes such as glycerol, urea, and H_2O_2 along with water, all of which are vital to the physiological performance of plants (Bárcana et al., 2014). Apart from these roles, AQPs also exert a potential influence on redox events in plants under unfavorable conditions. Additionally, since AQPs can be gated through reversible phosphorylation and dephosphorylation, this offers a way to control the water fluxes and movement dynamics across the cellular and vacuolar membranes.

Salt Stress

The strategic effect of mycorrhization on water acquisition and transport in salt-stressed and non-stressed plants strongly suggests its impact on AQP channels that mediate water transport in plants (Chen et al., 2017). The expression profile of AQPs in mycorrhizal plants experiencing salinity stress has revealed strikingly different results in different studies. Ouziad et al. (2006) reported that the transcript levels of *LePIP1* and *LeTIP* exhibited a significant reduction in the roots and a slight upregulation in the leaves of the salt-stressed tomato colonized with *Glomus* spp. The tissue-specific expression suggests that fungi might acquire the function of water mobilization and transportation from roots to shoots, rather than the water uptake by roots from the soil, as the latter would allow the ingress of toxic Na^+ and Cl^- ions into the roots along with the inflow of water; as few AQPs are reported to have Na^+ conduction properties, like *AtPIP2;1* and *AtPIP2;2* of *Arabidopsis* (Kourghi et al., 2017). In another study, the transcript levels of *LsPIP1* and *LsPIP2* were inhibited by mycorrhization (*G. intraradices*) in non-stressed *Lactuca sativa* plants; however, the expression of *LsPIP1* got enhanced with 100 mM high-salinity NaCl dose, while no stark difference in that of *LsPIP2* was notable between AM and non-AM plants (Jahromi et al., 2008).

The dynamism of AQP responses depends on the strain of AMF and the host type, the intrinsic nature and mode of the osmotic stress applied, and the isoform of AQP, adding layers of complexity to the AMF-mediated regulation of AQP expression (Jahromi et al., 2008; Ruiz-Lozano and Aroca, 2010). Four PIP genes analyzed in *Phaseolus vulgaris* plants colonized with *G. intraradices*, and subjected to salinity, low temperature, or drought stress, revealed contrasting results (Aroca et al., 2007). Three of the genes investigated exhibited differential regulation by mycorrhization under each stress regime. Salinity stress resulted in the upregulation of all the PIP genes in both sets of plants (AM and non-AM), with a considerable enhancement in

TABLE 2 | Summary of studies on effects of AM symbiosis on aquaporin (AQP) gene expression under non-stressed, water-deficit (salt and drought stress), and heavy metal (HM) stress conditions. The consequences of AMF-mediated AQP regulation on physiology of stressed plants are also included.

Abiotic conditions	Plant species	AMF Species	AMF-mediated effects on Aquaporin gene expression	Consequences on physiology of AM Plants	References
No Stress	<i>Petroselinum crispum</i>	<i>Glomus fasciculatum</i>	↑ <i>PcTIP</i>	Plant water status not measured	Roussel et al., 1997
	<i>Medicago truncatula</i>	<i>G. mosseae</i>	↑ <i>MtTIP</i>	Plant water status not studied	Krajinski et al., 2000
	<i>Poplar tremula</i> × <i>tremuloides</i>	<i>G. mosseae</i>	↑ <i>PttPIP1.1</i> , ↑ <i>PttPIP2.3</i> , ↑ <i>PttPIP2.5</i>	Increased L _o in AM plants	Marjanović et al., 2005
	<i>Medicago truncatula</i>	Unpublished data	↑ <i>MtPIP2;1</i> and ↑ <i>MtNIP1</i>	Plant water status not taken into account	Uehlein et al., 2007
Salt stress	<i>Lycopersicon esculentum</i>	<i>G. geosporum</i> and <i>G. intraradices</i>	↓ <i>LePIP1</i> and ↓ <i>LeTIP</i> =↓ <i>LePIP2</i>	Plant water status analysis not conducted	Ouziad et al., 2006
	<i>Phaseolus vulgaris</i>	<i>G. intraradices</i>	↑ <i>PvPIP1.1</i> , ↑ <i>PvPIP1.3</i> , ↑ <i>PvPIP2.1</i> , ↓ <i>PvPIP1;2</i>	Increase in J _v , L _o , and RWC	Aroca et al., 2007
	<i>Lactuca sativa</i>	<i>G. intraradices</i>	↑ <i>LsPIP1</i> and =↓ <i>LsPIP2</i>	Increased RWC, lower ABA accumulation in roots, lower proline content	Jahromi et al., 2008
	<i>Robinia pseudoacacia</i>	<i>Rhizophagus irregularis</i>	Roots: ↑ <i>RpPIP1;3</i> , ↑ <i>RpPIP2;1</i> , ↑ <i>RpTIP1;1</i> , ↓ <i>RpTIP1;3</i> Leaves: ↓ <i>RpPIP2;1</i> and ↓ <i>RpTIP1;3</i> ↑ <i>RpPIP1;1</i> and ↑ <i>RpPIP1;3</i>	Increase in the RWC at 200 mM NaCl by 9%, increased WST, improved Pn by 106% at 100 mM NaCl and by 81% at 200 mM NaCl, Higher WUE, Lower Ci, higher Gs than non-AM plants	Chen et al., 2017
	<i>Poncirus trifoliata</i>	<i>Paraglomus occultum</i>	↓ <i>PtTIP1;1</i> , ↓ <i>PtTIP1;2</i> and ↓ <i>PtTIP1;3</i> =↓ <i>PtPIP2;1</i> , =↑ <i>PtTIP4;1</i> (no salt)	Spurred root water absorption, accelerated	Ding et al., 2020
	<i>Lactuca sativa</i>	<i>F. mosseae</i> and <i>Claroideoglomus lamellosum</i>	↑↑ <i>PtTIP5;1</i> (salt stress) No effect on PIP1, ↑PIP2 (↑PIP2A, ↑PIP2B, ↑PIP2C) abundance	Leaf Ψ by 9.14%, significantly higher RWC	Santander et al., 2021
Drought stress	<i>Glycine max</i> , <i>Lactuca sativa</i>	<i>R. intraradices</i>	↓ <i>GmPIP1</i> , ↓ <i>GmPIP2</i> ↓ <i>LsPIP1</i> , ↓ <i>LsPIP2</i>	Higher leaf Ψ and RWC	Porcel et al., 2006
	<i>Phaseolus vulgaris</i>	<i>G. mosseae</i>	↓ <i>PvPIP1;1</i> , ↓ <i>PvPIP1;2</i> , ↓ <i>PvPIP1;3</i> ↓ <i>PvPIP2;1</i>	Increased RWC and J _v	Aroca et al., 2007
	<i>Zea mays</i>	<i>R. intraradices</i> Isolate BEG 121	↑ <i>ZmPIP1;1</i> , ↑ <i>ZmPIP1;2</i> ↑ <i>ZmPIP2;5</i> , ↑ <i>ZmPIP2;6</i> ↓ <i>ZmPIP2;2</i>	Application of exogenous ABA enhanced J _v and L _o in AM and non-AM plants, regardless of the water regime	Ruiz-Lozano et al., 2009
	<i>Z. mays</i>	<i>R. intraradices</i>	Short term drought: ↑ <i>ZmPIP1;1</i> , ↑ <i>ZmPIP1;2</i> ↑ <i>ZmPIP1;3</i> , ↑ <i>ZmPIP1;4</i> ↑ <i>ZmPIP1;6</i> , ↑ <i>ZmPIP2;2</i> ↑ <i>ZmPIP2;4</i> , ↑ <i>ZmPIP1;1</i> ↑ <i>ZmPIP1;2</i> , ↑ <i>ZmTIP1;1</i> , ↑ <i>ZmTIP1;2</i> ↓ <i>ZmNIP2;1</i> , ↓ <i>ZmNIP2;2</i> Sustained drought: ↓ <i>ZmPIP1;1</i> , ↓ <i>ZmPIP1;1</i> ↓ <i>ZmPIP1;3</i> , ↓ <i>ZmPIP1;4</i> , ↓ <i>ZmPIP2;2</i> , ↓ <i>ZmPIP2;4</i> ↓ <i>ZmNIP2;1</i> , ↓ <i>ZmNIP2;2</i> ↓ <i>ZmTIP1;1</i> , ↓ <i>ZmTIP1;2</i> Roots: ↓ <i>RpTIP1;1</i> ↓ <i>RpPIP1;3</i> Stem: ↑ <i>RpTIP1;1</i> , ↑ <i>RpTIP2;1</i> , ↑ <i>RpPIP2;1</i> , ↓ <i>RpPIP1;1</i> , ↓ <i>RpPIP1;3</i> Leaves: ↑ <i>RpTIP2;1</i> , ↑ <i>RpPIP2;1</i> ↓ <i>RpTIP1;3</i> , ↓ <i>RpPIP1;3</i>	Increased J _v and L _o values Increased L _h values under drought Decreased J _v and L _o values, reduced sap flow, decreased Si uptake, decreased B uptake, enhanced RLWC, Leaf Ψ , and ABA level in roots and better plant growth performance	Bárcana et al., 2014
	<i>Robinia pseudoacacia</i>	<i>R. irregularis</i>	Roots: ↓ <i>RpTIP1;1</i> ↓ <i>RpPIP1;3</i> Stem: ↑ <i>RpTIP1;1</i> , ↑ <i>RpTIP2;1</i> , ↑ <i>RpPIP2;1</i> , ↓ <i>RpPIP1;1</i> , ↓ <i>RpPIP1;3</i> Leaves: ↑ <i>RpTIP2;1</i> , ↑ <i>RpPIP2;1</i> ↓ <i>RpTIP1;3</i> , ↓ <i>RpPIP1;3</i>	Higher dry mass and lower WSD and electrolyte leakage, increased leaf Pn and Gs	He et al., 2016

(Continued)

TABLE 2 | Continued

Abiotic conditions	Plant species	AMF Species	AMF-mediated effects on Aquaporin gene expression	Consequences on physiology of AM Plants	References
	<i>Poncirus trifoliata</i>	<i>G. mosseae</i>	↑ <i>PtTIP1</i> ;2, ↑ <i>PtTIP1</i> ;3, ↑ <i>PtTIP4</i> ;1 ↓ <i>PtTIP2</i> ;1 and ↓ <i>PtTIP5</i> ;1; <i>PtTIP1</i> ;1 and <i>PtTIP2</i> ;2 unaffected	Significantly enhanced leaf RWC, Leaf Ψ , and plant growth performance (plant height, stem diameter, leaf number, and biomass), elevated root ABA levels	Jia-Dong et al., 2019
	<i>Z. mays</i>	<i>R. irregularis</i>	↑ <i>ZmPIP2</i> ;1, ↑ <i>ZmPIP2</i> ;6	Enhanced Pn, gs, Ci, and water permeability of mycorrhizal plants	Quiroga et al., 2019a
	<i>Z. mays</i>	<i>F. mosseae</i>	↓ <i>PtPIPs</i> , unaltered <i>PtTIPs</i> , ↓↓ <i>PtNIP1</i> ;1, ↓↓ <i>PtNIP5</i> ;1, ↓↓ <i>PtNIP6</i> ;1	Enhanced Pn, gs, E, leaf Ψ , and RWC; lower Lt. Increased plant growth performance of AM plants	Zou et al., 2019
Heavy Metal Stress (low, moderate and high Cu stress)	<i>Salix purpurea</i> L.	<i>R. irregularis</i>	↑ <i>TIP2</i> ;2 in roots of inoculated plants, ↑ <i>PIP1</i> ;2 in low-Cu treatment samplings, <i>PIP2</i> ;2 steady among all Cu-treatments	Increased L_o , moderated K_L , increased antioxidant capacity (↑SOD and ↑APX)	Almeida-Rodríguez et al., 2016

J_v , sap flow rate; L_h , hydrostatic root hydraulic conductance; RWC, relative water content; ABA, abscisic acid; L_o , root hydraulic conductance; Lt, leaf temperature; Si, silicon; B, boron; RLWC, relative leaf water content; Ci, intracellular CO₂ concentration; Pn, net photosynthetic rate; WSD, water saturation deficit; Gs, stomatal conductance; WUE, water-use efficiency; Ψ , water potential; SOD, superoxide dismutase; APX, ascorbate peroxidase; K_L , leaf-specific conductivity.

AM plants (Table 2). Such a kind of differential AQP expression registered under each stress based on the presence of AMF may be indicative of the following: (1) difference at the level of regulation under the specified set of stresses, (2) different functions performed by each PIP isoform analyzed under each stress examined, and (3) substitution/compensation of the roles performed by plant AQPs by that of fungal AQPs depending on the particular stress foisted. However, to confirm the latter hypothesis, a simultaneous expression profile of fungal AQPs under different stress episodes needs to be generated.

Chen et al. (2017) detected an upregulation of four AQP genes, *RpPIP1*;1, *RpPIP1*;3, *RpPIP2*;1, and *RpTIP2*;1, in all the mycorrhizal *Robinia pseudoacacia* plants at high-salinity dosage (200 mM of NaCl). In this experiment, AM plants were found to exhibit lower values of intracellular (carbon dioxide) CO₂ concentration (Ci) under all doses of salinity. Higher Ci value reflects demolition of the photosynthetic apparatus and passivation of enzymes involved in CO₂ fixation that results in reduced CO₂ assimilation (Sheng et al., 2008). Besides, AM plants also experienced less reduction in Gs (stomatal conductance) values under salinity, which indicated better water status of mycorrhized plants, enabled by “maintained” CO₂ diffusion through stomata by the regulation of leaf AQPs. Likewise, in another recent study on *P. trifoliata*, inoculation with *P. occultum* resulted in differential expression of TIP isoforms and spurred water absorption (↑RWC) and accelerated the leaf Ψ (water potential) by 9.14% (Ding et al., 2020). In *L. sativa* [two cultivars, Grand Rapids (GR) and Lollo Bionda (LB)] inoculated with a consortium of *F. mosseae* and *Claroideoglomus lamellosum*, no alteration in PIP1 abundance was observed, which the authors attribute to the conservative nature of these proteins. In salt-stressed AM cultivars, increased phosphorylation levels of PIP2A, PIP2B, and PIP2C were observed compared to the non-inoculated counterparts. Also, an increased level of PIP2 proteins was observed in the membrane of inoculated GR plants as opposed to non-inoculated ones subjected to salt stress (Santander et al., 2021) (Table 2). All the studies analyzed,

hitherto, are congruent with the findings of Valot et al. (2005) that the inoculation of a plant (*M. truncatula*) with mycorrhiza (*G. intraradices*) differentially regulates several plasma membrane proteins, some of them are inhibited while some of them are induced.

There are currently two schools of thought that explain the diverse expression of AQPs registered in response to AMF under desiccation stress. The first one is premised on the induced expression of AQPs under dehydration stress, which is plausibly explained as a mechanism to boost membrane permeability and facilitate the water uptake and conduction in planta. The second is premised on the fact that the expression of AQPs is inhibited in plants experiencing dehydration stress; this might be an efficient strategy to facilitate water conservation by decreasing membrane permeability to water as well an endeavor of the plant to conserve metabolic energy under stressful conditions (Sonah et al., 2017).

Drought Stress

Since AQPs are inherently regulated at transcriptional and posttranscriptional levels, it becomes important to delineate the exact role of AMF in the modulation of AQP-encoding genes as a mechanism to enhance the stress tolerance of plants under water-deficit conditions. However, the dynamics of AMF-mediated AQP regulation remain enigmatic to date. Abiotic stress, mycorrhization, or an interplay between the both evokes a signaling relay that begins with the generation of stress messengers and/or stress-induced hormones. We posit that AMF, under stress, might influence the AQP expression at transcriptional, translational, and post-transcriptional (phosphorylation, multimerization, cycling, and internalization of AQPs) levels that contributes to the overall increment in AQP expression and protein abundance, thus boosting the conduction of H₂O, CO₂, glycerol, NH₃, etc., in stressed plants. AMF-reinforced regulation of AQP expression and transcript abundance can fortify tolerance of plants to water-deficit conditions arising from different stresses by improving root

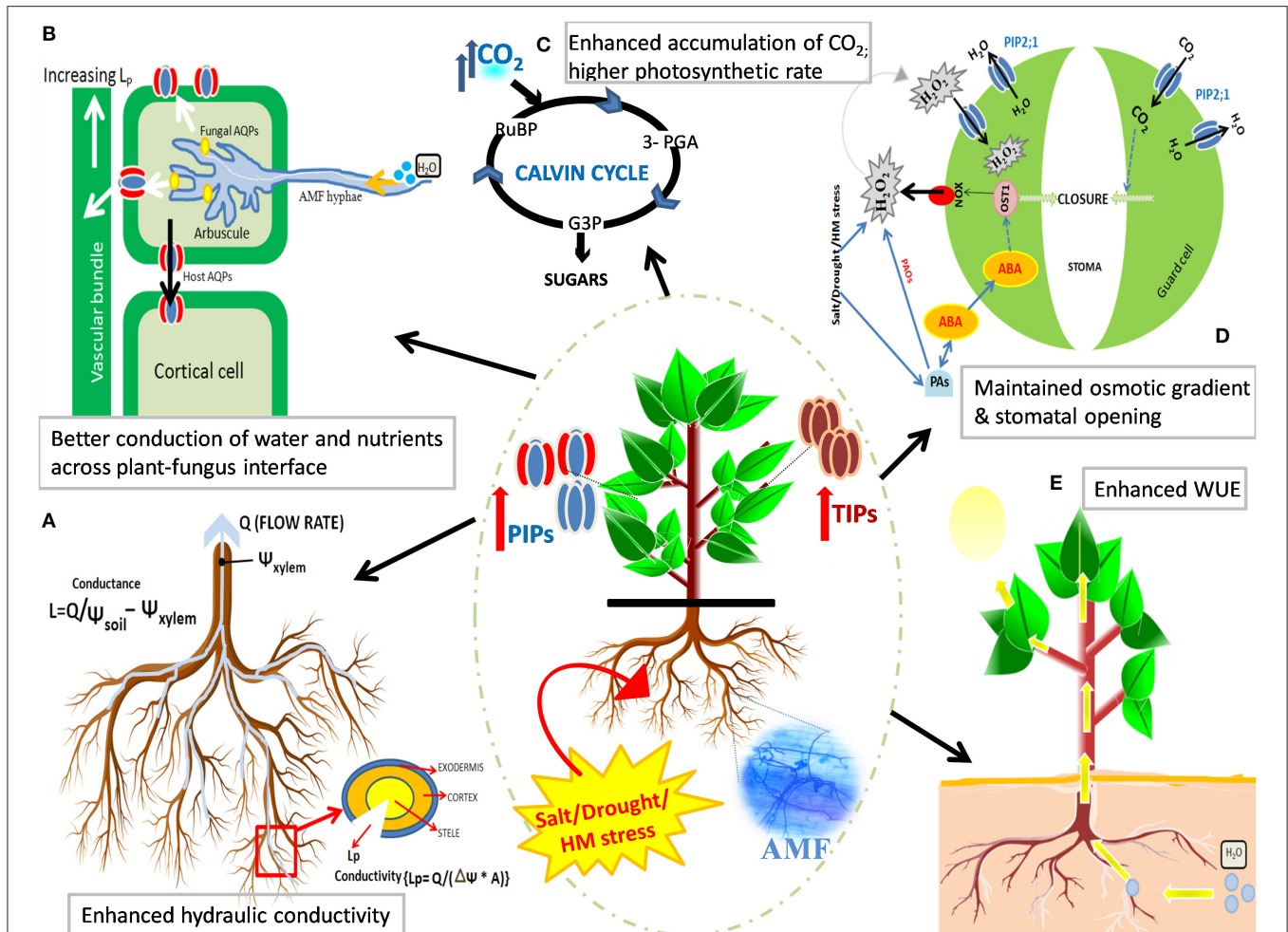


FIGURE 4 | Effect of mycorrhizal symbiosis and abiotic stress on the regulation of aquaporin (AQP) expression and improving the stress tolerance in plants. AMF strengthen the regulation of AQP genes and modulates AQP protein to fortify tolerance of plants to water-deficit conditions arising from salt, drought, or metal stress. AMF-mediated upregulation of AQP activity [especially plasma membrane intrinsic proteins (PIPs) and tonoplast intrinsic proteins (TIPs)] results in: **(A)** an improved root hydraulic conductivity, **(B)** a better exchange of substrates and boosted nitrogen uptake across plant-fungal interface, **(C)** the accumulation of intracellular CO_2 , thus aiding in more photo-fixation of carbon (higher photosynthetic rates), **(D)** maintained osmotic gradient (and cell turgidity) and stomatal opening, and **(E)** improved transcellular conduction of water and plant water status by maintaining water-use efficiency (WUE).

hydraulic conductivity, better exchange of nutrients across the plant–fungus interface, higher photosynthetic rates, maintaining cell turgidity and stomatal opening, and improving water-use efficiency of the plant (Figure 4).

As proposed by Javot and Maurel (2002), the ability of AMF to accelerate the water uptake in roots should imply enhanced permeability of water, which can be derived from higher activity of AQP genes; which is why mycorrhization should account for the upregulation of AQPs to promote transcellular water conduction. However, the results obtained in mycorrhized water-stressed soybean and lettuce plants were incongruous to the aforementioned hypothesis, as *PIP* expression was downregulated in both the plants colonized by mycorrhiza (*Glomus* spp.), although the leaf ψ and RWC were higher in AM plants than their non-AM counterparts (Porcel et al., 2006). Such an effect of AM symbiosis might be conceived as a

regulatory mechanism operative in AM plants to: (1) conserve metabolic energy under stressful conditions or (2) restrict water loss from the tissues into the hypertonic soil milieu. However, the downregulation of *PIP* genes was observed only in the case of plants inoculated with *G. mosseae* and not with *G. intraradices*. The results, somehow, correspond with the study of Marulanda et al. (2003), wherein the soil-water uptake capacity of *G. intraradices* was the highest and *G. mosseae* showed reduced efficiency thereof. This speaks for different strategies employed by the two fungi in order to safeguard plants from water stress. *G. intraradices* might be conditioned to enhance water permeability of roots and, thus, maintain higher expression of PIP aquaporins in the inoculated plants; while *G. mosseae* seems to be directing the conservation of already absorbed water in the plants, hence decreasing membrane water permeability by downregulating PIP gene expression (Ruiz-Lozano and Aroca, 2010).

In another study conducted by Porcel et al. (2005), the symbiotic efficiency (measured in terms of plant biomass), and not the root colonization efficiency, of the mycorrhized *NtAQP1* antisense tobacco plants was compromised on silencing the *NtAQP1* gene under drought stress. The results obtained have led to suggest the following: (1) *NtAQP1* is irrelevant in the colonization process, (2) the reduction in *NtAQP1* transcripts might have been compensated by other AQPs, and/or (3) *NtAQP1* is involved in CO₂ diffusion in tobacco accounting to the promotion of photosynthesis and augmented production of photosynthates, resulting in better growth of plants (Uehlein et al., 2003; Bárzana et al., 2012). Symbiosis by *Rhizophagus irregularis* enhanced drought tolerance in *R. pseudoacacia* plants by calibrating the expression of *RpAQP* genes, and by improving plant biomass, cellular water status, net photosynthetic rate, and stomatal conductance (He et al., 2016). The expression levels of *RpTIP2;1* and *RpPIP2;1* were consistently augmented by AMF in all three tissues, leaves, stem, and roots during stress, thereby canalizing water fluxes toward plant tissues of significant importance in physiology. Differential expression of AQPs has also been reported in AMF-inoculated *Zea mays* (Bárzana et al., 2014) and *P. trifoliata* (Jia-Dong et al., 2019), manifested in altered physiology and plant growth performance under different regimes of drought stress (Table 2). Changes in the AQP gene expression profile under drought stress has been noticed not only in plants associated with AMF but also in the colonizing AM species thereof. The expression of two AQP genes, *GintAQP1* and *GintAQP2*, of *G. intraradices*, was significantly induced in response to water-deficit conditions, thus reinforcing the postulation of AMF involvement in directing plant tolerance to cellular desiccation by increasing water-use efficiency (WUE) (Li et al., 2013).

In maize colonized by *R. intraradices*, *ZmPIP2;2* and *ZmPIP2;6* were induced by the AMF along with the induced expression of fungal *GintAQP2* under drought stress. AM inoculation resulted in an increased photosynthetic rate, an enhanced stomatal conductance, and a higher photosynthetic capacity. The phosphorylation levels of PIP2 were also found to be enhanced that might have translated into increased AQP activities in the mycorrhizal plants (Quiroga et al., 2019a). In mycorrhizal maize plants, the abundance of phosphorylated AQP proteins (PIP1, PIP2, PIP2A, PIP2B, and PIP2C) decreased in well-watered plants subjected to IAA or 6FI (inhibitor of IAA). However, not so significant changes were observed in the phosphorylation levels of AQPs in drought-stressed mycorrhizal plants (Quiroga et al., 2020) (Table 2). Similar observations were obtained in a previous study by Quiroga et al. (2019b), wherein no change in *ZmAQP* accumulation was observed in drought-stressed AM plants in the presence of sodium azide (metabolic inhibitor of AQPs). This could be attributed to the already higher apoplastic water flow and root hydraulic conductance in AM plants (over non-AM plants). Thus, the inhibitory effect of sodium azide on root hydraulic conductance in AM plants was lesser, which, together with increased apoplastic water flow, suggested a compensatory mechanism for the inhibition of AQP activity in these plants under stress (Quiroga et al., 2019b). Even so, Zou et al. (2019) observed that the expression of all *PtPIPs*

in drought-stressed trifoliolate orange was downregulated by the AMF treatment, while the *PtTIPs* expression remained unaltered under the same set of conditions. Increased overexpression of *PIPs* may accelerate wilting in plants; hence the decreased *PIP* expression in AMF-inoculated drought-stressed plants can be speculated as a mechanism to minimize water loss in stressed plants (Ruiz-Lozano and Aroca, 2017). Besides, a dramatic reduction in the root *PtNIP1;1*, *PtNIP5;1* and *PtNIP6;1* levels were observed in water-stressed AM plants. As fungal hyphae can directly provide boron to the host plant, the decrease of NIPs is seen as a potential avoidance mechanism to the ensuing toxicity by otherwise excessive boron accumulation in the plant (Ruiz-Lozano and Aroca, 2017). In yet another study by Jia-Dong et al. (2019), both drought stress and AMF notably amplified the relative level of expression of root *PtTIP1;2*, *PtTIP1;3*, and *PtTIP4;1* in *P. trifoliata*, which might have accounted for an increased active water absorption. Contrastingly, the expression levels of *PtTIP2;1* and *PtTIP5;1* were found to be reduced in stressed AM plants. Since fungal hyphae can also meet the water needs of the host plant, this might be a strategy of AM plants to not rely on extensive host AQP expression and conserve energy. Moreover, increased ABA content observed in these plants could have hastened the water retention, thereby diminishing the requirement for overexpression of all the root-TIPs to absorb water (Table 2).

Taken together, it can be summarized that different AQP genes exhibit upregulation or downregulation under the same stress regime and those belonging to the same subgroup may register distinct expression profiles under different stress regimes (Bárzana et al., 2014). The expression pattern of same isoforms also differs with the fungal strain under study and the tissue analyzed. Despite vast experimental evidence about the implication of AQP genes in various stress responses, the explicit role of individual genes or specific AQP subfamilies is still baffling, owing to their extremely complex and integrated roles in response to various environmental stimuli, and active participation in fundamental growth and developmental processes. A comprehensive understanding of how AMF regulates AQP gene expression has pragmatic implications for crop improvement and management under stress.

Heavy Metal Stress

As aforementioned, the regulation of AQPs is governed by various factors including abiotic conditions like HMs. Metal ions influence the AQP activity by binding to the sulfhydryl groups of the proteins, inducing conformational changes (gating), and thus declining their water conduction capacity (Agre et al., 1998). Metal stress also affects the plant water status by impairing root growth, influencing stomatal density, decreasing vessel and tracheid size, all of which result in deceleration of short-distance and long-distance water conduction (Rucińska-Sobkowiak, 2016).

The regulatory influence of HMs on AQP activity and expression has been validated in several plants, like Hg-stressed *Pisum sativum* (Beaudette et al., 2007), Cu-stressed *Mesembryanthemum crystallinum* (Kholodova et al., 2011), arsenic [As (III)] exposed *Brassica juncea* (Srivastava et al.,

2013), HMs (Zn^{2+} , Pb^{2+} , Cd^{2+} , and Hg^{2+}) stressed *Allium cepa* that accounted to metal-induced drop in water permeability in the order of $\text{Hg}^{2+} > \text{Cd}^{2+} > \text{Pb}^{2+} > \text{Zn}^{2+}$ (Przedpelska-Wasowicz and Wierzbicka, 2011), Zn-stressed poplar (Ariani et al., 2019), and *Brassica rapa* (Fatemi et al., 2020). Multiple reports validate the significance of AQP activity as efflux transporters that excrete HMs out of the cellular space (Vats et al., 2021). For instance, *Oryza sativa* *NIP2;1* mediates As(III) efflux (Zhao et al., 2010), and NIPs from Arabidopsis participate in the expulsion of As (III) from the cell (Xu et al., 2015). Increased internalization and relocation of AQP proteins are also presumably associated with HM sequestration, and thus providing tolerance to the mechanism in plants (Vats et al., 2021). *NIP2;1* is a multifunctional AQP whose role in the transportation of As, B, and Si has been experimentally ratified (Yamaji and Ma, 2007; Ma et al., 2008; Schnurbusch et al., 2010). Grapevine *VvXIP1* is involved in the transportation of Cu, As, Ni, along with H_2O_2 , thus might have a prospective role in metal homeostasis and signaling (Noronha et al., 2016).

Aquaporin response is the primary feedback to metal toxicity; and disturbed water balance is the first stress-triggered episode affecting the AQP confirmation (open/closed) (Przedpelska-Wasowicz and Wierzbicka, 2011). This offers the notion that AQPs have a key role to render under metal stress. Nevertheless, the clear mechanistic role furnished by different AQP isoforms under stress is yet to be categorically established owing to the complexity of integrated stress response that further varies with the plant species (Vats et al., 2021). Hence, the modulation of this recondite player could be a potential mechanism reinforced by AMF under HM stress, like that under salinity and drought. Unfortunately, the studies that corroborate this hypothesis and clarify the relation between plant–water relation and metal stress, and conceived role of AMF-mediated modulation of AQP activity in this context are slim to none. An exclusive study on *Salix purpurea* L. (a potential phytoremediator of trace elements) was conducted by Almeida-Rodríguez et al. (2016) wherein the symbiotic association of the host plant and *R. irregularis* was found to regulate a wide spectrum of metabolic and physiological outcomes upon Cu exposure. AMF-reinforced dynamic regulation of root AQPs, *PIP1;2*, together with an upregulation of root *TIP2;2* were held accountable for the maintained root hydraulic conductivity (L_p) and leaf-specific conductivity (K_L) under Cu stress. The observed translocation and accumulation of Cu ions in the cell wall and different organelles might be an outcome of osmoregulation encouraged by AQPs. TIPs and PIPs are key conduits of water conduction during this process (Table 2).

Although there has been profuse knowledge explosion in the domain of plant–AMF–HM interaction and multiple mechanisms have been ascribed to the ameliorative role of mycorrhizal association in assuaging HM stress, the arena of AMF-regulated AQP expression under metal stress is least explored. Hence, this might offer a potential area of research to decode the bona fide contribution of AMF to AQP regulation under metal stress. Moreover, the studies that deal with the functional characterization of fungal AQPs, which might serve as facultative AQPs to plants under stress, need to be performed.

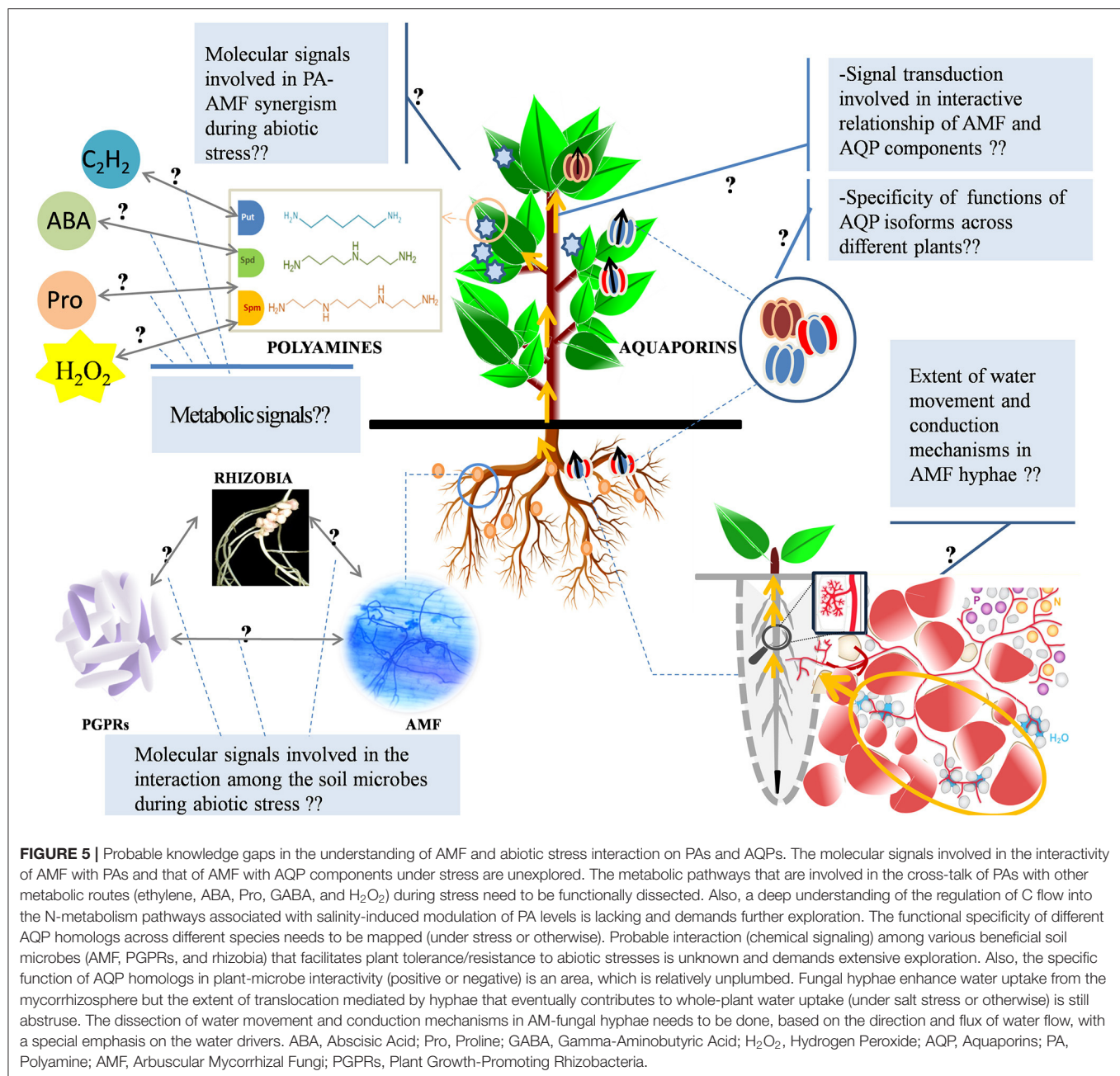
How fungal AQPs get affected under HM stress remains unexplored. Research flux should be trained toward studying the effect of metal toxicity on both host and fungal AQP genes and whether fungal AQPs are competent to counterbalance the metal-induced AQP downregulation in the host plants. More fungal AQP genes need to be identified and examined for their structure, function, and regulation under stress and otherwise.

CONCLUSION AND PERSPECTIVE FOR FUTURE STUDIES

Recurring episodes of environmental stresses affect arable land and stifle plant growth at both quantitative and qualitative levels. As opposed to biotic stress, which is controlled by monogenic trait, tolerance to abiotic stress is a multigenic complex trait that involves a multi-component signaling cascade, hence more difficult to monitor and engineer comparatively. Lately, several efforts are being invested in refining metal/salt/drought tolerance through the AMF application (mycoremediation) or genetic engineering. However, the complexity of tolerance mechanisms and difficulty in the transfer of technology to field conditions account to apparent inconsistency between theory and practice.

As reiterated in the paper, there exists a synergism between AMF and PA metabolism, which is exploited in plants under stressful conditions. However, the properties of signaling mechanisms, intricacies of cross-talk between PAs and other metabolic routes, and an exact mechanism underlying the putative role of AMF in mediating PA-induced alleviation of abiotic stress are far from being completely decoded owing to the increasing level of complexity. Considering that, the transcriptomic and metabolomic investigation in plants with knocked out/down PA synthesizing and catabolizing genes, interacting with AMF under stress, could offer a good outset for research direction. Besides, these approaches can be extrapolated to legumes wherein “rhizobia–AMF–PA” tripartite interaction can be investigated (Menéndez et al., 2019). The effect of PA knockout mutants can also be studied on other metabolic routes that are in a close-knit association with the PA metabolism under stress.

The cellular and molecular mechanisms of mycorrhizal influence on the AQP expression profile (increase/decrease) in response to water-deficit conditions (imposed by salt, drought, and HM stress) remain elusive and need exhaustive research (Figure 5). These voids in understanding offer potential research landscapes in quest of deeper insights and practical implications of AMF in stress. In order to advance a step forward in understanding AQP–abiotic stress relations in AM plants, the expression of the same AQP members (isoforms) should be investigated across different AMF species and types of stress in order to discern the specificity in AQP function and nature of regulation by AMF. Furthermore, probing the effect of the same AMF species on the AQP regulation across a combination of biotic and abiotic stresses could be another potential research line. Such studies can be conducted on multiple “HM/salt/drought-tolerant” AMF species that are naturally found in saline or metalliferous environments in association with



different crops, and then comparative analysis of their effects can be done in order to achieve the most efficacious combination of AMF species that can be employed as biofertilizers to dilute the ensuing toxic effects of stress on plants.

The fact that fungal hyphae enhance the water uptake from mycorrhizosphere to plant vasculature has been well-reviewed (Evelin et al., 2019), but the extent of translocation mediated by hyphae that eventually contributes to whole-plant water uptake (underwater deficit or otherwise) is still abstruse (Figure 5). On that account, the dissection of “water movement and conduction” mechanisms in AM-fungal hyphae needs to be done, based on the direction and flux of water flow with a special emphasis on the

water drivers (AQPs, etc.) (Wu et al., 2013). Functional genomics can be integrated to expand the dimensions of our understanding on these facets. Identification of key mycorrhizal genes and TFs that contribute to the modulation of AQP activity should be identified, gauged, and engineered to improve crop health under stress and otherwise.

In plants, the stress-triggered damage can be attenuated by exploring and exploiting the potential of beneficial soil microbes [plant growth-promoting rhizobacteria (PGPRs), rhizobia, and AMF], and then employing them discretely or in combination. Numerous studies evince the positive influence of dual/triple inoculation on plant growth and tolerance under abiotic stress

(Egamberdieva et al., 2013; Hashem et al., 2016; Hidri et al., 2019; Iggehon et al., 2020). Further research should focus on decoding the molecular signals that are involved in the interaction among the bio inoculants that ultimately influence plant physiology (Aroca and Ruiz-Lozano, 2009). The efficacy of multiple inoculations vs. single inoculation has also been investigated. Nonetheless, a lot more is there to understand about the fundamental mechanisms that are influenced by AMF and/or PGPR and/or rhizobia under stress (Yasmeen et al., 2019). A comprehensive molecular and physiological understanding of underlying mechanisms would enable us to better exploit the microbial bioresources as a potential tool for assuaging abiotic stress (Figure 5). Functional analysis of different growth-promoting microbes as first-generation plant biostimulants, when they are administered alone or in combination, would pave a way for the development of second-generation biostimulants with cooperative and complementary actions, mechanism of which can be functionally tailored. Besides, the employment of both microbial and non-microbial (seaweed extract or biochar) plant biostimulants should be promoted to reinforce sustainability efforts (González-González et al., 2020).

Global climate is changing rapidly than ever, menacing both plants and their symbiotic partners, thereby exerting significant direct and indirect effects on the growth and productivity of plants. Although AMF can attenuate the effects of abiotic stresses including climate change by fortifying the stress tolerance of host plants, their effects on AMF are given less attention, and hence poorly understood. Effects of these stresses on the symbiont could be direct or could be indirect through their impact on the host plant. It is expected that multiple abiotic stresses can directly affect the fitness, community composition, diversity, and symbiotic functioning of the fungi. Thus, keeping in mind the current scenario of global climate change and various other abiotic stresses, it is worth mentioning that studying the effects of abiotic stresses on AMF separately from plants will deliver a better understanding of the strengths and flaws of their ubiquitous relationship and for the employment of the AMF technology in sustainable agriculture.

In view of sustainable agriculture, the economical development of viable and potent AMF inocula to serve as bio fertilizer holds many practical applications; however, it is

still inadequate due to limited research capacity and technical complexities. Primary obstacles to large-scale production of AMF inocula are the following: (1) the obligate nature of the fungi, which makes the phase of crop cultivation a prerequisite and (2) the fear of contamination by weeds and pathogenic spores in soil-based inocula. Conducting an expansive open-field inoculation treatment seems economically prohibitive. However, if AM-friendly management (fall cover cropping and conservation tillage) is executed and AM-biodiversity is well-established, the fungal community will perpetuate and the hyphal networks will remain unaltered (Lehman et al., 2012; Berruti et al., 2016). A major step toward stable usage of AMF is to conduct wholesale field trials at diverse locations and analyze the cost-economy (Ceballos et al., 2013) in order to better aware the potential users of the advantages of AMF. Additionally, autonomous production of customized inocula from the native soil *per se* can be encouraged so as to make the strategy of bio fertilization and stress amelioration more economical for farmers of developing countries.

Taken together, AMF must be scouted on all levels to further examine their beneficial roles in the natural environment where plants are subjected to multiple stresses at a time; this would be imperative to make complete use of AMF as a bio fertilizer and stress mitigator in order to achieve sustainable agriculture production and guarantee food security.

AUTHOR CONTRIBUTIONS

KS and RK conceptualized the design, draft, and layout of the literature work. All the authors have critically reviewed the manuscript and approved it for submission.

ACKNOWLEDGMENTS

The authors jointly acknowledge the Science and Engineering Research Board (Grant no. SERB/CRG/2019/000836). RK acknowledges the Research Council of the University of Delhi for the publication support. KS, SG, and ST acknowledge the Council of Scientific and Industrial Research (CSIR) and PJ acknowledges the University Grants Commission (UGC) for fellowships.

REFERENCES

- Abdel-Fattah, G. M., Ibrahim, A. H., Al-Amri, S. M., and Shoker, A. E. (2013). Synergistic effect of arbuscular mycorrhizal fungi and spermine on amelioration of salinity stress of wheat (*Triticum aestivum* L. cv. gimiza 9). *Aust. J. Crop Sci.* 7, 1525–1532.
- Abeer, H., Abd Allah, E. F., Alqarawi, A. A., El-Didamony, G., Alwhibi, M., Egamberdieva, D., et al. (2014). Alleviation of adverse impact of salinity on faba bean (*Vicia faba* L.) by arbuscular mycorrhizal fungi. *Pak. J. Bot.* 46, 2003–2013.
- Agre, P., Bonhivers, M., and Borgnia, M. J. (1998). The aquaporins, blueprints for cellular plumbing systems. *J. Biol. Chem.* 273, 14659–14662. doi: 10.1074/jbc.273.24.14659
- Alam, M. Z., McGee, R., Hoque, M., Ahammed, G. J., and Carpenter-Boggs, L. (2019). Effect of arbuscular mycorrhizal fungi, selenium and biochar on photosynthetic pigments and antioxidant enzyme activity under arsenic stress in mung bean (*Vigna radiata*). *Front. Physiol.* 10:193. doi: 10.3389/fphys.2019.00193
- Alcázar, R., Altabella, T., Marco, F., Bortolotti, C., Reymond, M., Koncz, C., et al. (2010). Polyamines: molecules with regulatory functions in plant abiotic stress tolerance. *Planta* 231, 1237–1249. doi: 10.1007/s00425-010-1130-0
- Alcázar, R., García-Martínez, J. L., Cuevas, J. C., Tiburcio, A. F., and Altabella, T. (2005). Overexpression of ADC2 in *Arabidopsis* induces dwarfism and late-flowering through GA deficiency. *Plant J.* 43, 421–436. doi: 10.1111/j.1365-3113.2005.02465.x
- Almeida-Rodríguez, A. M., Gómez, M. P., Loubert-Hudon, A., Joly, S., and Labrecque, M. (2016). Symbiotic association between *Salix purpurea* L. and *Rhizophagus irregularis*: modulation of plant responses under copper stress. *Tree Physiol.* 36, 407–420. doi: 10.1093/treephys/tpv119
- Anwar, R., Mattoo, A. K., and Handa, A. K. (2015). “Polyamine interactions with plant hormones: crosstalk at several levels,” in *Polyamines*, eds T. Kusano, and H. Suzuki (Tokyo: Springer), 267–302. doi: 10.1007/978-4-431-55212-3_22

- Ariani, A., Barozzi, F., Sebastiani, L., di Toppi, L. S., di Sansebastiano, G. P., and Andreucci, A. (2019). AQUA1 is a mercury sensitive poplar aquaporin regulated at transcriptional and post-translational levels by Zn stress. *Plant Physiol. Biochem.* 135, 588–600. doi: 10.1016/j.plaphy.2018.10.038
- Aroca, R., Porcel, R., and Ruiz-Lozano, J. M. (2007). How does arbuscular mycorrhizal symbiosis regulate root hydraulic properties and plasma membrane aquaporins in *Phaseolus vulgaris* under drought, cold or salinity stresses? *New Phytol.* 173, 808–816. doi: 10.1111/j.1469-8137.2006.01961.x
- Aroca, R., and Ruiz-Lozano, J. M. (2009). “Induction of plant tolerance to semi-arid environments by beneficial soil microorganisms—a review”, in *Climate Change, Intercropping, Pest Control and Beneficial Microorganisms. Sustainable Agriculture Reviews*, ed E. Lichtfouse (Dordrecht: Springer), 121–135.
- Bai, J., Lin, X., Yin, R., Zhang, H., Junhua, W., Xueming, C., et al. (2008). The influence of arbuscular mycorrhizal fungi on As and P uptake by maize (*Zea mays* L.) from As-contaminated soils. *Appl. Soil Ecol.* 38, 137–145. doi: 10.1016/j.apsoil.2007.10.002
- Bárcana, G., Aroca, R., Bienert, G. P., Chaumont, F., and Ruiz-Lozano, J. M. (2014). New insights into the regulation of aquaporins by the arbuscular mycorrhizal symbiosis in maize plants under drought stress and possible implications for plant performance. *Mol. Plant Microbe Interact.* 27, 349–363. doi: 10.1094/MPMI-09-13-0268-R
- Bárcana, G., Aroca, R., Paz, J. A., Chaumont, F., Martínez-Ballesta, M. C., Carvajal, M., et al. (2012). Arbuscular mycorrhizal symbiosis increases relative apoplastic water flow in roots of the host plant under both well-watered and drought stress conditions. *Ann. Bot.* 109, 1009–1017. doi: 10.1093/aob/mcs007
- Beaudette, P. C., Chlup, M., Yee, J., and Emery, R. N. (2007). Relationships of root conductivity and aquaporin gene expression in *Pisum sativum*: diurnal patterns and the response to HgCl₂ and ABA. *J. Exp. Bot.* 58, 1291–1300. doi: 10.1093/jxb/erl289
- Berruti, A., Lumini, E., Balestrini, R., and Bianciotto, V. (2016). Arbuscular mycorrhizal fungi as natural biofertilizers: let's benefit from past successes. *Front. Microbiol.* 6:1559. doi: 10.3389/fmicb.2015.01559
- Berta, G., Sgorbati, S., Soler, V., Fusconi, A., Trotta, A., Citterio, A., et al. (1990). Variations in chromatin structure in host nuclei of a vesicular arbuscular mycorrhiza. *New Phytol.* 114, 199–205. doi: 10.1111/j.1469-8137.1990.tb00391.x
- Besford, R. T., Richardson, C. M., Campos, J. L., and Tiburcio, A. F. (1993). Effect of polyamines on stabilization of molecular complexes in thylakoid membranes of osmotically stressed oat leaves. *Planta* 189, 201–206. doi: 10.1007/BF00195077
- Bharti, A., Garg, S., Prakash, A., and Sharma, M. P. (2017). *Contribution of AMF in the Remediation of Drought Stress in Soybean Plants*. New Delhi: New India Publishing Agency.
- Ceballos, I., Ruiz, M., Fernández, C., Peña, R., Rodríguez, A., and Sanders, I. R. (2013). The *in vitro* mass-produced model mycorrhizal fungus, *Rhizophagus irregularis*, significantly increases yields of the globally important food security crop cassava. *PLoS ONE* 8:e70633. doi: 10.1371/journal.pone.0070633
- Chakraborty, K., Sairam, R. K., and Bhaduri, D. (2016). Effects of different levels of soil salinity on yield attributes, accumulation of nitrogen, and micronutrients in *Brassica* spp. *J. Plant. Nutr.* 39, 1026–1037. doi: 10.1080/01904167.2015.1109105
- Chen, D., Shao, Q., Yin, L., Younis, A., and Zheng, B. (2019). Polyamine function in plants: metabolism, regulation on development, and roles in abiotic stress responses. *Front. Plant Sci.* 9:1945. doi: 10.3389/fpls.2018.01945
- Chen, J., Zhang, H., Zhang, X., and Tang, M. (2017). Arbuscular mycorrhizal symbiosis alleviates salt stress in black locust through improved photosynthesis, water status, and K⁺/Na⁺ homeostasis. *Front. Plant Sci.* 8:1739. doi: 10.3389/fpls.2017.01739
- Chen, X., Bründl, M., Friesacher, T., and Sary-Weininger, A. (2020). Computational insights into voltage dependence of polyamine block in a strong inwardly rectifying K⁺ channel. *Front. Pharmacol.* 11:721. doi: 10.3389/fphar.2020.00721
- Chen, X., Li, H., Chan, W. F., Wu, C., Wu, F., Wu, S., et al. (2012). Arsenite transporters expression in rice (*Oryza sativa* L.) associated with arbuscular mycorrhizal fungi (AMF) colonization under different levels of arsenite stress. *Chemosphere* 89, 1248–1254. doi: 10.1016/j.chemosphere.2012.07.054
- Christie, P., Li, X., and Chen, B. (2004). Arbuscular mycorrhiza can depress translocation of zinc to shoots of host plants in soils moderately polluted with zinc. *Plant Soil* 261, 209–217. doi: 10.1023/B:PLSO.0000035542.79345.1b
- Cicatelli, A., Lingua, G., Todeschini, V., Biondi, S., Torrigiani, P., and Castiglione, S. (2010). Arbuscular mycorrhizal fungi restore normal growth in a white poplar clone grown on heavy metal-contaminated soil, and this is associated with upregulation of foliar metallothionein and polyamine biosynthetic gene expression. *Ann. Bot.* 106, 791–802. doi: 10.1093/aob/mcq170
- Cicatelli, A., Torrigiani, P., Todeschini, V., Biondi, S., Castiglione, S., and Lingua, G. (2014). Arbuscular mycorrhizal fungi as a tool to ameliorate the phytoremediation potential of poplar: biochemical and molecular aspects. *iForest* 7, 333–349. doi: 10.3832/ifor1045-007
- Cuevas, J. C., Lopez-Cobollo, R., Alcazar, R., Zarza, X., Koncz, C., Altabella, T., et al. (2008). Putrescine is involved in *Arabidopsis* freezing tolerance and cold acclimation by regulating abscisic acid levels in response to low temperature. *Plant Physiol.* 148, 1094–1105. doi: 10.1104/pp.108.122945
- Ding, Y. E., Fan, Q. F., He, J. D., Wu, H. H., Zou, Y. N., Wu, Q. S., et al. (2020). Effects of mycorrhizas on physiological performance and root TIPs expression in trifoliate orange under salt stress. *Arch. Agron. Soil Sci.* 66, 182–192. doi: 10.1080/03650340.2019.1607313
- Dubois, M., Van den Broeck, L., and Inzé, D. (2018). The pivotal role of ethylene in plant growth. *Trends Plant Sci.* 23, 311–323. doi: 10.1016/j.tplants.2018.01.003
- Echeverría, M., Sannazzaro, A. I., Ruiz, O. A., and Menéndez, A. B. (2013). Modulatory effects of *Mesorhizobium tianshanense* and *Glomus intraradices* on plant proline and polyamine levels during early plant response of *Lotus tenuis* to salinity. *Plant Soil* 364, 69–79. doi: 10.1007/s11104-012-1312-6
- Egamberdieva, D., Jabborova, D., and Wirth, S. (2013). “Alleviation of salt stress in legumes by co-inoculation with *Pseudomonas* and *Rhizobium*”, in *Plant Microbe Symbiosis: Fundamentals and Advances*, ed N. Aroca (New Delhi: Springer), 291–303.
- El Ghachtouli, N., Paynot, M., Morandi, D., Martin-Tanguy, J., and Gianinazzi, S. (1995). The effect of polyamines on endomycorrhizal infection of wild-type *Pisum sativum*, cv. Frisson (nod⁺ myc⁺) and two mutants (nod[−] myc⁺ and nod[−] myc[−]). *Mycorrhiza* 5, 189–192. doi: 10.1007/s005720050058
- Espasandin, F. D., Maiale, S. J., Calzadilla, P., Ruiz, O. A., and Sansberro, P. A. (2014). Transcriptional regulation of 9-cis-epoxycarotenoid dioxygenase (NCED) gene by putrescine accumulation positively modulates ABA synthesis and drought tolerance in *Lotus tenuis* plants. *Plant Physiol. Biochem.* 76, 29–35. doi: 10.1016/j.plaphy.2013.12.018
- Evelin, H., Devi, T. S., Gupta, S., and Kapoor, R. (2019). Mitigation of salinity stress in plants by arbuscular mycorrhizal symbiosis: current understanding and new challenges. *Front. Plant Sci.* 10:470. doi: 10.3389/fpls.2019.00470
- Evelin, H., Giri, B., and Kapoor, R. (2012). Contribution of *Glomus intraradices* inoculation to nutrient acquisition and mitigation of ionic imbalance in NaCl-stressed *Trigonella foenum-graecum*. *Mycorrhiza* 22, 203–217. doi: 10.1007/s00572-011-0392-0
- Evelin, H., Giri, B., and Kapoor, R. (2013). Ultrastructural evidence for AMF mediated salt stress mitigation in *Trigonella foenum-graecum*. *Mycorrhiza* 23, 71–86. doi: 10.1007/s00572-012-0449-8
- Evelin, H., Kapoor, R., and Giri, B. (2009). Arbuscular mycorrhizal fungi in alleviation of salt stress: a review. *Ann. Bot.* 104, 1263–1280. doi: 10.1093/aob/mcp251
- Even-Chen, Z., Mattoo, A. K., and Goren, R. (1982). Inhibition of ethylene biosynthesis by aminoethoxyvinylglycine and by polyamines shunts label from 3, 4-[14C] methionine into spermidine in aged orange peel discs. *Plant Physiol.* 69, 385–388. doi: 10.1104/pp.69.2.385
- Ezawa, T., Smith, S. E., and Smith, F. A. (2002). P metabolism and transport in AM fungi. *Plant Soil* 244, 221–230. doi: 10.1023/A:1020258325010
- FAO (2017) *The Future of Food and Agriculture – Trends and Challenges*. Rome.
- Fatemi, H., Zaghdoud, C., Nortes, P. A., Carvajal, M., and Martínez-Ballesta, M. D. C. (2020). Differential aquaporin response to distinct effects of two Zn concentrations after foliar application in pak choi (*Brassica rapa* L.) plants. *Agronomy* 10:450. doi: 10.3390/agronomy10030450
- Flowers, T. J., Garcia, A., Koyama, M., and Yeo, A. R. (1997). Breeding for salt tolerance in crop plants—the role of molecular biology. *Acta Physiol. Plant* 19, 427–433. doi: 10.1007/s11738-997-0039-0
- Flowers, T. J., and Yeo, A. R. (1995). Breeding for salinity resistance in crop plants: where next? *Funct. Plant Biol.* 22, 875–884. doi: 10.1071/PP950875

- Foo, E., McAdam, E. L., Weller, J. L., and Reid, J. B. (2016). Interactions between ethylene, gibberellins, and brassinosteroids in the development of rhizobial and mycorrhizal symbioses of pea. *J. Exp. Bot.* 67, 2413–2424. doi: 10.1093/jxb/erw047
- Freschi, L. (2013). Nitric oxide and phytohormone interactions: current status and perspectives. *Front. Plant Sci.* 4:398. doi: 10.3389/fpls.2013.00398
- Galston, A. W., and Sawhney, R. K. (1990). Polyamines in plant physiology. *Plant Physiol.* 94, 406–410. doi: 10.1104/pp.94.2.406
- Garg, N., and Bhandari, P. (2016). Interactive effects of silicon and arbuscular mycorrhiza in modulating ascorbate-glutathione cycle and antioxidant scavenging capacity in differentially salt-tolerant *Cicer arietinum* L. genotypes subjected to long-term salinity. *Protoplasma* 253, 1325–1345. doi: 10.1007/s00709-015-0892-4
- Garg, N., and Saroy, K. (2020). Interactive effects of polyamines and arbuscular mycorrhiza in modulating plant biomass, N₂ fixation, ureide, and trehalose metabolism in *Cajanus cajan* (L.) Millsp. genotypes under nickel stress. *Environ. Sci. Pollut. Res.* 27, 3043–3064. doi: 10.1007/s11356-019-07300-6
- Goicoechea, N., Szalai, G., Antolín, M. C., Sánchez-Díaz, M., and Paldi, E. (1998). Influence of arbuscular mycorrhizae and *Rhizobium* on free polyamines and proline levels in water-stressed alfalfa. *J. Plant Physiol.* 153, 706–711. doi: 10.1016/S0176-1617(98)80224-1
- Gonzalez, M. E., Marco, F., Minguet, E. G., Carrasco-Sorli, P., Blázquez, M. A., Carbonell, J., et al. (2011). Perturbation of spermine synthase gene expression and transcript profiling provide new insights on the role of the tetraamine spermine in *Arabidopsis* defense against *Pseudomonas viridiflava*. *Plant Physiol.* 156, 2266–2277. doi: 10.1104/pp.110.171413
- González-González, M. F., Ocampo-Alvarez, H., Santacruz-Ruvalcaba, F., Sánchez-Hernández, C. V., Casarrubias-Castillo, K., Becerril-Espinosa, A., et al. (2020). Physiological, ecological, and biochemical implications in tomato plants of two plant biostimulants: arbuscular mycorrhizal fungi and seaweed extract. *Front. Plant Sci.* 11:999. doi: 10.3389/fpls.2020.00999
- Groppa, M. D., Tomaro, M. L., and Benavides, M. P. (2007). Polyamines and heavy metal stress: the antioxidant behavior of spermine in cadmium- and copper-treated wheat leaves. *Biomolecules* 20, 185–195. doi: 10.1007/s10534-006-9026-y
- Gupta, S., Thokchom, S. D., and Kapoor, R. (2021). Arbuscular mycorrhiza improves photosynthesis and restores alteration in sugar metabolism in *Triticum aestivum* L. grown in arsenic contaminated soil. *Front. Plant Sci.* 12:334. doi: 10.3389/fpls.2021.640379
- Gusman, G. S., Oliveira, J. A., Farnese, F. S., and Cambraia, J. (2013). Mineral nutrition and enzymatic adaptation induced by arsenate and arsenite exposure in lettuce plants. *Plant Physiol. Biochem.* 71, 307–314. doi: 10.1016/j.plaphy.2013.08.006
- Hanfrey, C., Sommer, S., Mayer, M. J., Burtin, D., and Michael, A. J. (2001). *Arabidopsis* polyamine biosynthesis: absence of ornithine decarboxylase and the mechanism of arginine decarboxylase activity. *Plant J.* 27, 551–560. doi: 10.1046/j.1365-3113X.2001.01100.x
- Hashem, A., Abd-Allah, E. F., Alqarawi, A. A., Al-Huqail, A. A., Wirth, S., and Egamberdieva, D. (2016). The interaction between arbuscular mycorrhizal fungi and endophytic bacteria enhances plant growth of *Acacia gerrardii* under salt stress. *Front. Microbiol.* 7:1089. doi: 10.3389/fmicb.2016.01089
- Hashem, A., Alqarawi, A. A., Radhakrishnan, R., Al-Arjani, A. B. F., Aldehaish, H. A., Egamberdieva, D., et al. (2018). Arbuscular mycorrhizal fungi regulate the oxidative system, hormones and ionic equilibrium to trigger salt stress tolerance in *Cucumis sativus* L. *Saudi J. Biol. Sci.* 25, 1102–1114. doi: 10.1016/j.sjbs.2018.03.009
- He, F., Zhang, H., and Tang, M. (2016). Aquaporin gene expression and physiological responses of *Robinia pseudoacacia* L. to the mycorrhizal fungus *Rhizophagus irregularis* and drought stress. *Mycorrhiza* 26, 311–323. doi: 10.1007/s00572-015-0670-3
- Hidri, R., Mahmoud, O. M. B., Debez, A., Abdelli, C., Barea, J. M., and Azcon, R. (2019). Modulation of C: N: P stoichiometry is involved in the effectiveness of a PGPR and AM fungus in increasing salt stress tolerance of *Sulla carnosa* Tunisian provenances. *Appl. Soil Ecol.* 143, 161–172. doi: 10.1016/j.apsoil.2019.06.014
- Hu, Y., and Chen, B. (2020). Arbuscular mycorrhiza induced putrescine degradation into γ -aminobutyric acid, malic acid accumulation, and improvement of nitrogen assimilation in roots of water-stressed maize plants. *Mycorrhiza* 30, 329–339. doi: 10.1007/s00572-020-00952-0
- Hu, Y., Xie, W., and Chen, B. (2020). Arbuscular mycorrhiza improved drought tolerance of maize seedlings by altering photosystem II efficiency and the levels of key metabolites. *Chem. Biol. Technol. Agric.* 7, 1–14. doi: 10.1186/s40538-020-00186-4
- Hura, T., Dziurka, M., Hura, K., Ostrowska, A., and Dziurka, K. (2015). Free and cell wall-bound polyamines under long-term water stress applied at different growth stages of *Triticosecale Wittm.* *PLoS ONE* 10:e0135002. doi: 10.1371/journal.pone.0135002
- Ibrahim, A. H., Abdel-Fattah, G. M., Eman, F. M., Abd El-Aziz, M. H., and Shohr, A. E. (2011). Arbuscular mycorrhizal fungi and spermine alleviate the adverse effects of salinity stress on electrolyte leakage and productivity of wheat plants. *Phyton Int. J. Exp. Bot.* 51, 261–276.
- Igiehon, N. O., Babalola, O. O., Cheseto, X., and Torto, B. (2020). Effects of rhizobia and arbuscular mycorrhizal fungi on yield, size distribution and fatty acid of soybean seeds grown under drought stress. *Microbiol. Res.* 242:126640. doi: 10.1016/j.micres.2020.126640
- Iqbal, N., Umar, S., Khan, N. A., and Khan, M. I. R. (2014). A new perspective of phytohormones in salinity tolerance: regulation of proline metabolism. *Environ. Exp. Bot.* 100, 34–42. doi: 10.1016/j.envexpbot.2013.12.006
- Ishikawa, F., Suga, S., Uemura, T., Sato, M. H., and Maeshima, M. (2005). Novel type aquaporin SIPs are mainly localized to the ER membrane and show cell-specific expression in *Arabidopsis thaliana*. *FEBS Lett.* 579, 5814–5820. doi: 10.1016/j.febslet.2005.09.076
- Jahromi, F., Aroca, R., Porcel, R., and Ruiz-Lozano, J. M. (2008). Influence of salinity on the *in vitro* development of *Glomus intraradices* and on the *in vivo* physiological and molecular responses of mycorrhizal lettuce plants. *Microb. Ecol.* 55:45. doi: 10.1007/s00248-007-9249-7
- Jaleel, C. A., Manivannan, P., Wahid, A., Farooq, M., Al-Juburi, H. J., Somasundaram, R., et al. (2009). Drought stress in plants: a review on morphological characteristics and pigments composition. *Int. J. Agric. Biol.* 11, 100–105.
- Javot, H., and Maurel, C. (2002). The role of aquaporins in root water uptake. *Ann. Bot.* 90, 301–313. doi: 10.1093/aob/mcf199
- Jia-Dong, H., Tao, D., Hui-Hui, W., Ying-Ning, Z., Qiang-Sheng, W., and Kamil, K. (2019). Mycorrhizas induce diverse responses of root TIP aquaporin gene expression to drought stress in trifoliate orange. *Sci. Hortic.* 243, 64–69. doi: 10.1016/j.scienta.2018.08.010
- Juniper, S., and Abbott, L. (1993). Vesicular-arbuscular mycorrhizas and soil salinity. *Mycorrhiza* 4, 45–57. doi: 10.1007/BF00204058
- Kakkar, R. K., and Sawhney, V. K. (2002). Polyamine research in plants—a changing perspective. *Physiol. Plant* 116, 281–292. doi: 10.1034/j.1399-3054.2002.1160302.x
- Kaplan, R., Vaziri, M., and Zwiazek, J. J. (2018). Regulation of aquaporins in plants under stress. *Biol. Res.* 51, 1–11. doi: 10.1186/s40659-018-0152-0
- Khalid, M., Saeed, U. R., Hassani, D., Hayat, K., Pei, Z. H. O. U., and Nan, H. U. I. (2021). Advances in fungal-assisted phytoremediation of heavy metals: a review. *Pedosphere* 31, 475–495. doi: 10.1016/S1002-0160(20)60091-1
- Khalloufi, M., Martínez-Andújar, C., Lachaâl, M., Karray-Bourouai, N., Pérez-Alfocea, F., and Albacete, A. (2017). The interaction between foliar GA3 application and arbuscular mycorrhizal fungi inoculation improves growth in salinized tomato (*Solanum lycopersicum* L.) plants by modifying the hormonal balance. *J. Plant Physiol.* 214, 134–144. doi: 10.1016/j.jplph.2017.04.012
- Kholodova, V., Volkov, K., Abdeyeva, A., and Kuznetsov, V. (2011). Water status in *Mesembryanthemum crystallinum* under heavy metal stress. *Environ. Exp. Bot.* 71, 382–389. doi: 10.1016/j.envexpbot.2011.02.007
- Khoshbakt, D., Asghari, M. R., and Haghighi, M. (2018). Influence of foliar application of polyamines on growth, gas-exchange characteristics, and chlorophyll fluorescence in *Bakrai citrus* under saline conditions. *Photosynthetica* 56, 731–742. doi: 10.1007/s11099-017-0723-2
- Kourghy, M., Nourmohammadi, S., Pei, J. V., Qiu, J., McGaughey, S., Tyerman, S. D., et al. (2017). Divalent cations regulate the ion conductance properties of diverse classes of aquaporins. *Int. J. Mol. Sci.* 18:2323. doi: 10.3390/ijms18112323
- Krajinski, F., Biela, A., Schubert, D., Gianinazzi-Pearson, V., Kaldenhoff, R., and Franken, P. (2000). Arbuscular mycorrhiza development regulates the mRNA abundance of *Mtaqp1* encoding a mercury-insensitive aquaporin of *Medicago truncatula*. *Planta* 211, 85–90. doi: 10.1007/s004250000263

- Lazzarato, L., Trebbi, G., Pagnucco, C., Franchin, C., Torrigiani, P., and Betti, L. (2009). Exogenous spermidine, arsenic and β -aminobutyric acid modulate tobacco resistance to tobacco mosaic virus, and affect local and systemic glucosyl salicylic acid levels and arginine decarboxylase gene expression in tobacco leaves. *J. Plant Physiol.* 166, 90–100. doi: 10.1016/j.jplph.2008.01.011
- Lehman, R. M., Taheri, W. I., Osborne, S. L., Buyer, J. S., and Douds, D. D. Jr. (2012). Fall cover cropping can increase arbuscular mycorrhizae in soils supporting intensive agricultural production. *Appl. Soil Ecol.* 61, 300–304. doi: 10.1016/j.apsoil.2011.11.008
- Li, M., Lu, J., Tao, M., Li, M., Yang, H., Xia, E. H., et al. (2020). Genome-wide identification of seven polyamine oxidase genes in *Camellia sinensis* (L.) and their expression patterns under various abiotic stresses. *Front. Plant Sci.* 11:1338. doi: 10.3389/fpls.2020.544933
- Li, S., Jin, H., and Zhang, Q. (2016). The effect of exogenous spermidine concentration on polyamine metabolism and salt tolerance in *Zoysiagrass* (*Zoysia japonica* Steud.) subjected to short-term salinity stress. *Front. Plant Sci.* 7:1221. doi: 10.3389/fpls.2016.01221
- Li, T., Hu, Y. J., Hao, Z. P., Li, H., and Chen, B. D. (2013). Aquaporin genes *GintAQPF1* and *GintAQPF2* from *Glomus intraradices* contribute to plant drought tolerance. *Plant Signal. Behav.* 8:e24030. doi: 10.4161/psb.24030
- Li, Z., Zhang, Y., Peng, D., Peng, Y., Zhang, X., Ma, X., et al. (2018). The inhibition of polyamine biosynthesis weakens the drought tolerance in white clover (*Trifolium repens*) associated with the alteration of extensive proteins. *Protoplasma* 255, 803–817. doi: 10.1007/s00709-017-1186-9
- Li, Z., Zhang, Y., Zhang, X., Peng, Y., Merewitz, E., Ma, X., et al. (2016). The alterations of endogenous polyamines and phytohormones induced by exogenous application of spermidine regulate antioxidant metabolism, metallothionein and relevant genes conferring drought tolerance in white clover. *Environ. Exp. Bot.* 124, 22–38. doi: 10.1016/j.envexpbot.2015.12.004
- Lin, C. C., and Kao, C. H. (1999). Excess copper induces an accumulation of putrescine in rice leaves. *Bot. Bull. Acad. Sin.* 40, 213–218.
- Liu, C., Zhao, X., Yan, J., Yuan, Z., and Gu, M. (2020). Effects of salt stress on growth, photosynthesis, and mineral nutrients of 18 pomegranate (*Punica granatum*) cultivars. *Agronomy* 10:27. doi: 10.3390/agronomy10010027
- Liu, K., Fu, H., Bei, Q., and Luan, S. (2000). Inward potassium channel in guard cells as a target for polyamine regulation of stomatal movements. *Plant Physiol.* 124, 1315–1326. doi: 10.1104/pp.124.3.1315
- Liu, M., Chen, J., Guo, Z., and Lu, S. (2017). Differential responses of polyamines and antioxidants to drought in a centipede grass mutant in comparison to its wild type plants. *Front. Plant Sci.* 8:792. doi: 10.3389/fpls.2017.00792
- Liu, T., Kim, D. W., Niitsu, M., Berberich, T., and Kusano, T. (2014). *Oryza sativa* polyamine oxidase 1 back-converts tetraamines, spermine and thermospermine, to spermidine. *Plant Cell Rep.* 33, 143–151. doi: 10.1007/s00299-013-1518-y
- Liu, Y., Liang, H., Lv, X., Liu, D., Wen, X., and Liao, Y. (2016). Effect of polyamines on the grain filling of wheat under drought stress. *Plant Physiol. Biochem.* 100, 113–129. doi: 10.1016/j.plaphy.2016.01.003
- Luo, Y. (2009). *Effects of AMF on Cell Membrane, Endogenous Polyamines, and Salicylic Acid in Citrus Under Drought Stress*. Huazhong Agriculture University.
- Ma, J. F., Yamaji, N., Mitani, N., Xu, X. Y., Su, Y. H., McGrath, S. P., et al. (2008). Transporters of arsenite in rice and their role in arsenic accumulation in rice grain. *Proc. Natl. Acad. Sci. U.S.A.* 105, 9931–9935. doi: 10.1073/pnas.0802361105
- Marjanović, Ž., Uehlein, N., Kaldenhoff, R., Zwiazek, J. J., Weiß, M., Hampp, R., et al. (2005). aquaporins in poplar: what a difference a symbiont makes!. *Planta* 222, 258–268. doi: 10.1007/s00425-005-1539-z
- Marulanda, A., Azcon, R., and Ruiz-Lozano, J. M. (2003). Contribution of six arbuscular mycorrhizal fungal isolates to water uptake by *Lactuca sativa* plants under drought stress. *Physiol. Plant* 119, 526–533. doi: 10.1046/j.1399-3054.2003.00196.x
- Mattoo, A. K., and White, W. B. (2018). *Regulation of Ethylene Biosynthesis in Plant Hormone Ethylene*. Boca Raton, FL: CRC Press, 21–42.
- Maurel, C. (2007). Plant aquaporins: novel functions and regulation properties. *FEBS Lett.* 581, 2227–2236. doi: 10.1016/j.febslet.2007.03.021
- Mekonnen, D. W., Flügge, U. I., and Ludewig, F. (2016). Gamma-aminobutyric acid depletion affects stomata closure and drought tolerance of *Arabidopsis thaliana*. *Plant Sci.* 245, 25–34. doi: 10.1016/j.plantsci.2016.01.005
- Menéndez, A. B., Calzadilla, P. I., Sansberro, P. A., Espasandin, F. D., Gazquez, A., Bordenave, C. D., et al. (2019). Polyamines and legumes: Joint stories of stress, nitrogen fixation and environment. *Front. Plant Sci.* 10:1415. doi: 10.3389/fpls.2019.01415
- Merlos, M. A., Zitka, O., Vojtech, A., Azcón-Aguilar, C., and Ferrol, N. (2016). The arbuscular mycorrhizal fungus *Rhizophagus irregularis* differentially regulates the copper response of two maize cultivars differing in copper tolerance. *Plant Sci.* 253, 68–76. doi: 10.1016/j.plantsci.2016.09.010
- Mo, H., Wang, X., Zhang, Y., Zhang, G., Zhang, J., and Ma, Z. (2015). Cotton polyamine oxidase is required for spermine and camalexin signaling in the defence response to *Verticillium dahliae*. *Plant J.* 83, 962–975. doi: 10.1111/tpj.12941
- Mohapatra, S., Minocha, R., Long, S., and Minocha, S. C. (2009). Putrescine overproduction negatively impacts the oxidative state of poplar cells in culture. *Plant Physiol. Biochem.* 47, 262–271. doi: 10.1016/j.plaphy.2008.12.007
- Nahar, K., Hasanuzzaman, M., Alam, M. M., Rahman, A., Suzuki, T., and Fujita, M. (2016). Polyamine and nitric oxide crosstalk: antagonistic effects on cadmium toxicity in mung bean plants through upregulating the metal detoxification, antioxidant defense and methylglyoxal detoxification systems. *Ecotoxicol. Environ. Saf.* 126, 245–255. doi: 10.1016/j.ecoenv.2015.12.026
- Niemi, K., Sutela, S., Häggman, H., Scagel, C., Vuosku, J., Jokela, A., et al. (2006). Changes in polyamine content and localization of *Pinus sylvestris* ADC and *Suillus variegatus* ODC mRNA transcripts during the formation of mycorrhizal interaction in an *in vitro* cultivation system. *J. Exp. Bot.* 57, 2795–2804. doi: 10.1093/jxb/erl049
- Nogales, A., Aguirreolea, J., Santa María, E., Camprubí, A., and Calvet, C. (2009). Response of mycorrhizal grapevine to *Armillaria mellea* inoculation: disease development and polyamines. *Plant Soil* 317:177. doi: 10.1007/s11104-008-9799-6
- Noronha, H., Araújo, D., Conde, C., Martins, A. P., Soveral, G., Chaumont, F., et al. (2016). The grapevine uncharacterized intrinsic protein 1 (*VvXIP1*) is regulated by drought stress and transports glycerol, hydrogen peroxide, heavy metals but not water. *PLoS ONE* 11:e0160976. doi: 10.1371/journal.pone.0160976
- Ouziad, F., Wilde, P., Schmelzer, E., Hildebrandt, U., and Bothe, H. (2006). Analysis of expression of aquaporins and Na^+/H^+ transporters in tomato colonized by arbuscular mycorrhizal fungi and affected by salt stress. *Environ. Exp. Bot.* 57, 177–186. doi: 10.1016/j.envexpbot.2005.05.011
- Ozawa, R., Berteza, C. M., Foti, M., Narayana, R., Arimura, G. I., Muroi, A., et al. (2009). Exogenous polyamines elicit herbivore-induced volatiles in lima bean leaves: involvement of calcium, H_2O_2 and Jasmonic acid. *Plant Cell Physiol.* 50, 2183–2199. doi: 10.1093/pcp/pcp153
- Pál, M., Csász, G., Szalai, G., Oláh, T., Khalil, R., Yordanova, R., et al. (2017). Polyamines may influence phytochelatin synthesis during Cd stress in rice. *J. Hazard. Mater.* 340, 272–280. doi: 10.1016/j.jhazmat.2017.07.016
- Pál, M., Szalai, G., and Janda, T. (2015). Speculation: polyamines are important in abiotic stress signaling. *Plant Sci.* 237, 16–23. doi: 10.1016/j.plantsci.2015.05.003
- Parádi, I. (2003). Influence of arbuscular mycorrhiza and cadmium on the polyamine contents of Ri T-DNA transformed *Daucus carota* L. root cultures. *Acta Biol. Szeged* 47, 31–36.
- Parvin, S., Lee, O. R., Sathiyaraj, G., Khorolragchaa, A., Kim, Y. J., and Yang, D. C. (2014). Spermidine alleviates the growth of saline-stressed ginseng seedlings through antioxidative defense system. *Gene* 537, 70–78. doi: 10.1016/j.gene.2013.12.021
- Podlešáková, K., Ugena, L., Spíchal, L., Doležal, K., and De Diego, N. (2018). Phytohormones and polyamines regulate plant stress responses by altering GABA pathway. *New Biotechnol.* 48, 53–65. doi: 10.1016/j.nbt.2018.07.003
- Popovic, R. B., Kyle, D. J., Cohen, A. S., and Zalík, S. (1979). Stabilization of thylakoid membranes by spermine during stress-induced senescence of barley leaf discs. *Plant Physiol.* 64, 721–726. doi: 10.1104/pp.64.5.721
- Porcel, R., Aroca, R., Azcon, R., and Ruiz-Lozano, J. M. (2006). PIP aquaporin gene expression in arbuscular mycorrhizal *Glycine max* and *Lactuca sativa* plants in relation to drought stress tolerance. *Plant Mol. Biol.* 60, 389–404. doi: 10.1007/s11103-005-4210-y
- Porcel, R., Aroca, R., and Ruiz-Lozano, J. M. (2012). Salinity stress alleviation using arbuscular mycorrhizal fungi: a review. *Agro Sustain. Dev.* 32, 181–200. doi: 10.1007/s13593-011-0029-x

- Porcel, R., Gómez, M., Kaldenhoff, R., and Ruiz-Lozano, J. M. (2005). Impairment of *NtAQPI* gene expression in tobacco plants does not affect root colonisation pattern by arbuscular mycorrhizal fungi but decreases their symbiotic efficiency under drought. *Mycorrhiza* 15, 417–423. doi: 10.1007/s00572-005-0346-5
- Priya, M., Sharma, L., Kaur, R., Bindumadhava, H., Nair, R. M., Siddique, K. H. M., et al. (2019). GABA (γ -aminobutyric acid), as a thermo-protectant, to improve the reproductive function of heat-stressed mungbean plants. *Sci. Rep.* 9, 1–14. doi: 10.1038/s41598-019-44163-w
- Przedpelska-Wasowicz, E. M., and Wierzbicka, M. (2011). Gating of aquaporins by heavy metals in *Allium cepa* L. epidermal cells. *Protoplasma* 248, 663–671. doi: 10.1007/s00709-010-0222-9
- Quiroga, G., Erice, G., Aroca, R., Chaumont, F., and Ruiz-Lozano, J. M. (2019a). Contribution of the arbuscular mycorrhizal symbiosis to the regulation of radial root water transport in maize plants under water deficit. *Environ. Exp. Bot.* 167, 103–821. doi: 10.1016/j.envexpbot.2019.103821
- Quiroga, G., Erice, G., Aroca, R., Zamarreño, Á. M., García-Mina, J. M., and Ruiz-Lozano, J. M. (2020). Radial water transport in arbuscular mycorrhizal maize plants under drought stress conditions is affected by indole-acetic acid (IAA) application. *J. Plant Physiol.* 246:153115. doi: 10.1016/j.jplph.2020.153115
- Quiroga, G., Erice, G., Ding, L., Chaumont, F., Aroca, R., and Ruiz-Lozano, J. M. (2019b). The arbuscular mycorrhizal symbiosis regulates aquaporins activity and improves root cell water permeability in maize plants subjected to water stress. *Plant Cell Environ.* 42, 2274–2290. doi: 10.1111/pce.13551
- Radhakrishnan, R., and Lee, I. J. (2013a). Regulation of salicylic acid, jasmonic acid and fatty acids in cucumber (*Cucumis sativus* L.) by spermidine promotes plant growth against salt stress. *Acta Physiol. Plant* 35, 3315–3322. doi: 10.1007/s11738-013-1364-0
- Radhakrishnan, R., and Lee, I. J. (2013b). Spermine promotes acclimation to osmotic stress by modifying antioxidant, abscisic acid, and jasmonic acid signals in soybean. *J. Plant Growth Regul.* 32, 22–30. doi: 10.1007/s00344-012-9274-8
- Rady, M. M., and Hemida, K. A. (2015). Modulation of cadmium toxicity and enhancing cadmium-tolerance in wheat seedlings by exogenous application of polyamines. *Ecotoxicol. Environ. Saf.* 119, 178–185. doi: 10.1016/j.ecoenv.2015.05.008
- Rangan, P., Subramani, R., Kumar, R., Singh, A. K., and Singh, R. (2014). Recent advances in polyamine metabolism and abiotic stress tolerance. *BioMed Res. Int.* 2014:239621. doi: 10.1155/2014/239621
- Ren, C. G., Kong, C. C., and Xie, Z. H. (2018). Role of abscisic acid in strigolactone-induced salt stress tolerance in arbuscular mycorrhizal *Sesbania cannabina* seedlings. *BMC Plant Biol.* 18:74. doi: 10.1186/s12870-018-1292-7
- Rezvanpour, S., Hatamzadeh, A., Elahinia, S. A., and Asghari, H. R. (2015). Exogenous polyamines improve mycorrhizal development and growth and flowering of *Freesia hybrida*. *J. Hortic. Res.* 23, 17–25. doi: 10.2478/johr-2015-0013
- Roser, M., Ritchie, H., and Ortiz-Ospina, E. (2013). *World Population Growth. Our World in Data*. Available online at: <https://ourworldindata.org/world-population-growth>
- Roussel, H., Bruns, S., Gianinazzi-Pearson, V., Hahlbrock, K., and Franken, P. (1997). Induction of a membrane intrinsic protein-encoding mRNA in arbuscular mycorrhiza and elicitor-stimulated cell suspension cultures of parsley. *Plant Sci.* 126, 203–210. doi: 10.1016/S0168-9452(97)00106-4
- Rucińska-Sobkowiak, R. (2016). Water relations in plants subjected to heavy metal stresses. *Acta Physiol. Plant* 38:257. doi: 10.1007/s11738-016-2277-5
- Ruiz-Lozano, J. M., and Aroca, R. (2010). “Host response to osmotic stresses: stomatal behaviour and water use efficiency of arbuscular mycorrhizal plants,” in *Arbuscular Mycorrhizas: Physiology and Function*, eds H. Koltai, and Y. Kapulnik (Dordrecht: Springer), 239–256. doi: 10.1007/978-90-481-9489-6_11
- Ruiz-Lozano, J. M., and Aroca, R. (2017). “Plant aquaporins and mycorrhizae: their regulation and involvement in plant physiology and performance,” in *Plant Aquaporins. Signaling and Communication in Plants*, eds F. Chaumont, and S. Tyerman (Cham: Springer), 333–353. doi: 10.1007/978-3-319-49395-4_15
- Ruiz-Lozano, J. M., del Mar Alguacil, M., Bárzana, G., Vernieri, P., and Aroca, R. (2009). Exogenous ABA accentuates the differences in root hydraulic properties between mycorrhizal and non mycorrhizal maize plants through regulation of PIP aquaporins. *Plant Mol. Biol.* 70:65. doi: 10.1007/s11103-009-9492-z
- Sade, D., Brotman, Y., Eybishtz, A., Cuadros-Inostroza, Á., Fernie, A. R., Willmitzer, L., et al. (2013). Involvement of the hexose transporter gene *LeHT1* and of sugars in resistance of tomato to tomato yellow leaf curl virus. *Mol. Plant* 6, 1707–1710. doi: 10.1093/mp/sst036
- Sannazzaro, A. I., Echeverría, M., Albertó, E. O., Ruiz, O. A., and Menéndez, A. B. (2007). Modulation of polyamine balance in *Lotus glaber* by salinity and arbuscular mycorrhiza. *Plant Physiol. Biochem.* 45, 39–46. doi: 10.1016/j.plaphy.2006.12.008
- Santander, C., Aroca, R., Cartes, P., Vidal, G., and Cornejo, P. (2021). Aquaporins and cation transporters are differentially regulated by two arbuscular mycorrhizal fungi strains in lettuce cultivars growing under salinity conditions. *Plant Physiol. Biochem.* 158, 396–409. doi: 10.1016/j.plaphy.2020.11.025
- Saroy, K., and Garg, N. (2021). Relative effectiveness of arbuscular mycorrhiza and polyamines in modulating ROS generation and ascorbate-glutathione cycle in *Cajanus cajan* under nickel stress. *ESPR* 1–18. doi: 10.1007/s11356-021-13878-7
- Schnurbusch, T., Hayes, J., Hrmova, M., Baumann, U., Ramesh, S. A., Tyerman, S. D., et al. (2010). Boron toxicity tolerance in barley through reduced expression of the multifunctional aquaporin HvNIP2; 1. *Plant Physiol.* 153, 1706–1715. doi: 10.1104/pp.110.158832
- Sequera-Mutiozabal, M., Antoniou, C., Tiburcio, A. F., Alcázar, R., and Fotopoulos, V. (2017). Polyamines: emerging hubs promoting drought and salt stress tolerance in plants. *Curr. Mol. Biol. Rep.* 3, 28–36. doi: 10.1007/s40610-017-0052-z
- Serafini-Fracassini, D., Torrigiani, P., and Branca, C. (1984). Polyamines bound to nucleic acids during dormancy and activation of tuber cells of *Helianthus tuberosus*. *Physiol. Plant* 60, 351–357. doi: 10.1111/j.1399-3054.1984.tb06075.x
- Shahid, M. A., Balal, R. M., Khan, N., Rossi, L., Rathinasabapathi, B., Liu, G., et al. (2018). Polyamines provide new insights into the biochemical basis of Cr-tolerance in Kinnow mandarin grafted on diploid and double-diploid rootstocks. *Environ. Exp. Bot.* 156, 248–260. doi: 10.1016/j.envexpbot.2018.09.015
- Sharma, A., Shahzad, B., Kumar, V., Kohli, S. K., Sidhu, G. P. S., and Bali, A. S. (2019). Phytohormones regulate accumulation of osmolytes under abiotic stress. *Biomolecules* 9, 285–297. doi: 10.3390/biom9070285
- Sharma, S., Anand, G., Singh, N., and Kapoor, R. (2017). Arbuscular mycorrhiza augments arsenic tolerance in wheat (*Triticum aestivum* L.) by strengthening antioxidant defense system and thiol metabolism. *Front. Plant Sci.* 8:906. doi: 10.3389/fpls.2017.00906
- Shaul-Keinan, O., Gadkar, V., Grünzweig, J. M., Chet, I., Elad, Y., et al. (2002). Hormone concentrations in tobacco roots change during arbuscular mycorrhizal colonization with *Glomus intraradices*. *New Phytol.* 1542, 501–507. doi: 10.1046/j.1469-8137.2002.00388.x
- Shekoofeh, E., Sepideh, H., and Roya, R. (2012). Role of mycorrhizal fungi and salicylic acid in salinity tolerance of *Ocimum basilicum* resistance to salinity. *Afr. J. Biotechnol.* 11, 2223–2235. doi: 10.5897/AJB11.1672
- Shelp, B. J., Bozzo, G. G., Trobacher, C. P., Chiu, G., and Bajwa, V. S. (2012). Strategies and tools for studying the metabolism and function of γ -aminobutyrate in plants. I. Pathway structure. *Botany* 90, 651–668. doi: 10.1139/b2012-030
- Sheng, M., Tang, M., Chen, H., Yang, B., Zhang, F., and Huang, Y. (2008). Influence of arbuscular mycorrhizae on photosynthesis and water status of maize plants under salt stress. *Mycorrhiza* 18, 287–296. doi: 10.1007/s00572-008-0180-7
- Siemens, J. A., and Zwiazek, J. J. (2004). Changes in root water flow properties of solution culture grown trembling aspen (*Populus tremuloides*) seedlings under different intensities of water-deficit stress. *Physiol. Plant* 121, 44–49. doi: 10.1111/j.0031-9317.2004.00291.x
- Singh, P., Basu, S., and Kumar, G. (2018). “Polyamines metabolism: a way ahead for abiotic stress tolerance in crop plants,” in *Biochemical, Physiological and Molecular Avenues for Combating Abiotic Stress Tolerance in Plants* (Elsevier).
- Singh, S., Parihar, P., Singh, R., Singh, V. P., and Prasad, S. M. (2016). Heavy metal tolerance in plants: role of transcriptomics, proteomics, metabolomics, and ionomics. *Front. Plant Sci.* 6:1143. doi: 10.3389/fpls.2015.01143
- Slocum, R. D., Kaur-Sawhney, R., and Galston, A. W. (1984). The physiology and biochemistry of polyamines in higher plants. *Arch. Biochem. Biophys.* 235, 283–303. doi: 10.1016/0003-9861(84)90201-7
- Smith, S. E., and Read, D. J. (2008). *Mycorrhizal Symbiosis*. San Diego, CA: Academic Press.
- Sonah, H., Deshmukh, R. K., Labbé, C., and Bélanger, R. R. (2017). Analysis of aquaporins in Brassicaceae species reveals high-level of conservation and

- dynamic role against biotic and abiotic stress in canola. *Sci. Rep.* 7, 1–17. doi: 10.1038/s41598-017-02877-9
- Souza, L. A., Camargos, L. S., Schiavinato, M. A., and Andrade, S. A. L. (2014). Mycorrhization alters foliar soluble amino acid composition and influences tolerance to Pb in *Calopogonium mucunoides*. *Theor. Exp. Plant Physiol.* 26, 211–216. doi: 10.1007/s40626-014-0019-x
- Srivastava, S., Srivastava, A. K., Suprasanna, P., and D'Souza, S. F. (2013). Quantitative real-time expression profiling of aquaporins-isoforms and growth response of *Brassica juncea* under arsenite stress. *Mol. Biol. Rep.* 40, 2879–2886. doi: 10.1007/s11033-012-2303-7
- Taie, H. A., El-Yazal, M. A. S., Ahmed, S. M., and Rady, M. M. (2019). Polyamines modulate growth, antioxidant activity, and genomic DNA in heavy metal-stressed wheat plant. *Environ. Sci. Pollut. Res.* 26, 22338–22350. doi: 10.1007/s11356-019-05555-7
- Taylor, A., and Bhatla, S. C. (2021). Polyamine homeostasis modulates plasma membrane and tonoplast-associated aquaporin expression in etiolated salt-stressed sunflower (*Helianthus annuus* L.) seedlings. *Protoplasma* 258, 661–672. doi: 10.1007/s00709-020-01589-8
- Taylor, A., Tandon, R., and Bhatla, S. C. (2019). Nitric oxide modulates polyamine homeostasis in sunflower seedling cotyledons under salt stress. *Plant Signal. Behav.* 14:1667730. doi: 10.1080/15592324.2019.1667730
- Tajti, J., Janda, T., Majláth, I., Szalai, G., and Pál, M. (2018). Comparative study on the effects of putrescine and spermidine pre-treatment on cadmium stress in wheat. *Ecotoxicol. Environ. Saf.* 148, 546–554. doi: 10.1016/j.ecoenv.2017.10.068
- Talaat, N. B., and Shawky, B. T. (2013). Modulation of nutrient acquisition and polyamine pool in salt-stressed wheat (*Triticum aestivum* L.) plants inoculated with arbuscular mycorrhizal fungi. *Acta Physiol. Plant* 3, 2601–2610. doi: 10.1007/s11738-013-1295-9
- Tseng, T. Y., Ou, J. F., and Wang, C. Y. (2013). Role of the ascorbate-glutathione cycle in paraquat tolerance of rice. *Weed Sci.* 61, 361–373. doi: 10.1614/WS-D-12-00133.1
- Tun, N. N., Santa-Catarina, C., Begum, T., Silveira, V., Handro, W., Floh, E. I. S., et al. (2006). Polyamines induce rapid biosynthesis of nitric oxide (NO) in *Arabidopsis thaliana* seedlings. *Plant Cell Physiol.* 47, 346–354. doi: 10.1093/pcp/pci252
- Uehlein, N., Fileschi, K., Eckert, M., Bienert, G. P., Bertl, A., and Kaldenhoff, R. (2007). Arbuscular mycorrhizal symbiosis and plant aquaporin expression. *Phytochemistry* 68, 122–129. doi: 10.1016/j.phytochem.2006.09.033
- Uehlein, N., Lovisolo, C., Siefritz, F., and Kaldenhoff, R. (2003). The tobacco aquaporin NtAQP1 is a membrane CO₂ pore with physiological functions. *Nature* 425, 734–737. doi: 10.1038/nature02027
- Urano, K., Yoshida, Y., Nanjo, T., Igarashi, Y., Seki, M., Sekiguchi, F., et al. (2003). Characterization of *Arabidopsis* genes involved in biosynthesis of polyamines in abiotic stress responses and developmental stages. *Plant Cell Environ.* 26, 1917–1926. doi: 10.1046/j.1365-3040.2003.01108.x
- Valot, B., Dieu, M., Recorbet, G., Raes, M., Gianinazzi, S., and Dumas-Gaudot, E. (2005). Identification of membrane-associated proteins regulated by the arbuscular mycorrhizal symbiosis. *Plant Mol. Biol.* 59, 565–580. doi: 10.1007/s11103-005-8269-2
- Vats, S., Sudhakaran, S., Bhardwaj, A., Mandlik, R., Sharma, Y., Kumar, S., et al. (2021). Targeting aquaporins to alleviate hazardous metal (loid) s imposed stress in plants. *J. Hazard. Mater.* 408:124910. doi: 10.1016/j.jhazmat.2020.124910
- Wang, B., and Qiu, Y. L. (2006). Phylogenetic distribution and evolution of mycorrhizas in land plants. *Mycorrhiza* 16, 299–363. doi: 10.1007/s00572-005-0033-6
- Wang, D., Li, L., Xu, Y., Limwachiranon, J., Li, D., Ban, Z., et al. (2017). Effect of exogenous nitro oxide on chilling tolerance, polyamine, proline, and γ -aminobutyric acid in Bamboo Shoots (*Phyllostachys praecox* f. *prevernalis*). *J. Agric. Food Chem.* 65, 5607–5613. doi: 10.1021/acs.jafc.7b02091
- Wen, X. P., Pang, X. M., Matsuda, N., Kita, M., Inoue, H., Hao, Y. J., et al. (2008). Over-expression of the apple spermidine synthase gene in pear confers multiple abiotic stress tolerance by altering polyamine titers. *Transgenic Res.* 17, 251–263. doi: 10.1007/s11248-007-9098-7
- Wu, J., Shu, S., Li, C., Sun, J., and Guo, S. (2018). Spermidine-mediated hydrogen peroxide signaling enhances the antioxidant capacity of salt-stressed cucumber roots. *Plant Physiol. Biochem.* 128, 152–162. doi: 10.1016/j.plaphy.2018.05.002
- Wu, Q. S., He, X. H., Zou, Y. N., Liu, C. Y., Xiao, J., and Li, Y. (2012b). Arbuscular mycorrhizas alter root system architecture of *Citrus tangerine* through regulating metabolism of endogenous polyamines. *Plant Growth Regul.* 68, 27–35. doi: 10.1007/s10725-012-9690-6
- Wu, Q. S., Peng, Y. H., Zou, Y. N., and Liu, C. Y. (2010). Exogenous polyamines affect mycorrhizal development of *Glomus mosseae*-colonized citrus (*Citrus tangerine*) seedlings. *Sci. Asia* 36, 254–258. doi: 10.2306/scienceasia1513-1874.2010.36.254
- Wu, Q. S., Srivastava, A. K., and Zou, Y. N. (2013). AMF-induced tolerance to drought stress in citrus: a review. *Sci. Hortic.* 164, 77–87. doi: 10.1016/j.scienta.2013.09.010
- Wu, Q. S., Zou, Y. N., Liu, C. Y., and Ting, L. U. (2012a). Interacted effect of arbuscular mycorrhizal fungi and polyamines on root system architecture of citrus seedlings. *J. Integr. Agric.* 11, 1675–1681. doi: 10.1016/S2095-3119(12)60170-1
- Xu, W., Dai, W., Yan, H., Li, S., Shen, H., Chen, Y., et al. (2015). Arabidopsis NIP3; 1 plays an important role in arsenic uptake and root-to-shoot translocation under arsenite stress conditions. *Mol. Plant* 8, 722–733. doi: 10.1016/j.molp.2015.01.005
- Yamaji, N., and Ma, J. F. (2007). Spatial distribution and temporal variation of the rice silicon transporter Lsi1. *Plant Physiol.* 143, 1306–1313. doi: 10.1104/pp.106.093005
- Yamasaki, H., and Cohen, M. F. (2006). NO signal at the crossroads: polyamine-induced nitric oxide synthesis in plants? *Trends Plant Sci.* 11, 522–524. doi: 10.1016/j.tplants.2006.09.009
- Yang, H., Shi, G., Wang, H., and Xu, Q. (2010). Involvement of polyamines in adaptation of *Potamogeton crispus* L. to cadmium stress. *Aquat. Toxicol.* 100, 282–288. doi: 10.1016/j.aquatox.2010.07.026
- Yasmeen, T., Tariq, M., Iqbal, S., Arif, M. S., Riaz, M., Shahzad, S. M., et al. (2019). “Ameliorative capability of plant growth promoting rhizobacteria (PGPR) and arbuscular mycorrhizal fungi (AMF) against salt stress in plant,” in *Plant Abiotic Stress Tolerance*, eds M. M. Hasanuzzaman, K. Hakeem, K. Nahar, and H. Alharby (Cham: Springer), 409–448. doi: 10.1007/978-3-030-06118-0_17
- Yong, Z., Liyuan, X., Bingquan, X., Ming, Z., Jianfu, L., Dong, Y., et al. (2003). Effect of polyamine on growth and development of arbuscular mycorrhizal fungi in vitro culture condition. *Mycosystema* 22, 417–423.
- Zapata, P. J., Serrano, M., Pretel, M. T., Amorós, A., and Botella, M. Á. (2004). Polyamines and ethylene changes during germination of different plant species under salinity. *Plant Sci.* 167, 781–788. doi: 10.1016/j.plantsci.2004.05.014
- Zhang, F., Zou, Y. N., Wu, Q. S., and Kuča, K. (2020). Arbuscular mycorrhizas modulate root polyamine metabolism to enhance drought tolerance of trifoliate orange. *Environ. Exp. Bot.* 171:103926. doi: 10.1016/j.envexpbot.2019.103926
- Zhao, X. Q., Mitani, N., Yamaji, N., Shen, R. F., and Ma, J. F. (2010). Involvement of silicon influx transporter OsNIP2; 1 in selenite uptake in rice. *Plant Physiol.* 153, 1871–1877. doi: 10.1104/pp.110.157867
- Zou, Y. N., Wu, H. H., Giri, B., Wu, Q. S., and Kuča, K. (2019). Mycorrhizal symbiosis down-regulates or does not change root aquaporin expression in trifoliate orange under drought stress. *Plant Physiol. Biochem.* 144, 292–299. doi: 10.1016/j.plaphy.2019.10.001
- Zou, Y. N., Zhang, F., Srivastava, A. K., Wu, Q. S., and Kuča, K. (2021). Arbuscular mycorrhizal fungi regulate polyamine homeostasis in roots of trifoliate orange for improved adaptation to soil moisture deficit stress. *Front. Plant Sci.* 11, 2046–2059. doi: 10.3389/fpls.2020.600792

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.

Copyright © 2021 Sharma, Gupta, Thokchom, Jangir and Kapoor. This is an open-access article distributed under the terms of the Creative Commons Attribution License (CC BY). The use, distribution or reproduction in other forums is permitted, provided the original author(s) and the copyright owner(s) are credited and that the original publication in this journal is cited, in accordance with accepted academic practice. No use, distribution or reproduction is permitted which does not comply with these terms.

GLOSSARY

ABA, Absciscic Acid; ADC, Arginine Decarboxylase; As, Arsenic; Al, Aluminum; AM, Arbuscular Mycorrhiza; AMF, Arbuscular Mycorrhizal Fungi; APOX, Ascorbate Peroxidase; AQPs, Aquaporins; C, Carbon; Cad, Cadaverine; Cd, Cadmium; CHA, Cyclohexylamine; Ci, Intracellular CO₂ Concentration; Cl⁻, Chloride Ion; cNMP, cyclic Nucleotide Monophosphate; Co, Cobalt; CO₂, Carbon Dioxide; Cr, Chromium; Cu, Copper; CuAO, Copper Amine Oxidase; DAO, Diamine Oxidases; DFMA, α -Difluoromethyl Arginine; DFMO, α -Difluoromethyl Ornithine; DHAR, Dehydroascorbate Reductase; DNA, Deoxyribonucleic Acid; ER, Endoplasmic Reticulum; Fe, Iron; GA, Gibberellic Acid; GABA, Gamma-Aminobutyric Acid; GABAT, Gamma-Aminobutyric Acid Transaminase; Gs, Stomatal Conductance; GSH, Glutathione Reduced; GSSH, Glutathione Oxidized; H₂O, Water; H₂O₂, Hydrogen Peroxide; Hg, Mercury; HMs, Heavy Metals; *HMT*, Heavy Metal Tolerance Gene; J_v, Sap Flow Rate; KIRC, Inward Rectifying K⁺ Channels; K_L, Leaf-Specific Conductivity; L_h, Hydrostatic

Root Hydraulic Conductance; L_o, Root Hydraulic Conductance; L_t, Leaf Temperature; MDHAR, Monodehydroascorbate Reductase; MIPs, Major Intrinsic Proteins; Mn, Manganese; Mo, Molybdenum; MT, Metallothionien; MVBs, Multivesicular Bodies; N, Nitrogen; Na⁺, Sodium Ion; NaCl, Sodium Chloride; *NCED: 9-cis-epoxycarotenoid dioxygenase*; NH₂, Amine Group; NH₃, Ammonia; Ni, Nickel; NIPs, Nodulin-26 like Intrinsic Proteins; NO, Nitric Oxide; ODC, Ornithine Decarboxylase; PAO, Polyamine Oxidase; PAs, Polyamines; Pb, Lead; PCs, Phytochelatins; PGPRs, Plant Growth-Promoting Rhizobacteria; PIPs, Plasma Membrane Intrinsic Proteins; POP, Persistent Organic Pollutants; Put, Putrescine; RNA, Ribonucleic Acid; RWC, Relative Water Content; SA, Salicylic Acid; SAM, S-Adenosyl Methionine; SAMDC, SAM Decarboxylase; SAR, Sodium Adsorption Ratio; SIPs, Small Basic Intrinsic Protein; Si, Silicon; Spd, Spermidine; SPDS, Spermidine Synthase; Spm, Spermine; SPMS, Spermine Synthase; TCA, Tricarboxylic Acid Cycle; TGN, Trans-Golgi Network; TIPs, Tonoplast Intrinsic Proteins; WUE, Water-Use Efficiency; Zn, Zinc; Ψ , Water Potential.



Mycorrhizal-Assisted Phytoremediation and Intercropping Strategies Improved the Health of Contaminated Soil in a Peri-Urban Area

María Teresa Gómez-Sagasti^{1*}, Carlos Garbisu², Julen Urrea², Fátima Míguez¹, Unai Artetxe¹, Antonio Hernández¹, Juan Vilela³, Itziar Alkorta⁴ and José M. Becerril¹

OPEN ACCESS

Edited by:

Juan Manuel Ruiz-Lozano,
Consejo Superior de Investigaciones
Científicas (CSIC), Spain

Reviewed by:

Engracia Madejon,
Institute of Natural Resources
and Agrobiological of Seville (CSIC),
Spain
Bhoopander Giri,
University of Delhi, India

*Correspondence:

María Teresa Gómez-Sagasti
maria.teresa.gomez@ehu.eus

Specialty section:

This article was submitted to
Plant Abiotic Stress,
a section of the journal
Frontiers in Plant Science

Received: 09 April 2021

Accepted: 19 May 2021

Published: 02 July 2021

Citation:

Gómez-Sagasti MT, Garbisu C,
Urrea J, Míguez F, Artetxe U,
Hernández A, Vilela J, Alkorta I and
Becerril JM (2021)
Mycorrhizal-Assisted
Phytoremediation and Intercropping
Strategies Improved the Health
of Contaminated Soil in a Peri-Urban
Area. *Front. Plant Sci.* 12:693044.
doi: 10.3389/fpls.2021.693044

Soils of abandoned and vacant lands in the periphery of cities are frequently subjected to illegal dumping and can undergo degradation processes such as depletion of organic matter and nutrients, reduced biodiversity, and the presence of contaminants, which may exert an intense abiotic stress on biological communities. Mycorrhizal-assisted phytoremediation and intercropping strategies are highly suitable options for remediation of these sites. A two-year field experiment was conducted at a peri-urban site contaminated with petroleum hydrocarbons and polychlorinated biphenyls, to assess the effects of plant growth (spontaneous plant species, *Medicago sativa*, and *Populus × canadensis*, alone vs. intercropped) and inoculation of a commercial arbuscular mycorrhizal and ectomycorrhizal inoculum. Contaminant degradation, plant performance, and biodiversity, as well as a variety of microbial indicators of soil health (microbial biomass, activity, and diversity parameters) were determined. The rhizosphere bacterial and fungal microbiomes were assessed by measuring the structural diversity and composition via amplicon sequencing. Establishment of spontaneous vegetation led to greater plant and soil microbial diversity. Intercropping enhanced the activity of soil enzymes involved in nutrient cycling. The mycorrhizal treatment was a key contributor to the establishment of intercropping with poplar and alfalfa. Inoculated and poplar-alfalfa intercropped soils had a higher microbial abundance than soils colonized by spontaneous vegetation. Our study provided evidence of the potential of mycorrhizal-assisted phytoremediation and intercropping strategies to improve soil health in degraded peri-urban areas.

Keywords: alfalfa, poplar, mycorrhizal inoculation, organic contaminants, plant diversity, soil microbial properties

INTRODUCTION

Abandonment of agricultural and industrial sites in the periphery of many cities often results in the presence of randomly distributed vacant areas that are highly vulnerable and susceptible to uncontrolled deposition of wastes, thus contributing to soil degradation and contamination (Németh and Langhorst, 2014; Gómez-Sagasti et al., 2018; Míguez et al., 2020). Properly managed, these abandoned sites not only can be a valuable ecological resource, for instance by providing ecosystem services (Nassauer and Raskin, 2014; Herrmann et al., 2016) and harboring biodiversity (Delgado de la Flor et al., 2020; Perry et al., 2020), but can also afford an opportunity for land redevelopment and hence economic and social benefits (Kim et al., 2018). Implementation of sustainable, cost-efficient *in-situ* interventions, such as phytoremediation strategies, can improve soil health and thus contribute to the redevelopment of degraded peri-urban vacant lands (Míguez et al., 2020).

Phytoremediation is a gentle remediation option (Kidd et al., 2015) that uses plants and associated microbes to restore soils contaminated with inorganic and/or organic compounds. A variety of characteristics make phytoremediation an attractive alternative to conventional physicochemical methods of soil remediation: low capital and maintenance costs, easy start-up, non-invasiveness, environmentally friendly character, high public acceptance, etc. (Alkorta et al., 2010). The success of phytoremediation depends on the ability of certain plant species, together with their associated rhizosphere microorganisms, to stabilize (phytostabilization) and/or extract (phytoextraction) inorganic (metal) contaminants and/or degrade organic compounds (rhizoremediation). Thus, phytoremediation can gradually alleviate the potential environmental risks caused by contaminants and improve soil health (Vangronsveld et al., 2009).

Nitrogen-fixing herbaceous species such as alfalfa (*Medicago sativa* L.) and fast-growing trees such as poplar (*Populus* L.) are highly suitable candidates for phytoremediation initiatives. Alfalfa, an important perennial forage crop, has been widely used for phytoremediation of soils contaminated with metals (Bonfranceschi et al., 2009), petroleum hydrocarbons (Phillips et al., 2009; Panchenko et al., 2017), and aromatic hydrocarbons (Fan et al., 2008; Teng et al., 2011; Marchand et al., 2016). This leguminous plant species has many advantages for phytoremediation purposes, such as a deep root system favorable for the establishment of rhizosphere microorganisms (Kirk et al., 2005), high yield, and drought tolerance (Zhang M. et al., 2019). Furthermore, alfalfa requires little maintenance (it does not need annual plowing and sowing) and can fix up to 230 kg N ha⁻¹ yr⁻¹ (Tisdale and Nelson, 1974). Because of their high biomass production rates, poplars are increasingly chosen for reforestation of abandoned agricultural lands, revegetation of areas that have been degraded by industrial contamination (Tognetti et al., 2013; Foulon et al., 2016), and phytoremediation and phytomanagement strategies (Ciadamidaro et al., 2017). Many poplars are pioneer species, a trait that is inherently linked to their fast growth rate and deep root system (Hu et al., 2019). As with alfalfa, poplars have been extensively used for

phytoremediation of metal- and hydrocarbon-contaminated soils (Lingua et al., 2008).

Although most phytoremediation studies have been conducted using a single plant species, the potential economic and environmental benefits of intercropping with trees and herbaceous species are well established (Taghiyari and Sisi, 2012). In particular, a poplar-alfalfa intercropping system has high potential for *in-situ* phytoremediation of contaminated soils.

The low availability of nutrients and contaminants in soil, as well as the unacceptably long time period required for effective remediation, are often the most important limitations of phytoremediation in the field. Two soil-management practices (used singly or in combination) that can assist phytoremediation processes are application of organic amendments (Gómez-Sagasti et al., 2018; Míguez et al., 2020) and inoculation of microorganisms such as mycorrhizal fungi, which can form symbiotic associations with most land plants (Miransari, 2011). Under natural conditions, *Populus* species and hybrids have the unique property of being colonized by both endo- and ectomycorrhizal (arbuscular) fungi, making them unique model systems for study of the interactions between plants and microorganisms (Martin et al., 2004; Karliński et al., 2010). In addition to improving plant mineral nutrition and health, mycorrhizal interactions are involved in protecting plants against soil-borne stresses (Szuba, 2015).

The objective of remediation processes must be not only to remove contaminants (and their associated risks) from the soil, but to recover soil health (Epelde et al., 2009; Gómez-Sagasti et al., 2012). Reliable and sensitive indicators of soil health are needed to properly assess the efficiency of phytoremediation processes. Besides the traditional physicochemical indicators, biological indicators of soil health (including plant, animal, and microbial parameters) are widely used to evaluate the effectiveness of phytoremediation processes (Burgess et al., 2016; Lacalle et al., 2018; Anza et al., 2019).

This study investigated the benefits of two phytoremediation strategies for the physicochemical and biological properties of a contaminated peri-urban vacant soil: (i) intercropping of alfalfa with hybrid black poplar vs. monocultures of each species; and (ii) inoculation with a commercial mycorrhizal inoculum vs. non-inoculation. Treatment effects were evaluated in terms of the degradation of soil organic contaminants, plant performance (i.e., plant diversity, plant growth, and ecophysiological traits), and the status of soil microbial communities (i.e., microbial biomass, activity, and functional and structural diversity). We hypothesized that the selected treatments (mycorrhizal-assisted phytoremediation and intercropping) would enhance plant performance and improve the health of a contaminated soil in a peri-urban area.

MATERIALS AND METHODS

Site Description and Experimental Design

A field experiment was conducted on abandoned vacant land in the Júndiz Industrial Park, located in the peri-urban belt

of Vitoria-Gasteiz (42°50'N; 2°40'W, northern Spain, elevation 508 m a.s.l.). This area has a temperate Mediterranean climate with dry summers, cold winters, and a mean annual rainfall of 700–800 mm. Prior to the experiment, the soil surface was cleared by removing construction and demolition waste and other inert residues that were illegally dumped. Then, the soil was thoroughly mixed and leveled with a backhoe loader. Physicochemical analyses showed that the soil was slightly alkaline (pH > 8.5) and loamy, with low contents of topsoil organic matter (1.0%) and major nutrients (0.1% N DW⁻¹ soil, 7.2 mg available P kg⁻¹ DW soil, 85 mg available K⁺ kg⁻¹ DW soil). Because of these low organic matter (OM) and nutrient contents, the soil was amended with compost (75 t ha⁻¹) in order to facilitate plant growth and stimulate soil microbial activity. The compost was acquired from BIOCUMPOST de Álava U.T.E., a municipal solid-waste treatment plant located near the study area. The compost application rate was established following the recommendations regarding water content, OM content, C:N ratio, and metal contents of the Spanish legislation for organic fertilizers (Royal Decree 999/2017 on inorganic and organic fertilizers, transposition of EU Regulation No. 2003/2003) (Government of Spain, 2017). We used the recommended rate of application of a previous study performed in this area (Míguez et al., 2020). In this way, we ensure not only immediate correction of soil organic matter content (it was raised from 1 to 8.6%), but also its long-term positive effect without the need to intervene again and destroy the herbaceous community that could be potentially established. The compost was carefully mixed into the topsoil (0–30 cm depth), using a rotary tiller. The physicochemical characteristics of the compost-amended soil are detailed in **Table 1**. The compost amendment did not alter the soil pH but markedly increased N, P and K content, as expected.

In March 2017, one month after application of the compost, the following 6 treatments were performed in triplicate (18

plots of 5 m × 5 m): (1) no planting (control plot), where we followed the development of spontaneous vegetation (v); (2) alfalfa (*Medicago sativa*) (a); (3) non-inoculated hybrid black poplar (*Populus × canadensis*, hereafter “poplar”) (Pv); (4) poplar inoculated with a commercial mycorrhizal inoculum (Piv); (5) non-inoculated poplar intercropped with alfalfa (Pa); and (6) inoculated poplar intercropped with alfalfa (Pia). Alfalfa (ecotype “Tierra de Campos”) was sown in the corresponding plots at a seed rate of 20 kg ha⁻¹. Unrooted poplar cuttings, provided by Biopoplar s.r.l.¹ were rooted in a greenhouse prior to transplantation in the field. The rooted cuttings were planted at a spacing of 1.5 m × 1.0 m. The commercial mycorrhizal inoculum (Micoplus, bioFlower Products) consisted of a consortium of ectomycorrhizae (*Rhizopogon* spp., *Scleroderma* spp., and *Pisolithus tinctorius* at 2,106 spores g⁻¹) and endomycorrhizae (*Glomus intraradices* and *Glomus mosseae* at > 60 infective propagules g⁻¹) attached to an inert medium bed. Following the manufacturer's instructions, the mycorrhizae were inoculated in the corresponding treatments as follows: (i) during the rooting period of poplar cuttings in the greenhouse; (ii) in the field, just after the rooted poplar cuttings were planted; and (iii) three and (iv) 12 months after the rooted poplar cuttings were planted. The use of standardized mycorrhizal inoculum allows a more reproducible inoculation over both spatial and temporal scales. The experiment was monitored in the first (July 2017) and second (July 2018) growing seasons. A drought event occurred in August 2017.

Determination of Soil Physicochemical Properties

In the first (July 2017) and second (2018) growing seasons, soil samples were collected from the top 20 cm, air-dried at room temperature, and passed through a 2-mm mesh. Physicochemical analyses of the soil samples were performed at the University of Santiago de Compostela (Spain), according to standard methods. The total metal concentrations in the soil samples were determined according to the microwave-heating EPA method 3050B for Inductively Coupled Plasma-Optical Emission Spectrophotometer (ICP-OES) analysis (U.S. Environmental Protection Agency (US-EPA), 1996). Soil samples for the determination of organic contaminants were taken just after implementation of the treatments (March 2017; t₀) and at the end of the experiment (July 2018; t_f). The concentrations of organic contaminants (i.e., total petroleum hydrocarbons, TPHs C10–C40; total polychlorinated biphenyls, PCBs; and polycyclic aromatic hydrocarbons, PAHs) were determined by a certified laboratory (SYNLAB Analytics & Services B.V., Rotterdam, Netherlands). Total C₁₀–C₄₀ hydrocarbon fractions were analyzed according to ISO 16703 (2004) standard using hexane/acetone extraction and Gas Chromatography-Flame Ionization Detector. The PCBs and PAHs contents were determined in accordance with an internal method accredited by the Dutch RvA (Raad voor Accreditatie) and ISO 18287 (2006) standard, respectively, followed by Gas Chromatography-Mass Spectrometry (GC-MS) detection.

¹www.biopoplar.com

TABLE 1 | Soil physicochemical properties.

Total clay (%)	36.6
Coarse sand (%)	6.8
Fine sand (%)	13.8
Total silt (%)	42.8
Texture class (USDA)	Clay loam
pH (1:2.5)	8.5
Organic matter (% DW)	8.6
Total N (% DW)	0.4
Organic C / Organic N	13.3
Available P (mg kg ⁻¹ DW)	223.5
Total K ⁺ (mg kg ⁻¹ DW)	3.6
Cadmium (mg kg ⁻¹ DW)	2.9
Copper (mg kg ⁻¹ DW)	44.8
Nickel (mg kg ⁻¹ DW)	19.5
Lead (mg kg ⁻¹ DW)	76.2
Chromium (mg kg ⁻¹ DW)	21.1
Zinc (mg kg ⁻¹ DW)	168.9
Cobalt (mg kg ⁻¹ DW)	14.1
Manganese (mg kg ⁻¹ DW)	176.6

Determination of Plant Parameters

Plant diversity, biomass, and ecophysiological parameters were determined 3 months after the treatment implementation (first growing season, July 2017) and at the end of the experiment (second growing season, July 2018).

For the study of plant diversity and biomass, three quadrats, each measuring 0.5 m × 0.5 m (0.25 m²) were placed at random in each plot. All vascular plant species within the quadrats were identified to species level. Then, shoots were removed and weighed (FW). Subsequently, the plants were dried at 40°C for 48 h and the dry mass (DW) was calculated.

Three fully expanded leaves from three poplar individuals in each replicate plot were sampled. The following biometric measurements were taken for these trees: (i) leaf dry weight (DW); (ii) specific leaf area (SLA), calculated as the coefficient between leaf area and DW; and (iii) total branch length, as the sum of the lengths of all branches. Leaves were maintained in the laboratory in 100% relative humidity, room temperature, and darkness for 12 h to mimic pre-dawn conditions. The maximum photochemical efficiency of PSII (F_v/F_m) was estimated using a portable fluorimeter (FluorPen FP100, Photon Systems Instruments). The maximum chlorophyll (Chl) fluorescence yield (F_m) was induced with a saturating pulse. The minimum (Chl) fluorescence (F_0) was recorded at low measuring light intensities. F_v/F_m was calculated as $(F_m - F_0)/F_m$ (García-Plazaola et al., 1999). The contents of chlorophylls, carotenoids, xanthophylls [violaxanthin (V), antheraxanthin (A), and zeaxanthin (Z)], and tocopherols were determined using the ultra-rapid UHPLC method, following Lacalle et al. (2020). Briefly, six discs (7.35 mm diameter) were collected from the youngest fully expanded leaf, frozen in liquid nitrogen, and stored at -80°C until analysis. The leaf discs were homogenized using a Tissue-Tearor in 1 mL pure acetone, the mixture was centrifuged at $13,200 \times g$ for 20 min, and 1.5 mL of the supernatant was filtered through a PTFE filter (0.2 µm) and analyzed using the AcquityTM UHPLC H-Class system (Waters®, Milford, MA, United States).

Determination of Soil Microbial Properties

In July 2017 and July 2018, soil samples were collected to determine the soil basal respiration (BR, indicator of soil microbial activity), substrate-induced respiration (SIR, indicator of potentially active microbial biomass), and bacterial functional diversity, as described by Epelde et al. (2008). Bacterial functional diversity was determined using Biolog EcoPlatesTM (Biolog Inc., Hayward, CA, United States), following Epelde et al. (2008). Average well color development (AWCD), Shannon's diversity index (H'), and substrate-consumption activity (SCA = area under the curve) at the mid-exponential growth phase (after 40 h of incubation) were calculated from the Biolog data.

At the end of the experiment (July 2018), soil samples were sieved to < 2 mm prior to the determination of total bacteria and total fungi (according to gene copy abundance values), soil enzyme activities, and microbial structural diversity, through amplicon sequencing.

For the quantification of total bacteria and fungi, DNA was extracted in triplicate from 0.25 g DW soil, using the Power Soil DNA Isolation Kit (MO Bio Laboratories, Carlsbad, CA, United States). Prior to DNA extraction, the soil samples were washed twice in 120 mM K₂HPO₄ (pH 8.0) to remove extracellular DNA (Kowalchuk et al., 1997). The amount of DNA in the samples was determined in a ND-1000 spectrophotometer (Thermo-Scientific, Wilmington, DE, United States). Real-time qPCR was carried out to determine total bacterial (16S rRNA) and fungal (internal transcribed spacer, ITS) gene-copy abundances for the estimation of total bacteria and fungi, respectively, according to Epelde et al. (2014).

The activities of the enzymes β-glucosidase (EC 3.2.1.21), β-glucosaminidase (EC 3.2.1.30), arylsulfatase (EC 3.1.6.8), acid phosphatase (EC 3.1.3.2), L-Ala-aminopeptidase (EC 3.4.11.12), and L-Leu-aminopeptidase (EC 3.4.11.1) were measured using fluorogenic substrates in microwell plates, following ISO/TS 22939 (International Organization for Standardization (ISO), 2010).

In order to assess the effect of treatments on the soil microbial structural diversity and composition, amplicon libraries were prepared using a dual indexing tag-tailed approach (D'Amore et al., 2016). The following sequence-specific primer pairs were used (Lanzén et al., 2016): 519F (CAGCMGCCGCGGTAA) from Øvreås et al. (1997) and 806R (GGACTACHVGGGTWTCTAAT) from Caporaso et al. (2012) for targeting the prokaryotic 16S rRNA hypervariable region V4; and ITS1F (CTTGGTCATTTAGAGGAAGTAA) and ITS2R (GCTGCGTTCTTCATCGATGC) (McGuire et al., 2013) for targeting the fungal ITS1 region. Libraries were sequenced by the Tecnalia Corporation (Miñano, Spain), using an Illumina MiSeq platform with the V2 kit and pair-ended 2 × 250 nt. The generated paired-end reads were merged, quality-filtered (i.e., primer trimming, removal of singletons and chimeric sequences), and grouped into operational taxonomic units (OTUs), following Lanzén et al. (2016). Finally, taxonomic assignments were made using CREST and SilvaMod v128 (Lanzén et al., 2012).

Statistical Analyses

All data are expressed as mean ± standard error ($n = 3$). First, data distribution and homoscedasticity were checked using the Kolmogorov-Smirnov and Levene tests, respectively. All original data showed normal distributions. Statistically significant effects ($p < 0.05$) of the treatments on plant and soil microbial (BR, SIR, bacterial functional diversity) parameters were determined using one-way analysis of variance (ANOVA). A three-way ANOVA was applied to examine the potential interactive effects of (1) time (2-year temporal evolution), (2) mycorrhizal inoculation, and (3) intercropping alfalfa-poplar on the above-mentioned parameters at a 0.05 level of significance. The statistical analysis was carried out using IBM SPSS Statistics for Windows (Version 24; IBM Corp., Armonk, NY, United States). Principal components analysis (PCA), executed with Canoco 5 software (ter Braak and Smilauer, 2012), was used to identify possible relationships among plant and soil microbial parameters.

The effects of the treatments on soil enzyme activities and total bacteria and fungi were analyzed by means of one-way ANOVA

and Duncan's multiple range test, using the agricolae package in the R software (Version 3.3.2). The values of all soil enzyme activities were used for the calculation of overall enzyme activity (OEA), following Epelde et al. (2014):

$$\text{OEA} = 10^{\log m} + \frac{\sum_{i=1}^n (\log n_i - \log m)}{n}$$

where m is the reference value (set to 100% for the mean value of each enzyme activity in the control soil) and n corresponds to the measured values for each enzyme activity as a percentage of the reference value. Soils from the plots where spontaneous vegetation was growing (v) were considered as controls. Significant differences in OEA values were analyzed by means of one-way ANOVA, with Duncan's post-hoc comparisons. In all tests, a p -value <0.05 was considered to be statistically significant.

Regarding the microbial structural diversity data, calculation of α -diversity indices (R richness: rarefied richness, H' : Shannon's index, J' : Pielou's evenness), multivariate statistics, and visualization of sequencing data from the 16S rRNA and ITS amplicon libraries were performed with the vegan package in the R software (Oksanen et al., 2015). Rarefied richness estimates, interpolating the expected richness at the lowest sample-specific sequencing depth, were used to compensate for variations in read numbers across samples (Lanzén et al., 2016). The function decostand was used to transform OTU distributions into relative abundances. Bray-Curtis dissimilarity matrices were calculated to compare the prokaryotic and fungal community composition between samples. These matrices were further used to perform non-metric multidimensional scaling (NMDS) with the function metaMDS. Permutational analyses of variance (PERMANOVA) were performed to assess the impacts of the treatments on the soil prokaryotic and fungal community composition, using the function adonis. Pairwise analyses of the relative abundances of each taxon at order level, representing more than 0.1% of the total reads for both prokaryotic and fungal communities, were performed by means of two-way ANOVA in order to detect significant differences among experimental factors.

RESULTS

Contaminant Degradation

The soil from the abandoned vacant land contained low concentrations of heavy metals (Cd, Cu, Zn, Pb) that did not exceed the Critical Reference Values for Ecosystem Protection established by Law 4/2015 of the Basque Country for agricultural settings (Government of the Basque Country, 2015). Nonetheless, although total metal concentrations did not exceed regulatory limits, we decided to quantify the effects of the treatments on these concentrations. At the end of the experiment, the concentrations of Cd, Cu, and Zn had decreased in all the treatments (75–88% reduction of Cd, 33–73% reduction of Cu, and 60–82% reduction of Zn), while the concentration of Pb was virtually unchanged (probably due to the very low mobility and availability of this metal in soil). The highest decrease in the total concentrations of the most mobile metals (Cd and Zn) in soil could be due to leaching and/or plant uptake.

In any case, total metal concentrations were very low for a contaminated soil. As indicated above, the soil was contaminated with TPHs, PCBs, and PAHs (Table 2). As is often the case in similar contaminated sites, this peri-urban vacant land had a highly heterogeneous distribution of soil contaminants among the plots. Therefore, in order to assess the effects of the treatments on contaminant degradation, we compared those plots with similar concentrations of contaminants just after treatment implementation (March 2017; t_0) vs. at the end of the experiment (July 2018; t_f).

At t_0 , TPHs were the most abundant contaminants in the soil, with concentrations ranging from 70–200 mg kg⁻¹, compared to PCBs (0–22 mg kg⁻¹) and PAHs (benzo[a]pyrene: 0.02–0.09 mg kg⁻¹; benzo[b]fluoranthene: 0.03–0.14 mg kg⁻¹) (Table 2). Overall, all these organic contaminants exceeded their respective Critical Reference Values established by the above-mentioned Basque legislation, and accordingly they must be remediated.

We did not observe phytotoxic symptoms caused by organic contaminants on plant communities developed in our studied plots, in comparison with the vegetation of non-contaminated sites within the study area. This could arise due to the low bioavailability of organic contaminants in soils resulted from decade-long aging processes. After the end of the experiment (t_f), alfalfa (a) proved to be the most effective treatment in terms of TPH and PAH degradation (Table 2). Mycorrhizal poplars, when planted alone, stimulated the degradation of TPHs, while non-mycorrhizal poplars enhanced PCB and PAH degradation. The low contaminant concentrations and, above all, their highly heterogeneous distribution in the soil hampered interpretation of the TPH degradation values. Likewise, the extremely low PAH concentrations (benzo[a]pyrene and benzo[b]fluoranthene) in

TABLE 2 | Concentration of soil organic contaminants.

Contaminant (mg kg ⁻¹)	Treatment					
	Sampling time	a	Pa	Pia	Pv	Piv v
TPH (C10–C40)	t_0	220.0	130.0	85.0	70.0	130.0 90.0
	t_f	108.3	173.3	83.3	146.7	118.3 105.0
Total PCBs	t_0	17.0	8.4	9.0	22.0	15.0 9.0
	t_f	16.7	8.9	18.7	15.0	13.7 7.4
Benzo[a]pyrene	t_0	0.06	0.03	0.05	0.09	0.04 0.02
	t_f	0.04	0.04	0.06	0.05	0.04 0.04
Benzo[b]fluoranthene	t_0	0.09	0.05	0.08	0.14	0.06 0.03
	t_f	0.07	0.06	0.1	0.08	0.07 0.07

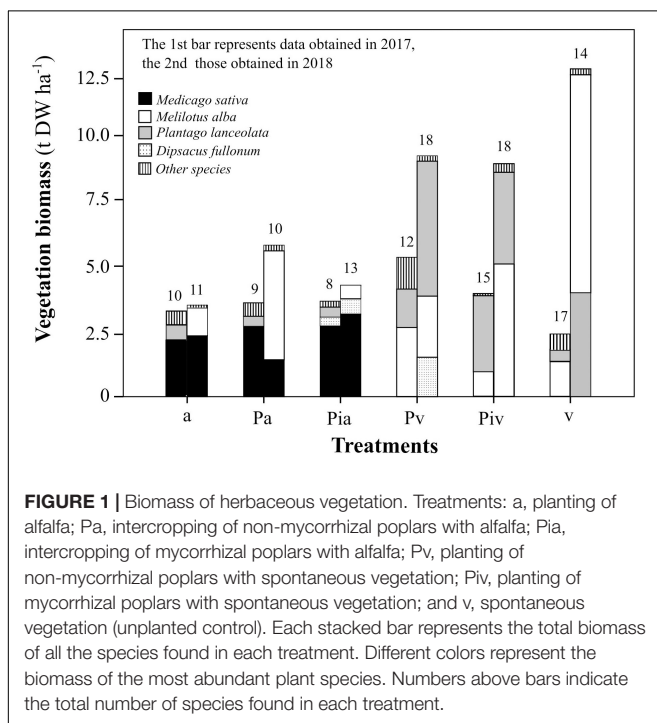
Concentrations (mg kg⁻¹) of Total Petroleum Hydrocarbons (TPH), Total PCBs, and PAHs (benzo[a]pyrene and benzo[b]fluoranthene) before (t_0) and after (t_f) application of the following treatments: a, planting of alfalfa; Pa, intercropping of non-mycorrhizal poplars with alfalfa; Pia, intercropping of mycorrhizal poplars with alfalfa; Pv, planting of non-mycorrhizal poplars with spontaneous vegetation; Piv, planting of mycorrhizal poplars with spontaneous vegetation; v, spontaneous vegetation (unplanted control). For reasons of spatial heterogeneity of organic contaminants, only those plots under different treatments with similar concentrations were compared. Critical Reference Values for Ecosystem Protection according to Basque legislation (mg kg⁻¹): TPH = 50; PCB = 0.01; Benzo[a]pyrene = 0.02; and Benzo[b]fluoranthene = 0.2.

some treatments, very close to the detection limit of the analytical technique used here for their quantification (0.02 mg kg^{-1}) and also close to the corresponding Critical Reference Values (0.02 and 0.2 mg kg^{-1} for benzo[a]pyrene and benzo[b]fluoranthene, respectively) further complicated the interpretation of the data.

Plant Performance and Diversity

Data on the biomass of the most abundant plant species, as well as the values for plant richness, are shown in **Figure 1**. All plots (planted and unplanted with alfalfa and/or poplar) were colonized by spontaneous vegetation. Some spontaneous plant species (*Melilotus albus*, *Plantago lanceolata*, and *Dipsacus fullonum*) were present in all plots and contributed substantially to the total biomass.

The presence of alfalfa, whether in a monoculture or in an intercropping system, limited the plant biodiversity. Plots where alfalfa had been sown showed the lowest levels of plant diversity, and this remained the case until the experiment ended. The highest levels of plant diversity were observed in plots where poplars (whether or not inoculated with the commercial mycorrhizal inoculum) had been transplanted, but only in the absence of alfalfa. This plant biodiversity was highest at the end of the experiment. Unplanted control plots were colonized by spontaneous vegetation and showed high plant diversity, dominated mainly by *M. albus*. This leguminous species colonized all plots, reaching its highest biomass in the unplanted control treatment. The significant growth of *M. albus* can probably explain the decrease in alfalfa biomass observed in the poplar/alfalfa intercropping system (Pa treatment) at the end of the experiment, most likely as a consequence of competition between these two leguminous species.



The performance of poplar trees during the first (July 2017) and second (July 2018) growing seasons is shown in **Figures 2, 3**. Regarding the biometric parameters measured here (**Figure 2**), the most important finding was probably the interspecific competition observed in the poplar/alfalfa intercropping treatment (Pa) in July 2017. More specifically, sowing alfalfa reduced the biomass of poplar branches in the first growing season (**Figure 2B**), but this adverse effect was not observed in the Pia treatment, where poplars were inoculated with the mycorrhizal inoculum and intercropped with alfalfa. No other differences in biometric parameters were observed in the first growing season. However, the levels of specific leaf area (**Figure 2A**) and total photosynthetic area (**Figure 2C**) in poplar trees increased in all treatments during the second growing season, indicating that the post-planting period is of critical importance for poplar tree performance.

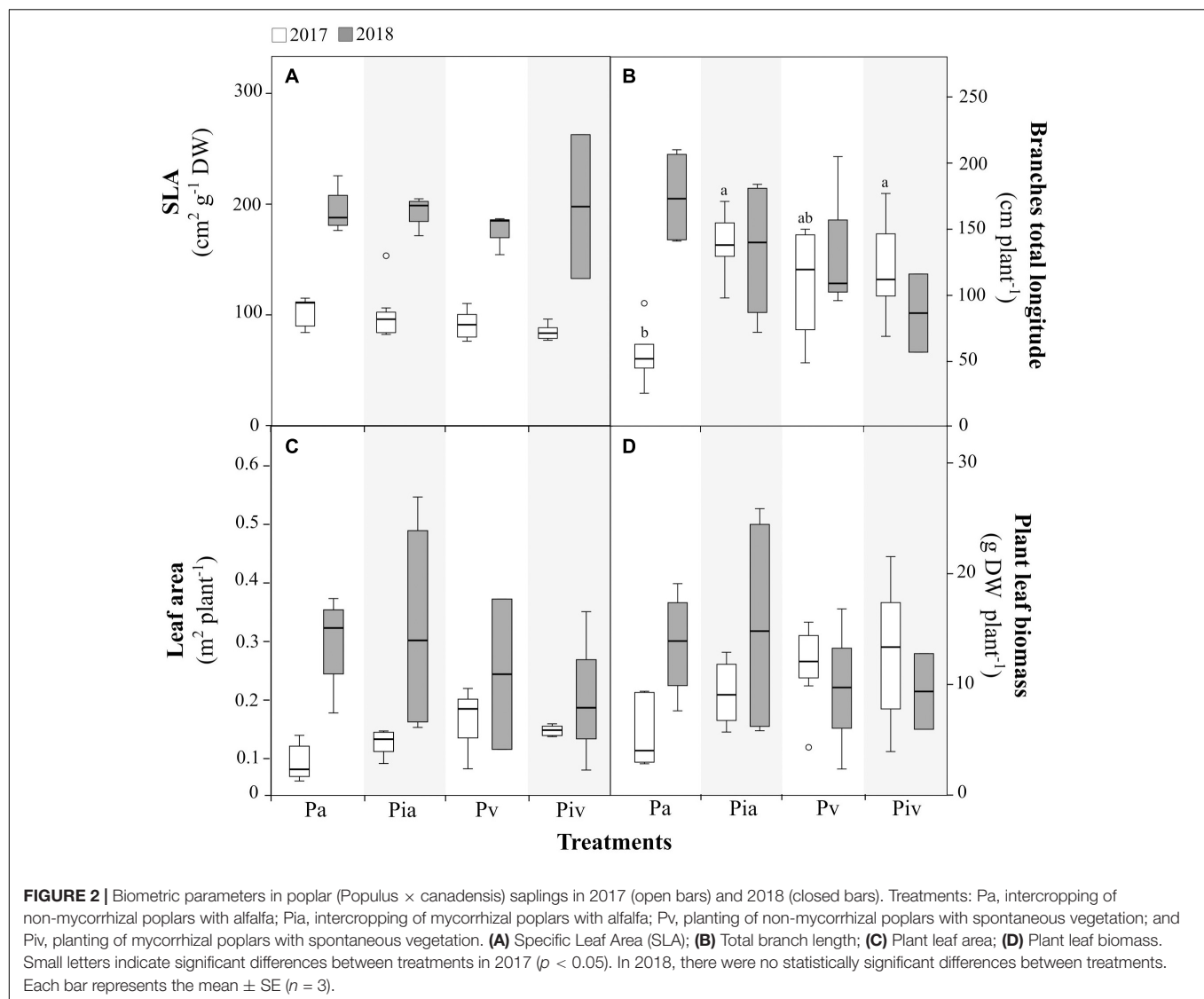
Regarding ecophysiological plant parameters, such as photochemical efficiency F_v/F_m (**Figure 3A**), VAZ content (**Figure 3B**), A+Z/VAZ de-epoxidation index (**Figure 3C**), tocopherol content (**Figure 3D**), total chlorophyll (**Figure 3E**), and carotenoids (**Figure 3F**), no significant differences were detected among the treatments. As was observed for some biometric parameters (**Figures 2A,C**), large differences in certain photoprotection indicators were observed between July 2017 and July 2018, i.e., the de-epoxidation index (**Figure 3C**) and the content of tocopherols (**Figure 3D**) were much higher in July 2017 than in July 2018. This observation reinforces the need to pay special attention to the initial steps in transplanting cuttings, in order to avoid possible episodes of plant stress, especially if poplar trees are to be intercropped with alfalfa.

Soil Microbial Properties

In the first growing season (July 2017), the levels of basal respiration (**Figure 4A**), substrate-induced respiration (**Figure 4B**), and bacterial functional diversity (**Figures 4C,D**) were lower than in July 2018. Soils in the poplar/alfalfa intercropping system (Pa treatment) showed significantly lower substrate-consumption activity (SCA) compared to the plots with poplar trees and spontaneous vegetation (Pv and Piv treatments). In the second growing season (July 2018), however, the levels of all these parameters increased 2–3-fold over the levels in July 2017. In July 2018, soils where alfalfa had been sown showed significantly higher abundances of bacterial (96%, **Figure 5A**; $p < 0.001$) and fungal (107%, **Figure 5B**; $p < 0.01$) gene copies compared to treatments with spontaneous vegetation, with the exception of the plots where mycorrhizal poplars were intercropped with alfalfa (Pia treatment; **Figure 5B**; $p < 0.05$).

Soil enzyme activities in general showed no significant differences among treatments (**Figure 6**); only the Pa treatment showed significantly higher activities. Indeed, the highest OEA levels (**Figure 6**) were detected in soils under the non-mycorrhizal poplars intercropped with alfalfa (Pa treatment). Intercropping significantly ($p < 0.05$) increased OEA values compared to monoculture plots, regardless of whether the poplar trees were inoculated with mycorrhizae (**Figure 6**).

Concerning soil microbial structural diversity, after singleton removal and quality filtering, amplicon sequencing resulted in



1,407,301 prokaryotic (16S rRNA) reads, which were clustered into 7,544 OTUs; while ITS amplicon sequencing resulted in 1,726,907 fungal (ITS1) reads, clustered into 1,233 OTUs, at a 3% dissimilarity level. The number of reads correlated significantly with total OTU richness for the 16S rRNA data ($p < 0.001$), suggesting an insufficient sequencing effort to obtain full coverage of the soil prokaryotic diversity. No significant correlation between the number of ITS1 reads and OTU richness was observed. However, considerable differences in the number of ITS1 reads were observed between samples: 72,753 and 121,363 ITS1 reads in the samples with the lowest and highest number of reads, respectively. At the end of the experiment (July 2018), no significant differences were observed between treatments regarding the values of the α -diversity indices for soil prokaryotic and fungal communities (Table 3). Plots where alfalfa had been sown showed significantly lower levels of prokaryotic ($p < 0.05$) and fungal ($p < 0.001$) rarefied richness, as well as significantly lower levels of fungal H' and J' ($p < 0.05$ for both),

in comparison with the plots where spontaneous plant species established themselves.

Regarding the soil microbial community composition (OTU profiles), NMDS analyses based on the Bray-Curtis dissimilarity matrix showed that both prokaryotic and fungal communities from the same soil samples tended to cluster together, indicating similar distribution patterns. On the other hand, at the end of the experiment (July 2018), the different treatments were separated according to both 16S rRNA and ITS amplicon data (PERMANOVA $p < 0.001$) (Supplementary Figure 1), indicating that they all had a strong effect on the composition of the soil prokaryotic and fungal communities. Unplanted control plots with their spontaneous vegetation showed the most differentiated soil prokaryotic and fungal communities, in both cases constituting individual clusters markedly separated from the rest.

In relation to soil microbial taxonomic classification, more than 88% of the 16S rRNA and ITS sequences were

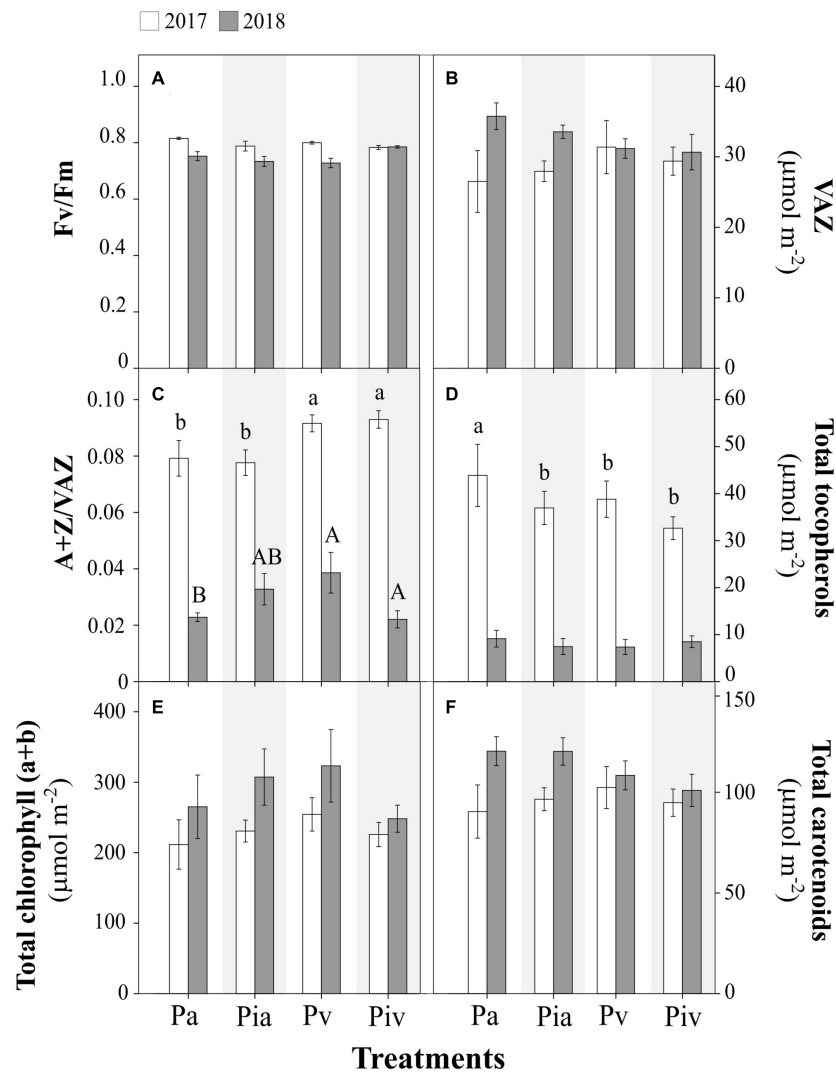


FIGURE 3 | Physiological parameters in poplar (*Populus x canadensis*) sapling leaves in 2017 (open bars) and 2018 (closed bars). Treatments: Pa, intercropping of non-mycorrhizal poplars with alfalfa; Pia, intercropping of mycorrhizal poplars with alfalfa; Pv, planting of non-mycorrhizal poplars with spontaneous vegetation; Piv, planting of mycorrhizal poplars with spontaneous vegetation. **(A)** Photochemical efficiency of PSII (F_v/F_m); **(B)** Total xanthophyll cycle pigments (VAZ); **(C)** De-epoxidation state of xanthophyll cycle pigments ($A+Z/VAZ$); **(D)** Total tocopherols; **(E)** Total chlorophyll; **(F)** Total carotenoids. Small and capital letters indicate significant differences between treatments in 2017 and 2018, respectively ($p < 0.05$). Each bar represents the mean $\pm$ SE ($n = 3$).

classified to order rank (for family rank, the classification of the ITS sequences dropped to 73%). Since the use of a deeper taxonomic resolution may not compensate for the loss of information regarding the rarest OTUs, we chose to use the order rank for our taxonomic analysis. At the order rank, the treatments were similar regarding the identity of the 30 most dominant prokaryotic (Supplementary Figure 2) and fungal (Supplementary Figure 3) taxa. Nevertheless, the abundances of these dominant taxa differed somewhat among treatments. At the end of the experiment, the most abundant bacterial orders were Xanthomonadales and Flavobacteriales (9.4 and 9.3% of the total prokaryotic community, respectively) in all the soils (Supplementary Figure 2). Nonetheless, the relative

abundance of these two orders was significantly lower in the soils where spontaneous vegetation was established (7.0 and 4.0%, respectively). In contrast, the soils with spontaneous vegetation showed higher abundances of Sphingomonadales (8.0%) and Pseudomonadales (8.3%) compared to the planted soils (6.3 and 2%, respectively) (Supplementary Figure 2). Regarding fungal communities, plots with spontaneous vegetation showed lower abundances of Ascomycota SH195297.07FU and Pleosporales (5.1 and 3.1% of the fungal community, respectively), compared to planted plots (12.8 and 8.3% of the fungal community, respectively), while showing a significantly higher abundance of Agaricales (13.5% vs 1.8%) (Supplementary Figure 3).

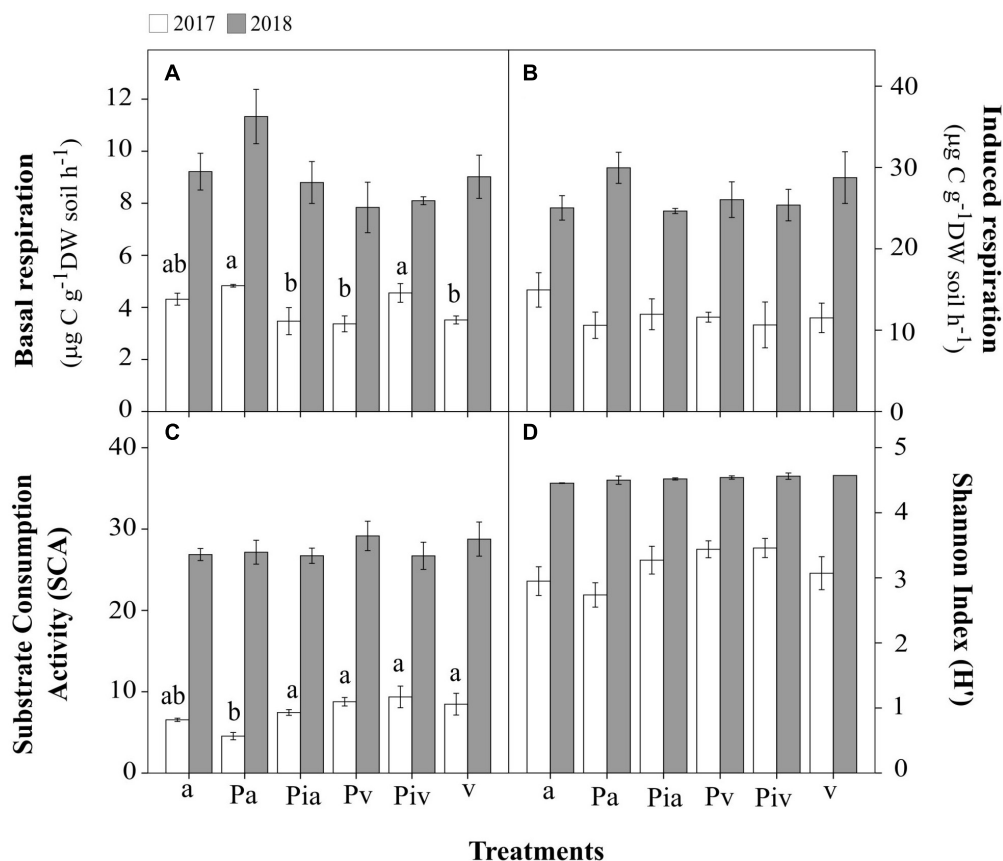


FIGURE 4 | Soil microbial activity, biomass, and bacterial functional diversity in 2017 (open bars) and 2018 (closed bars). Treatments: a, planting of alfalfa; Pa, intercropping of non-mycorrhizal poplars with alfalfa; Pia, intercropping of mycorrhizal poplars with alfalfa; Pv, planting of non-mycorrhizal poplars with spontaneous vegetation; Piv, planting of mycorrhizal poplars with spontaneous vegetation; v, spontaneous vegetation (unplanted control). Small letters indicate significant differences among treatments in 2017 ($p < 0.05$). **(A)** microbial activity in terms of basal respiration; **(B)** potentially active microbial biomass in terms of substrate-induced respiration; **(C)** Substrate Consumption Activity (SLA); **(D)** Shannon Index (H') for bacterial functional diversity. Small letters indicate significant differences between treatments in 2017 ($p < 0.05$). In 2018, there were no statistically significant differences between treatments. Each bar represents the mean $\pm$ SE ($n = 3$).

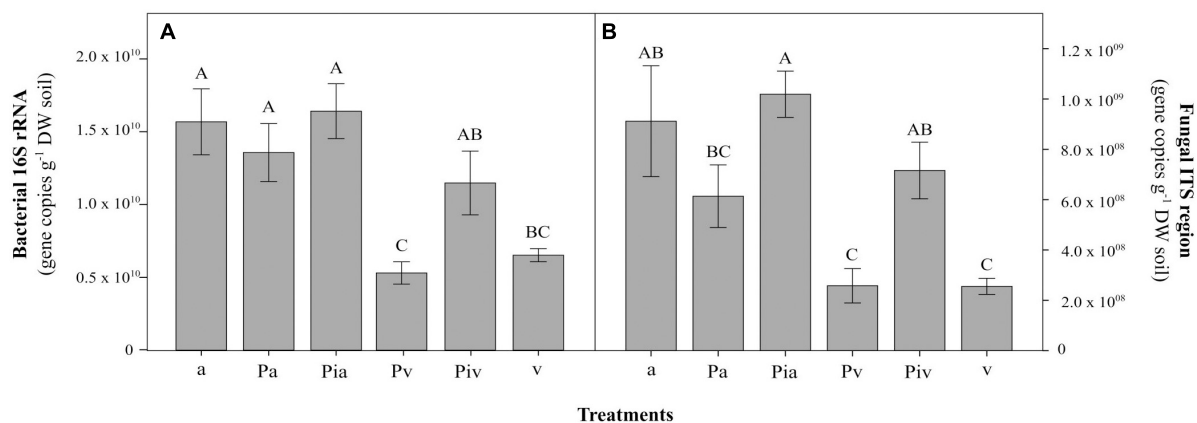
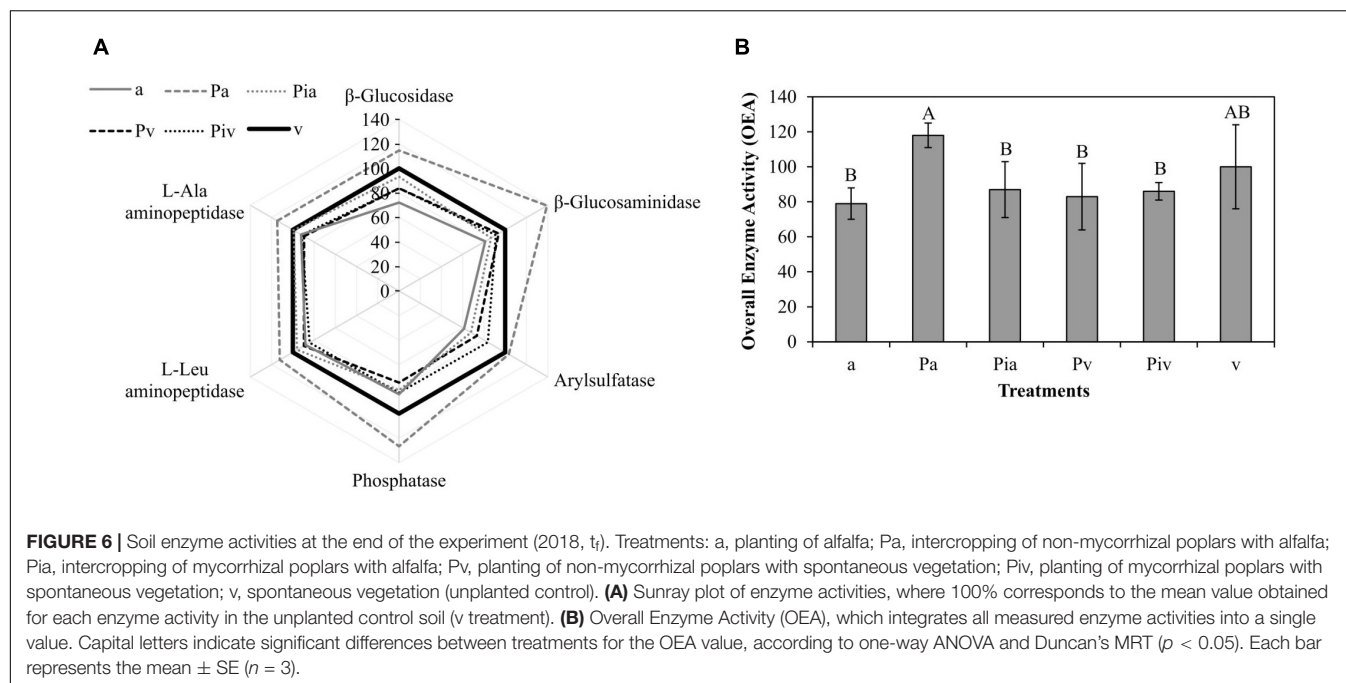


FIGURE 5 | Bacterial **(A)** and fungal **(B)** gene copy abundances at the end of the experiment (2018, t_f). Treatments: a, planting of alfalfa; Pa, intercropping of non-mycorrhizal poplars with alfalfa; Pia, intercropping of mycorrhizal poplars with alfalfa; Pv, planting of non-mycorrhizal poplars with spontaneous vegetation; Piv, planting of mycorrhizal poplars with spontaneous vegetation; v, spontaneous vegetation (unplanted control). Capital letters indicate significant differences between treatments according to one-way ANOVA and Duncan's MRT ($p < 0.05$). Each bar represents the mean $\pm$ SE ($n = 3$).

**TABLE 3 |** Structural diversity of soil microbial communities.

Treatment	Prokaryotic (16S rRNA)			Fungal (ITS)		
	R richness	H'	J'	R richness	H'	J'
a	3147 $\pm$ 147 ^{AB}	6.3 $\pm$.3 ^A	0.77 $\pm$.77 ^A	400 $\pm$ 00 ^E	3.23 $\pm$.23 ^B	0.53 $\pm$.53 ^A
Pa	3035 $\pm$ 035 ^B	6.2 $\pm$.2 ^A	0.77 $\pm$.77 ^A	453 $\pm$ 53 ^{CD}	3.43 $\pm$.43 ^{AB}	0.55 $\pm$.55 ^A
Pia	3155 $\pm$ 155 ^{AB}	6.3 $\pm$.3 ^A	0.78 $\pm$.78 ^A	426 $\pm$ 26 ^{DE}	3.40 $\pm$.40 ^{AB}	0.55 $\pm$.55 ^A
Pv	3205 $\pm$ 205 ^{AB}	6.2 $\pm$.2 ^A	0.75 $\pm$.75 ^A	521 $\pm$ 21 ^{AB}	3.70 $\pm$.70 ^A	0.59 $\pm$.59 ^A
Piv	3223 $\pm$ 223 ^{AB}	6.4 $\pm$.4 ^A	0.78 $\pm$.78 ^A	480 $\pm$ 80 ^{BC}	3.46 $\pm$.46 ^{AB}	0.56 $\pm$.56 ^A
v	3280 $\pm$ 280 ^A	6.1 $\pm$.1 ^A	0.75 $\pm$.75 ^A	537 $\pm$ 37 ^A	3.70 $\pm$.70 ^A	0.59 $\pm$.59 ^A

Rarefied richness (R richness), Shannon's index (H'), and Pielou's evenness (J') at the end of the experiment (t_1 , 2018). Treatments: a, planting of alfalfa; Pa, intercropping of non-mycorrhizal poplars with alfalfa; Pia, intercropping of mycorrhizal poplars with alfalfa; Pv, planting of non-mycorrhizal poplars with spontaneous vegetation; Piv, planting of mycorrhizal poplars with spontaneous vegetation; v, spontaneous vegetation (unplanted control). Different capital letters indicate significant differences between treatments ($p < 0.05$).

The 10 most abundant prokaryotic and fungal taxa in plots where alfalfa had been sown comprised 57 and 90% of the total community, respectively, compared to 54 and 86% for the unplanted control plots (Figures 7, 8). This result is in accordance with the lower values of microbial α -diversity in soils where alfalfa had been sown (Table 3). The order Pseudomonadales was not among the 10 most abundant prokaryotic taxa in the alfalfa (a) treatment (comprising only 1.8% of the total prokaryotic community), while in the unplanted control, this order comprised 4.3% of the total prokaryotic community (Figure 7A). Regarding soil fungal communities, the presence of alfalfa increased the abundances of the unidentified order Ascomycota SH195297.07FU and the order Pleosporales, compared to the unplanted control, which in turn showed higher abundances of the orders Pezizales and Agaricales (Figure 7B).

Mycorrhizal inoculation of poplar trees significantly increased the abundance of the prokaryotic orders Xanthomonadales,

Rhizobiales, and Micrococcales in comparison to soils where non-mycorrhizal poplars were planted (Figure 8A). On the other hand, mycorrhizal inoculation significantly reduced the abundances of the orders Sphingomonadales and Chitinophagales. Regarding the most abundant fungal taxa, mycorrhizal inoculation increased the abundances of two unidentified Ascomycota orders (SH195297.07FU and SH210351.07FU), which together comprised 25.2% of the total fungal community (Figure 8B). Within the non-mycorrhizal poplar plots, these two unidentified Ascomycota orders were less abundant, comprising 15.8% of the total fungal community. Mycorrhizal inoculation also led to a reduction in abundance of the orders Pleosporales, Pezizales, and Agaricales (Figure 8B). Mycorrhizal inoculation resulted in a higher number of significant differences in the abundance of prokaryotic taxa, with 15 taxa showing significant differences between inoculated and non-inoculated poplar plots (Supplementary Table 1). In comparison with prokaryotic taxa, the abundance of

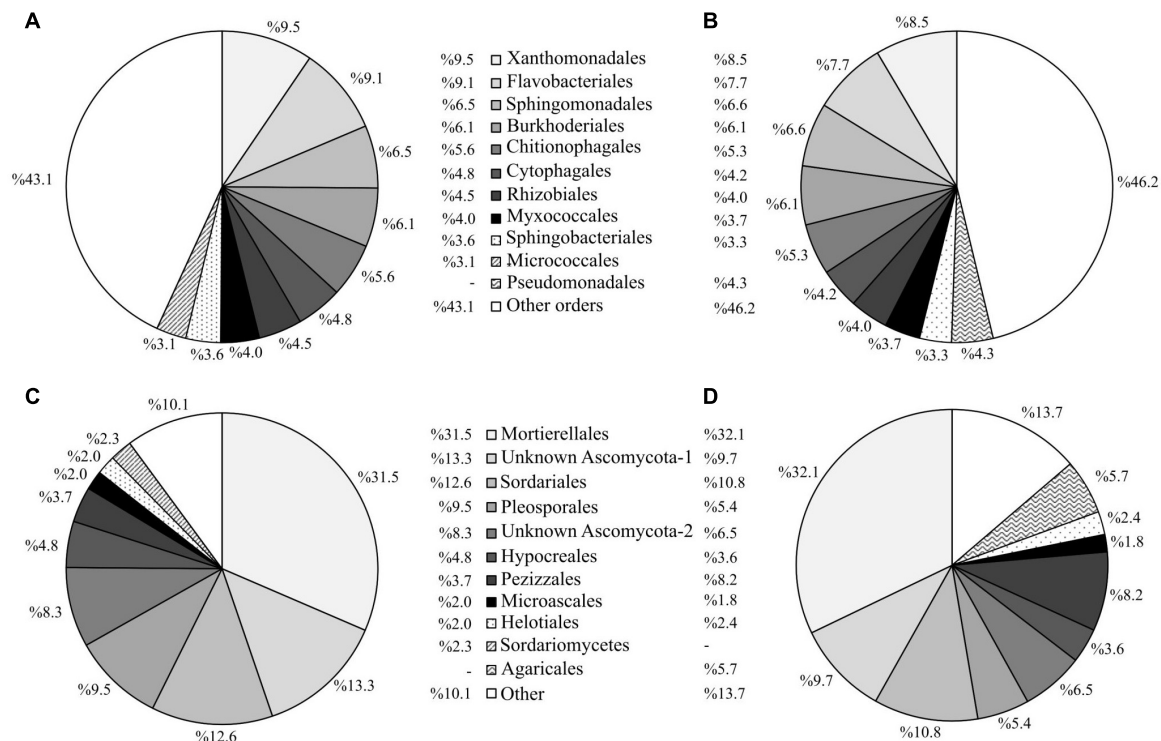


FIGURE 7 | Abundance profiles of the 10 most abundant prokaryotic and fungal taxa at order level for plots under the experimental factor “alfalfa sowing” at the end of the experiment. Abundance of prokaryotic taxa in plots with (A) and without (B) alfalfa. Abundance of fungal taxa in plots with (C) and without (D) alfalfa.

fungal taxa showed fewer differences among treatments, irrespective of the presence of alfalfa or mycorrhizal inoculation (Supplementary Table 1).

The PCA (Figure 9) indicated a marked separation of treatments with respect to the values of plant and soil microbial parameters. The presence of alfalfa and mycorrhizal inoculation (main components) explained 60% of the total variance in these parameters. Spontaneous vegetation appeared to be associated with plant diversity and biomass, as well as with soil substrate-induced respiration and bacterial functional diversity, toward the positive region of PC1; while plots where alfalfa had been sown (in monoculture or intercropping) appeared more closely linked to microbial activity and abundance parameters, toward the negative region of PC1. In contrast, the mycorrhizal poplars grouped along the positive region of PC2 and appeared to be closely associated with the microbial structural diversity data.

DISCUSSION

Contaminant Degradation

The presence of low concentrations of soil contaminants (although above regulatory limits, so that the soils are legally considered contaminated), together with low levels of contaminant availability in soil (due to the intrinsic persistence or recalcitrance of the contaminants, or to very long aging times) can greatly hinder the effectiveness of remediation strategies

(Mukherjee et al., 2014). In this respect, when soil organic contaminants have low water solubility and bioavailability, their microbial degradation is severely limited. In our study, the very long aging times, together with the low concentrations and hydrophobicity of the organic contaminants (TPHs, PCBs, and PAHs) probably resulted in their being tightly bound and retained in the soil matrix, thus limiting microbial degradation (Table 2). The short time elapsed since treatment implementation and the drought event that occurred in August 2017 likely also contributed to the low effectiveness of the treatments in reducing contaminant concentrations. Longer-term studies are needed to puzzle out whether these organic contaminants will be ultimately degraded or not. A thorough examination of the effects of mycorrhizal inoculation and intercropping on the phytoremediation of soils contaminated with organic compounds, especially at low concentrations, requires much additional research. After all, the majority of investigations dealing with the effect of mycorrhizal inoculation on phytoremediation efficiency have been conducted in metal-contaminated soils. Inoculation of mycorrhizal fungi has been used to improve the phytoremediation of a variety of organic contaminants, such as TPHs (Bois et al., 2005; Gunderson et al., 2007), PAHs (Joner et al., 2006), and explosives (Thompson et al., 1998; Thompson and Polebitski, 2010). However, in most cases, in agreement with our results, a low phytoremediation efficiency was observed. According to Coninx et al. (2017), the effect of mycorrhizal inoculants

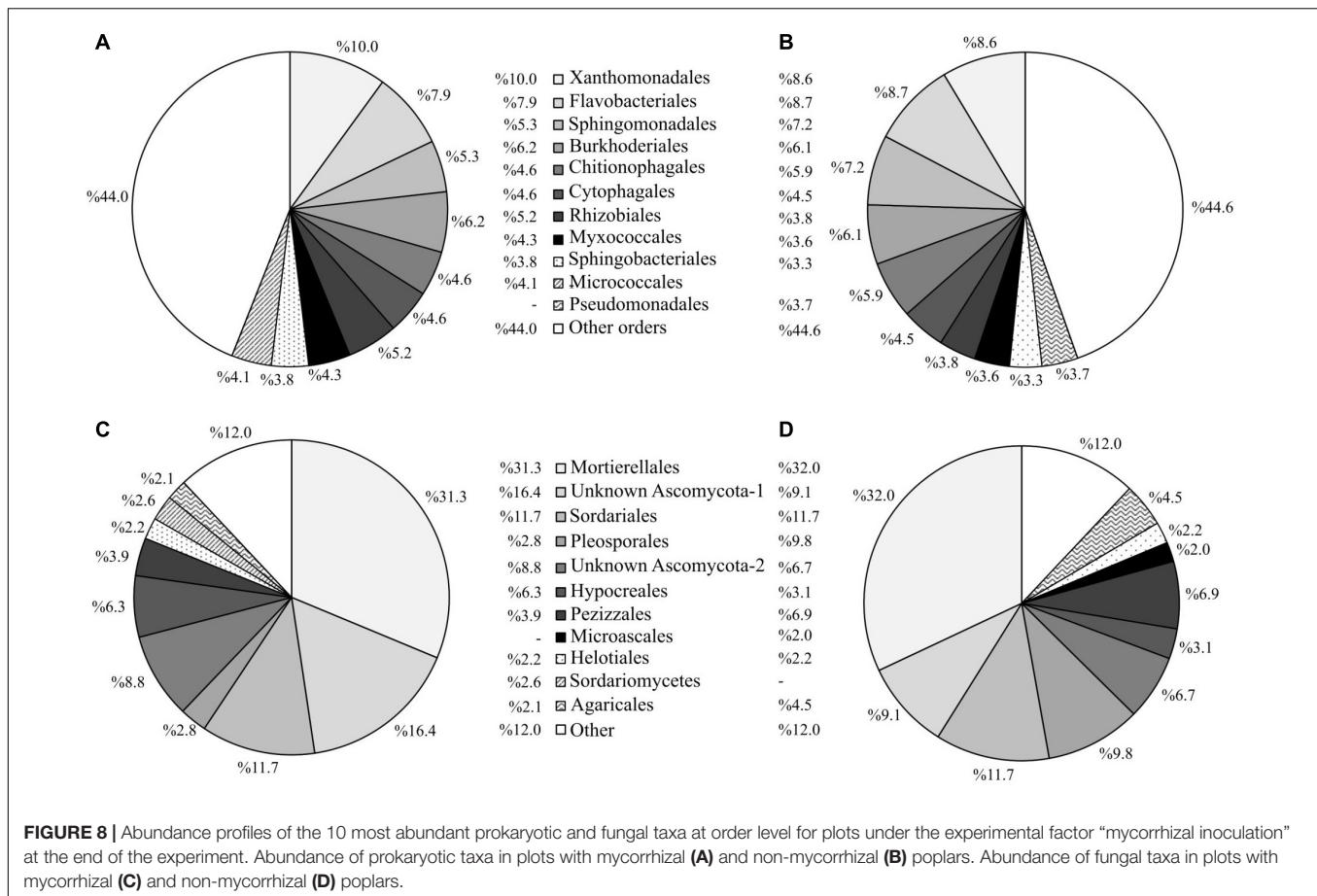


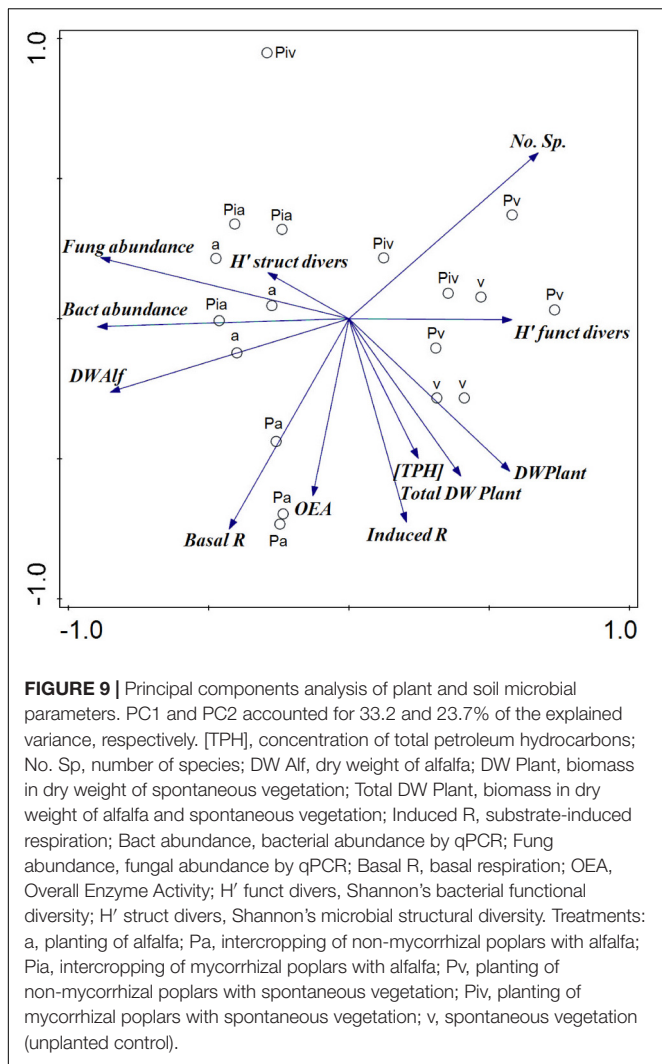
FIGURE 8 | Abundance profiles of the 10 most abundant prokaryotic and fungal taxa at order level for plots under the experimental factor “mycorrhizal inoculation” at the end of the experiment. Abundance of prokaryotic taxa in plots with mycorrhizal (A) and non-mycorrhizal (B) poplars. Abundance of fungal taxa in plots with mycorrhizal (C) and non-mycorrhizal (D) poplars.

is highly variable, as it depends on the nature of the contaminants, the specific mycorrhizal species, the plant species, the soil environmental conditions, and/or their potential interactions with the soil matrix and other soil organisms, etc. Furthermore, due to the well-known hydrophobicity and low bioavailability of TPHs (Garousin et al., 2021), PCBs (Ancona et al., 2017), and PAHs (Abraham et al., 2002), other strategies, such as the application of surfactants, could facilitate rhizodegradation processes, as these compounds can enclose colloidal particles of the organic contaminants, making them soluble in the aqueous phase and thereby increasing their bioavailability and hence their biodegradability (Martienssen and Schirmer, 2010).

Plant Performance and Diversity

Plant growth and diversity are important in the ecological recovery and restoration of degraded and contaminated soils. Selection of the most appropriate plant species is a crucial step to guarantee the success of phytoremediation processes. Forage crops (alfalfa) and fast-growing trees (poplars) have been previously used for phytoremediation of contaminated soils (Lingua et al., 2008; Panchenko et al., 2017), in many cases following a monoculture approach. However, nowadays agroforestry intercropping strategies are attracting much interest because of their potential environmental, economic, and social

benefits (Hotelier-Rous et al., 2020). In particular, poplar-alfalfa intercropping has high potential for *in-situ* phytoremediation of contaminated soils, since it combines the deep rooting system of poplar trees with the extensive colonization of the soil ecosystem by alfalfa roots, where nitrogen fixation takes place, contributing to lower maintenance (fertilization requirements) of the intercropping system and to enhancement of contaminant degradation. Another important issue is the presence and growth of spontaneous vegetation during the phytoremediation process. The potential interference of spontaneous vegetation with the growth of the planted vegetation (here, alfalfa and/or poplar), as well as potential synergistic or antagonistic effects regarding the impact of plants on soil physicochemical and biological properties, need to be carefully assessed. Our results indicated that the alfalfa crop limited colonization by spontaneous vegetation (Figure 1). Certain traits of alfalfa, such as its high growth rate, profuse root system, and nitrogen-fixation ability, can explain the capacity of this leguminous species to limit the growth of or even to outcompete other herbaceous species. On the other hand, the poplar plots showed high levels of plant diversity; interestingly, the number of plant species and the total plant biomass increased with time, the latter reaching levels as high as 10 t DW ha⁻¹. Among the spontaneous vegetation, *M. albus* appeared as a dominant pioneer species, showing the highest biomass productivity (12.5 t DW ha⁻¹) among all the



plant species. This leguminous species can compete effectively with alfalfa (see Pa treatment in **Figure 1**). The ability of *M. albus* to improve the health of degraded and contaminated soils (Halecki and Klatka, 2018), especially if associated with mycorrhizal fungi (Hernández-Ortega et al., 2012) or endophytic bacteria (Mitter et al., 2017, 2020), without requiring high levels of nutrients (low fertilization requirements) makes it a good candidate for long-term phytoremediation programs.

The growth of alfalfa when intercropped with poplar (**Figures 2A,C**) not only reduced the biomass and diversity of spontaneous vegetation but also decreased the woody biomass of poplar trees in the first growing season (Pa treatment, **Figure 2B**). The competition between the two crops for space and resources (e.g., water and nutrients) in the critical post-planting period can explain this observation. Interestingly, mycorrhizal inoculation of poplar trees counteracted the negative effect caused by alfalfa on woody biomass (Pia treatment, **Figure 2B**). The positive contribution of mycorrhizal fungi to plant establishment and growth in disturbed and stressed ecosystems is well known (Gould et al., 1996). According to

Quoreshi and Khalsa (2008), inoculation of seedlings or cuttings of poplars with mycorrhizal fungi ensures their optimal field performance. Under our experimental conditions, inoculation of poplar cuttings improved the establishment of young trees in the intercropping system (**Figure 2B**). The commercial mycorrhizal inoculum used here contains *Rhizopogon* spp., *Scleroderma* spp., and *Pisolithus tinctorius* (ectomycorrhizae), and *Glomus intraradices* and *Glomus mosseae* (arbuscular endomycorrhizae). These ectomycorrhizae are known to increase poplar biomass (Cripps, 2001; Gunderson et al., 2007; Ma et al., 2008). Analogous results have been reported with poplar trees growing in contaminated soils when inoculated with arbuscular endomycorrhizae belonging to Glomeromycota (Quoreshi and Khalsa, 2008; Cicatelli et al., 2010; Pallara et al., 2013; Zhang H. et al., 2019). Ectomycorrhizal and endomycorrhizal fungi can improve plant quality and performance because they (i) may alleviate the (oxidative) stress induced by metals (Godbold et al., 1998) and organic contaminants (Małachowska-Jutysz and Kalka, 2010), and (ii) facilitate soil exploration by roots for water and nutrient acquisition (Marschner and Dell, 1994; Giovannetti et al., 2001). The enhancement of stress tolerance in poplars derived from mycorrhizal inoculation is a key beneficial effect for phytoremediation purposes.

The above-mentioned differences among treatments in the first growing season (July 2017) disappeared in the second growing season (July 2018), indicating that once they are well established, poplar trees can effectively compete with the surrounding vegetation. In this regard, certain acclimation traits were observed during the more stressful period of the first growing season. Leaves are plastic organs that can vary greatly in morphology, anatomy, and physiology in response to environmental conditions. The increase in the sclerophylly of leaves, as reflected in the reduction of SLA values observed in all treatments in July 2017 (**Figure 2A**), is associated with enhanced water-use efficiency under stress (soil water stress) and is a common strategy of phenotypic adjustment in plants (Wellstein et al., 2017). Accordingly, in the second growing season (July 2018), when the environmental conditions were milder and the trees had become established, all poplar treatments showed a larger total leaf area per tree (**Figure 2C**). In 2017, in all poplar treatments, the transplant stress affected the values of the plant parameters determined here. This transplant stress was confirmed by the higher levels of the de-epoxidation index (**Figure 2C**) and tocopherol content (**Figure 3C**) in the poplar leaves. De-epoxidation of violaxanthin to antheraxanthin and then to zeaxanthin, as well as increased concentrations of antioxidants such as tocopherols in leaves, are photoprotective mechanisms observed in species of the genus *Populus* under stress conditions (Niinemets et al., 2003; Jia et al., 2020).

Soil Microbial Properties

The lower levels of soil microbial activity, biomass, and bacterial functional diversity observed in the first growing season (July 2017) could be at least partly due to the combined effect of the removal of vegetation cover and tilling just before the beginning

of the experiment, in order to prepare the site for the field experiment. Microbial soil properties have proved to be very sensitive to tilling practices (Kabiri et al., 2016). This early period was also critical for vegetation performance (Figures 2, 3) and plant diversity (Figure 1). In any case, this was a transient effect, as in the second growing season (July 2018), once the vegetation was well established and the soil structure had consolidated, the levels of these microbial parameters increased. Changes in soil environmental conditions are well known to influence microbial growth, activity, and diversity (Kabiri et al., 2016; Steinauer et al., 2016). Importantly, root exudates from the poplars, alfalfa, and spontaneous vegetation most likely contributed to this enhanced soil microbial activity and biomass. Successful phyto- and bio-remediation of contaminated soils requires acclimation by soil microorganisms (Abdollahinejad et al., 2020).

Soil enzyme activities control nutrient cycling and have often been used as bioindicators of soil health (Epelde et al., 2009; Gómez-Sagasti et al., 2012). In general, the soil enzyme activities showed no significant differences among treatments (Figure 6); only the Pa treatment resulted in significantly higher levels. Intercropping significantly ($p < 0.05$) increased OEA values compared to the monoculture plots, regardless of whether or not the poplar trees were inoculated with mycorrhizae (Figure 6). Intercropping of herbaceous and woody plant species has been reported to enhance soil enzyme activity compared to monoculture systems (Cui et al., 2018; Zeng et al., 2019). This enhancement is often attributed to an increase in the amount and diversity of root exudates, which provide not only energy but also a wide variety of easily available substrates for extracellular enzymes (Allison and Vitousek, 2005; Steinauer et al., 2016). Considering that all plots were initially amended with compost, and therefore one would expect higher soil microbial activity at the beginning of the experiment (induced by application of the organic amendment) followed by lower activity as time progressed (Keener et al., 2000), the observed stimulation of soil microbial communities caused by the established vegetation is a very interesting finding from the point of view of the targeted positive effect of plant treatments on soil microbial communities. Our results emphasize the need to consider both the plants and the soil microbial communities when designing and monitoring phytoremediation processes, in terms of their capacity to reduce contaminant concentrations and improve soil health.

The abundances of bacterial (Figure 5A) and fungal (Figure 5B) communities in soil samples, reflected in the gene-copy numbers, were increased by the presence of alfalfa and mycorrhizal poplars grown in the company of spontaneous vegetation. This stimulatory effect of soil microbial communities was not observed when analyzing the SIR data (Figure 4B) or community-level physiological profiles with Biolog EcoPlatesTM (Figure 4C), where no differences between treatments were observed. This could be explained by the changes in microbial structural biodiversity induced by alfalfa growth and mycorrhizal inoculation, as observed in the levels of abundance of specific bacterial and fungal taxa (Figures 7, 8) and the lower values of α -diversity (Table 3). Root exudates (Steinauer et al., 2016) and mycorrhization (Asmelash et al., 2016) can affect the structural and functional diversity of soil microbial communities.

The high functional redundancy of soil microbial communities (Girvan et al., 2005), as well as changes in the rhizosphere microbiome may explain the observed maintenance of microbial growth and activity.

At the beginning of the experiment, sowing alfalfa and transplanting poplar trees altered the composition of the existing native soil microbiota and initiated the development of a distinct soil microbial community. Mycorrhizal inoculation markedly affected the composition of the soil prokaryotic and fungal communities (Figure 9 and Supplementary Table 1). Alfalfa, on the other hand, increased the relative importance of certain abundant bacterial orders. Several dominant prokaryotic and fungal orders varied significantly, depending on the presence of alfalfa (Figure 7) and the use of the mycorrhizal inoculum (Figure 8). The phylum Proteobacteria dominated the prokaryotic rhizosphere communities of mycorrhizal poplar trees. Members of the orders Xanthomonadales (Gammaproteobacteria) and Rhizobiales (Alphaproteobacteria) were more abundant in soils where mycorrhizal poplars were planted, compared to soils with non-mycorrhizal poplars (Supplementary Figure 3). Members of the order Micrococcales (phylum Actinobacteria) were also abundant in the rhizosphere of poplar trees. These orders are likely to form part of the core rhizosphere microbiome of poplars, in both contaminated (Foulon et al., 2016) and non-contaminated soils (Shakya et al., 2013). Several members of these bacterial orders possess multifunctional plant growth-promoting traits, and therefore can promote plant growth *directly* by, for example, fixing nitrogen, solubilizing phosphorus and potassium from a limited nutrient pool, and producing phytohormones and siderophores; or *indirectly* by producing ACC deaminase activity, antibiotics, antifungal compounds, etc. (Hayat et al., 2010; Yadav et al., 2018). The existence of plant growth-promoting bacteria in their rhizosphere facilitates the establishment and growth of poplar trees in degraded and/or contaminated sites. Additionally, some unidentified orders of Ascomycota were detected in high abundance in soils where alfalfa and mycorrhizal poplars were present. Some studies have recognized the key role of the phylum Ascomycota in environments contaminated with organics (Aranda, 2016) and metals (Gil-Martínez et al., 2021). However, the role of Ascomycota in the transformation of soil contaminants remains poorly understood. Interestingly, members of Ascomycota have been found to be essential drivers of OM decomposition in soils where hybrid poplars are planted (Zheng et al., 2017). We can therefore conclude that our mycorrhizal inoculum enhanced the abundance of Proteobacteria, Actinobacteria, and Ascomycota, which in turn benefited not only plant growth but also soil OM mineralization and nutrient cycling, thus improving the fertility of the soil studied here.

Our study supports the existence of an intimate relationship between above-ground plant diversity and below-ground microbial diversity, as reported for other contaminated soils (Burgess et al., 2017). Soils colonized by spontaneous vegetation showed not only the highest plant diversity (Figures 1, 9) but also the highest richness of soil bacteria and fungi (Table 3). Some treatments, such as alfalfa sowing (Figure 7) and mycorrhizal

inoculation (**Figure 8**), reduced plant and soil microbial diversity but favored the abundance of specific microbial taxa. These changes in soil microbial composition did not affect the overall soil microbial activity, probably owing to the above-mentioned high functional redundancy of soil microbial communities.

An important conclusion of the present study is that, although the concentration of organic contaminants was not reduced in this 2-year experiment by any of the phytoremediation treatments, soil health (reflected in the levels of microbial biomass, activity, and diversity parameters) was effectively improved. Our findings broaden current knowledge of the positive effects of alfalfa sowing on soil microbial biomass, and the benefits of intercropping for the stimulation of enzyme activity in soils contaminated with organic compounds. Finally, *Melilotus albus* appears to be a suitable candidate for phytoremediation of abandoned vacant lands contaminated with organic compounds.

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: European Nucleotide Archive [accession: <http://www.ebi.ac.uk/ena/data/view/PRJEB44358>].

AUTHOR CONTRIBUTIONS

JB and CG conceived and designed the study. MG-S, CG, IA, and JB had major roles in writing the manuscript. MG-S, JU, FM,

UA, and AH were actively involved in field and laboratory work, data analysis, and interpretation. JV, JB, and CG were responsible for acquiring funding. All authors meet all established criteria for inclusion in the authorship and have approved the submitted version.

FUNDING

This study was supported by the PhytoSUDOE (SOE1/P5/E0189) and Phy2SUDOE (SOE4/P5/E1021) projects funded by the Interreg Sudoe Programme through the European Regional Development Fund (ERDF), PRADA project (PID2019-110055RB-C21 and PID2019-110055RB-C22) from MINECO, and the Consolidated Research Group of the Basque Government (GV ITO18-16).

ACKNOWLEDGMENTS

The authors are grateful to Carmela Monterroso (University of Santiago de Compostela, Spain) for the soil physicochemical analyses and Lur Epelde and June Hidalgo from NEIKER-BRTA for their assistance with the bioinformatic treatment of sequencing data and its submission to the public repository.

SUPPLEMENTARY MATERIAL

The Supplementary Material for this article can be found online at: <https://www.frontiersin.org/articles/10.3389/fpls.2021.693044/full#supplementary-material>

REFERENCES

- Abdollahinejad, B., Pasalari, H., Jafari, A. J., Esrafil, A., and Farzadkia, M. (2020). Bioremediation of diesel and gasoline-contaminated soil by co-vermicomposting amended with activated sludge: diesel and gasoline degradation and kinetics. *Environ. Pollut.* 263:114584. doi: 10.1016/j.envpol.2020.114584
- Abraham, W.-R., Nogales, B., Golyshin, P. N., Pieper, D. H., and Timmis, K. N. (2002). Polychlorinated biphenyl-degrading microbial communities in soils and sediments. *Curr. Opin. Microbiol.* 5, 246–253.
- Alkorta, I., Becerril, J. M., and Garbisu, C. (2010). Phytostabilization of metal contaminated soils. *Rev. Environ. Health* 25, 135–146. doi: 10.1515/REVEH.2010.25.2.135
- Allison, S. D., and Vitousek, P. M. (2005). Responses of extracellular enzymes to simple and complex nutrient inputs. *Soil Biol. Biochem.* 37, 937–944. doi: 10.1016/j.soilbio.2004.09.014
- Ancona, V., Barra, A., Grenni, P., Di, M., Campanale, C., Calabrese, A., et al. (2017). Plant-assisted bioremediation of a historically PCB and heavy metal-contaminated area in Southern Italy. *Nat. Biotechnol.* 38, 65–73. doi: 10.1016/j.nbt.2016.09.006
- Anza, M., Salazar, O., Epelde, L., Becerril, J. M., Alkorta, I., and Garbisu, C. (2019). Remediation of organically contaminated soil through the combination of assisted phytoremediation and bioaugmentation. *Appl. Sci.* 9:4757. doi: 10.3390/app9224757
- Aranda, E. (2016). Promising approaches towards biotransformation of polycyclic aromatic hydrocarbons with Ascomycota fungi. *Curr. Opin. Biotechnol.* 38, 1–8. doi: 10.1016/j.copbio.2015.12.002
- Asmelash, F., Bekele, T., and Birhane, E. (2016). The potential role of arbuscular mycorrhizal fungi in the restoration of degraded lands. *Front. Microbiol.* 7:1095. doi: 10.3389/fmicb.2016.01095
- Bois, Y., Piché, G., Fung, M. Y. P., and Khalsa, D. P. (2005). Mycorrhizal inoculum potentials of pure reclamation materials and revegetated tailing sands from the Canadian oil sand industry. *Mycorrhiza* 15, 149–158. doi: 10.1007/s00572-004-0315-4
- Bonfranceschi, B. A., Flocco, C. G., and Donati, E. R. (2009). Study of the heavy metal phytoextraction capacity of two forage species growing in an hydroponic environment. *J. Hazard. Mater.* 165, 366–371. doi: 10.1016/j.jhazmat.2008.10.024
- Burges, A., Epelde, L., Benito, G., Artetxe, U., Becerril, J. M., and Garbisu, C. (2016). Enhancement of ecosystem services during endophyte-assisted aided phytostabilization of metal contaminated mine soil. *Sci. Total Environ.* 562, 480–492. doi: 10.1016/j.scitotenv.2016.04.080
- Burges, A., Epelde, L., Blanco, F., Becerril, J. M., and Garbisu, C. (2017). Ecosystem services and plant physiological status during endophyte-assisted phytoremediation of metal contaminated soil. *Sci. Total Environ.* 584–585, 329–338. doi: 10.1016/j.scitotenv.2016.12.146
- Caporaso, J. G., Lauber, C. L., Walters, W. A., Berg-Lyons, D., Huntley, J., Fierer, N., et al. (2012). Ultra-high-throughput microbial community analysis on the Illumina HiSeq and MiSeq platforms. *ISME J.* 6, 1621–1624. doi: 10.1038/ismej.2012.8
- Ciadamidaro, L., Girardclos, O., Bert, V., Zappellini, C., Yung, L., Foulon, J., et al. (2017). Poplar biomass production at phytomanagement sites is significantly enhanced by mycorrhizal inoculation. *Environ. Exp. Bot.* 139, 48–56. doi: 10.1016/j.envexpbot.2017.04.004

- Cicattelli, A., Lingua, G., Todeschini, V., Biondi, S., Torrigiani, P., and Castiglione, S. (2010). Arbuscular mycorrhizal fungi restore normal growth in a white poplar clone grown on heavy metal-contaminated soil, and this is associated with upregulation of foliar metallothionein and polyamine biosynthetic gene expression. *Ann. Bot.* 106, 791–802. doi: 10.1093/aob/mcq170
- Coninx, L., Martinova, V., and Rineau, F. (2017). Mycorrhiza-assisted phytoremediation. *Adv. Bot. Res.* 83, 127–188. doi: 10.1016/bs.abr.2016.12.005
- Cripps, C. L. (2001). “Mycorrhizal fungi of aspen forests: natural occurrence and potential applications,” in *Proceedings of the Sustaining Aspen in Western Landscapes: Symposium*, Rocky Mountain Research Station report RMRS-P-18 (Fort Collins, CO: USDA Forest Service), 285–298.
- Cui, T., Fang, L., Wang, M., Jiang, M., and Shen, G. (2018). Intercropping of gramineous pasture ryegrass (*Lolium perenne* L.) and leguminous forage alfalfa (*Medicago sativa* L.) increases the resistance of plants to heavy metals. *J. Chem.* 2018:7803408. doi: 10.1155/2018/7803408
- D’Amore, R., Ijaz, U. Z., Schirmer, M., Kenny, J. G., Gregory, R., Darby, A. C., et al. (2016). A comprehensive benchmarking study of protocols and sequencing platforms for 16S rRNA community profiling. *BMC Genomics* 17:55. doi: 10.1186/s12864-015-2194-9
- Delgado de la Flor, Y. A., Perry, K. I., Turo, K. J., Parker, D. M., Thompson, J. L., and Gardiner, M. M. (2020). Local and landscape-scale environmental filters drive the functional diversity and taxonomic composition of spiders across urban greenspaces. *J. Appl. Ecol.* 57, 1570–1580. doi: 10.1111/1365-2664.13636
- Epelde, L., Becerril, J. M., Hernández-Allica, J., Barrutia, O., and Garbisu, C. (2008). Functional diversity as indicator of the recovery of soil health derived from *Thlaspi caerulescens* growth and metal phytoextraction. *Appl. Soil Ecol.* 39, 299–310. doi: 10.1016/j.apsoil.2008.01.005
- Epelde, L., Burges, A., Mijangos, I., and Garbisu, C. (2014). Microbial properties and attributes of ecological relevance for soil quality monitoring during a chemical stabilization field study. *Appl. Soil Ecol.* 75, 1–12. doi: 10.1016/j.apsoil.2013.10.003
- Epelde, L., Ma Becerril, J., Alkorta, I., and Garbisu, C. (2009). “Heavy metal phytoremediation: microbial indicators of soil health for the assessment of remediation efficiency,” in *Advances in Applied Bioremediation. Soil Biology*, Vol. 17, eds A. Singh, R. Kuhad, and O. Ward (Berlin: Springer-Verlag), 299–313. doi: 10.1007/978-3-540-89621-0_16
- Fan, S., Li, P., Gong, Z., Ren, W., and He, N. (2008). Promotion of pyrene degradation in rhizosphere of alfalfa (*Medicago sativa* L.). *Chemosphere* 71, 1593–1598. doi: 10.1016/j.chemosphere.2007.10.068
- Foulon, J., Zappellini, C., Durand, A., Valot, B., Girardclos, O., Blaudez, D., et al. (2016). Environmental metabarcoding reveals contrasting microbial communities at two poplar phytomanagement sites. *Sci. Total Environ.* 571, 1230–1240. doi: 10.1016/j.scitotenv.2016.07.151
- García-Plazaola, J. I., Artetxe, U., Duñabeitia, M. K., and Becerril, J. M. (1999). Role of photoprotective systems of Holm-Oak (*Quercus ilex*) in the adaptation to winter conditions. *J. Plant Physiol.* 155, 625–630. doi: 10.1016/S0176-1617(99)80064-9
- Garousin, H., Pourbabaee, A. A., Alikhani, H. A., and Saleem, M. (2021). A combinational strategy mitigated old-aged petroleum contaminants: ineffectiveness of biostimulation as a bioremediation technique. *Front. Microbiol.* 12:642215. doi: 10.3389/fmicb.2021.642215
- Gil-Martínez, M., López-García, Á., Domínguez, M. T., Kjoller, R., Navarro-Fernández, C. M., Rosendahl, S., et al. (2021). Soil fungal diversity and functionality are driven by plant species used in phytoremediation. *Soil Biol. Biochem.* 153:108102. doi: 10.1016/j.soilbio.2020.108102
- Giovannetti, M., Fortuna, P., Citeresi, A. S., Morini, S., and Nuti, M. P. (2001). The occurrence of anastomosis formation and nuclear exchange in intact arbuscular mycorrhizal networks. *New Phytol.* 151, 717–724. doi: 10.1046/j.0028-646x.2001.00216.x
- Girvan, M. S., Campbell, C. D., Killham, K., Prosser, J. I., and Glover, L. A. (2005). Bacterial diversity promotes community stability and functional resilience after perturbation. *Environ. Microbiol.* 7, 301–313. doi: 10.1111/j.1462-2920.2005.00695.x
- Godbold, D. L., Jentschke, G., Winter, S., and Marschner, P. (1998). Ectomycorrhizas and amelioration of metal stress in forest trees. *Chemosphere* 36, 757–762. doi: 10.1016/S0045-6535(97)10120-5
- Gómez-Sagasti, M. T., Alkorta, I., Becerril, J. M., Epelde, L., Anza, M., and Garbisu, C. (2012). Microbial monitoring of the recovery of soil quality during heavy metal phytoremediation. *Water Ai. Soil Pollut.* 223, 3249–3262. doi: 10.1007/s11270-012-1106-8
- Gómez-Sagasti, M. T., Hernández, A., Artetxe, U., Garbisu, C., and Becerril, J. M. (2018). How valuable are organic amendments as tools for the phytomanagement of degraded soils? The knowns, known unknowns, and unknowns. *Front. Sustain. Food Syst.* 2:68. doi: 10.3389/fsufs.2018.00068
- Gould, A. B., Hendrix, J. W., and Ferriss, R. S. (1996). Relationship of mycorrhizal activity to time following reclamation of surface mine land in western Kentucky. I. Propagule and spore population densities. *Can. J. Bot.* 74, 247–261.
- Government of Spain (2017). Real Decreto 999/2017, de 24 de noviembre, por el que se modifica el Real Decreto 506/2013, de 28 de junio, sobre productos fertilizantes (in Spanish). *Off. Bull. Spain* 296, 119396–119450.
- Government of the Basque Country. (2015). Law 4/2015, of 25 June, for the Prevention and Correction of soil Pollution (in Spanish). *Official Bulletin of Spain BOE-A-2015-8272*. Madrid: Ministerio de Justicia.
- Gunderson, J. J., Knight, J. D., and Van Rees, K. C. J. (2007). Impact of ectomycorrhizal colonization of hybrid poplar on the remediation of diesel-contaminated soil. *J. Environ. Qual.* 34, 927–934. doi: 10.2134/jeq2006.0260
- Halecki, W., and Klatka, S. (2018). Long term growth of crop plants on experimental plots created among slag heaps. *Ecotoxicol. Environ. Saf.* 147, 86–92. doi: 10.1016/j.ecoenv.2017.08.025
- 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
- Hernández-Ortega, H. A., Alarcón, A., Ferrera-Cerrato, R., Zavaleta-Mancera, H. A., López-Delgado, H. A., and Mendoza-López, M. R. (2012). Arbuscular mycorrhizal fungi on growth, nutrient status, and total antioxidant activity of *Melilotus albus* during phytoremediation of a diesel-contaminated substrate. *J. Environ. Manage.* 95, S319–S324. doi: 10.1016/j.jenvman.2011.02.015
- Herrmann, D. L., Schwarz, K., Shuster, W. D., Berland, A., Chaffin, B. C., Garmestani, A. S., et al. (2016). Ecology for the Shrinking City. *Bioscience* 66, 965–973. doi: 10.1093/biosci/biw062
- Hotelier-Rous, N., Laroche, G., Durocher, È, Rivest, D., Olivier, A., Liagre, F., et al. (2020). Temperate agroforestry development: the case of Quebec and of France. *Sustainability* 12:7227. doi: 10.3390/su12177227
- Hu, Y., Gao, Z., Huang, Y., Chen, S., Yang, X., Su, J., et al. (2019). Impact of poplar-based phytomanagement on metal bioavailability in low-phosphorus calcareous soil with multi-metal contamination. *Sci. Total Environ.* 686, 848–855. doi: 10.1016/j.scitotenv.2019.06.072
- International Organization for Standardization (ISO) (2010). *ISO/TS 22939 Soil Quality – Measurement of Enzyme Activity Patterns in Soil Samples Using Fluorogenic Substrates in Micro-Well Plates*. Geneva: ISO.
- Jia, H., Wang, L., Li, J., Sun, P., Lu, M., and Hu, J. (2020). Comparative metabolomics analysis reveals different metabolic responses to drought in tolerant and susceptible poplar species. *Physiol. Plant.* 168, 531–546. doi: 10.1111/ppl.13036
- Joner, E. J., Leyval, C., and Colpaert, J. V. (2006). Ectomycorrhizas impede phytoremediation of polycyclic aromatic hydrocarbons (PAHs) both within and beyond the rhizosphere. *Environ. Pollut.* 142, 34–38. doi: 10.1016/j.envpol.2005.09.007
- Kabiri, V., Raiesi, F., and Ghazavi, M. A. (2016). Tillage effects on soil microbial biomass, SOM mineralization and enzyme activity in a semi-arid Calixerepts. *Agric. Ecosyst. Environ.* 232, 73–84. doi: 10.1016/j.agee.2016.07.022
- Karłinski, L., Rudawska, M., Kieliszewska-Rokicka, B., and Leski, T. (2010). Relationship between genotype and soil environment during colonization of poplar roots by mycorrhizal and endophytic fungi. *Mycorrhiza* 20, 315–324. doi: 10.1007/s00572-009-0284-8
- Keener, H. M., Dick, W. A., and Hoitink, H. A. J. (2000). “Composting and beneficial utilization of composted-by-product materials,” in *Land Application of Agricultural, Industrial, and Municipal By-Products*, eds J. F. Power, W. A. Dick, R. M. Kashmanian, J. T. Sims, R. J. Wright, M. D. Dawson, et al. (Madison, WI: Soil Science Society of America), 315–341.
- Kidd, P., Mench, M., Álvarez-López, V., Bert, V., Dimitriou, I., Friesl-Hanl, W., et al. (2015). Agronomic practices for improving gentle remediation of trace element-contaminated soils. *Int. J. Phytoremed.* 17, 1005–1037. doi: 10.1080/15226514.2014.1003788

- Kim, G., Miller, P. A., and Nowak, D. J. (2018). Urban vacant land typology: a tool for managing urban vacant land. *Sustain. Cities Soc.* 36, 144–156. doi: 10.1016/j.scs.2017.09.014
- Kirk, J. L., Klironomos, J. N., Lee, H., and Trevors, J. T. (2005). The effects of perennial ryegrass and alfalfa on microbial abundance and diversity in petroleum contaminated soil. *Environ. Pollut.* 133, 455–465. doi: 10.1016/j.envpol.2004.06.002
- Kowalchuk, G. A., Stephen, J. R., De Boer, W., Prosser, J. I., Embley, T. M., and Woldendorp, J. W. (1997). Analysis of ammonia-oxidizing bacteria of the β subdivision of the class *Proteobacteria* in coastal sand dunes by denaturing gradient gel electrophoresis and sequencing of PCR-amplified 16S ribosomal DNA fragments. *Appl. Environ. Microbiol.* 63, 1489–1497. doi: 10.1128/aem.63.4.1489-1497.1997
- Lacalle, R. G., Aparicio, J. D., Artetxe, U., Urionabarrenetxea, E., Polti, M. A., Soto, M., et al. (2020). Gentle remediation options for soil with mixed chromium (VI) and lindane pollution: biostimulation, bioaugmentation, phytoremediation and vermiremediation. *Heliyon* 6:e04550. doi: 10.1016/j.heliyon.2020.e04550
- Lacalle, R. G., Gómez-Sagasti, M. T., Artetxe, U., Garbisu, C., and Becerril, J. M. (2018). *Brassica napus* has a key role in the recovery of the health of soils contaminated with metals and diesel by rhizoremediation. *Sci. Total Environ.* 618, 347–356. doi: 10.1016/j.scitotenv.2017.10.334
- Lanzén, A., Epelde, L., Blanco, F., Martín, I., Artetxe, U., and Garbisu, C. (2016). Multi-targeted metagenetic analysis of the influence of climate and environmental parameters on soil microbial communities along an elevational gradient. *Sci. Rep.* 6:28257. doi: 10.1038/srep28257
- Lanzén, A., Jørgensen, S. L., Huson, D. H., Gorfer, M., Grindhaug, S. H., Jonassen, I., et al. (2012). CREST – classification resources for environmental sequence tags. *PLoS One* 7:e49334. doi: 10.1371/journal.pone.0049334
- Lingua, G., Franchin, C., Todeschini, V., Castiglione, S., Biondi, S., Burlando, B., et al. (2008). Arbuscular mycorrhizal fungi differentially affect the response to high zinc concentrations of two registered poplar clones. *Environ. Pollut.* 153, 137–147. doi: 10.1016/j.envpol.2007.07.012
- Ma, L., Wu, X., and Zheng, L. (2008). Mycorrhizal formation of nine ectomycorrhizal fungi on poplar cuttings. *Front. For. China* 3, 475–479. doi: 10.1007/s11461-008-0077-9
- Małachowska-Jutcz, A., and Kalka, J. (2010). Influence of mycorrhizal fungi on remediation of soil contaminated by petroleum hydrocarbons. *Fresenius Environ. Bull.* 19, 3217–3223.
- Marchand, C., Hogland, W., Kaczala, F., Jani, Y., Marchand, L., Augustsson, A., et al. (2016). Effect of *Medicago sativa* L. and compost on organic and inorganic pollutant removal from a mixed contaminated soil and risk assessment using ecotoxicological tests. *Int. J. Phytoremed.* 18, 1136–1147. doi: 10.1080/15226514.2016.1186594
- Marschner, H., and Dell, B. (1994). Nutrient uptake in mycorrhizal symbiosis. *Plant Soil* 159, 89–102. doi: 10.1007/BF00000098
- Martenssen, M., and Schirmer, M. (2010). Use of surfactants to improve the biological degradation of petroleum hydrocarbons in a field site study. *Environ. Technol.* 28, 573–582. doi: 10.1080/09593332808618816
- Martin, F., Tuskan, G. A., DiFazio, S. P., Lammers, P., Newcombe, G., and Podila, G. K. (2004). Symbiotic sequencing for the *Populus* mesocosm. *New Phytol.* 161, 330–335. doi: 10.1111/j.1469-8137.2004.00982.x
- McGuire, K. L., Payne, S. G., Palmer, M. L., Gillikin, C. M., Keefe, D., Kim, S. J., et al. (2013). Digging the New York City Skyline: soil fungal communities in green roofs and city parks. *PLoS One* 8:e58020. doi: 10.1371/journal.pone.0058020
- Míguez, F., Gómez-Sagasti, M. T., Hernández, A., Artetxe, U., Blanco, F., Castañeda, J. H., et al. (2020). In situ phytomanagement with *Brassica napus* and bio-stabilised municipal solid wastes is a suitable strategy for redevelopment of vacant urban land. *Urban For. Urban Green.* 47:126550. doi: 10.1016/j.ufug.2019.126550
- Miransari, M. (2011). Hyperaccumulators, arbuscular mycorrhizal fungi and stress of heavy metals. *Biotechnol. Adv.* 29, 645–653. doi: 10.1016/j.biotechadv.2011.04.006
- Mitter, E. K., de Freitas, J. R., and Germida, J. J. (2017). Bacterial root microbiome of plants growing in oil sands reclamation covers. *Front. Microbiol.* 8:849. doi: 10.3389/fmicb.2017.00849
- Mitter, E. K., de Freitas, J. R., and Germida, J. J. (2020). Hydrocarbon-degrading genes in root endophytic communities on oil sands reclamation covers. *Int. J. Phytoremed.* 22, 703–712. doi: 10.1080/15226514.2019.1707480
- Mukherjee, S., Juottonen, H., Siivonen, P., Lloret Quesada, C. L., Tuomi, P., Pulkkinen, P., et al. (2014). Spatial patterns of microbial diversity and activity in an aged creosote-contaminated site. *ISME J.* 8, 2131–2142. doi: 10.1038/ismej.2014.151
- Nassauer, J. I., and Raskin, J. (2014). Urban vacancy and land use legacies: a frontier for urban ecological research, design, and planning. *Landsc. Urban Plant.* 125, 245–253. doi: 10.1016/j.landurbplan.2013.10.008
- Németh, J., and Langhorst, J. (2014). Rethinking urban transformation: temporary uses for vacant land. *Cities* 40, 143–150. doi: 10.1016/j.cities.2013.04.007
- Niinemetts, Ü, Kollist, H., García-Plazaola, J. I., Hernández, A., and Becerril, J. M. (2003). Do the capacity and kinetics for modification of xanthophyll cycle pool size depend on growth irradiance in temperate trees? *Plant Cell Environ.* 26, 1787–1801. doi: 10.1046/j.1365-3040.2003.01096.x
- Oksanen, J., Blanchet, F. G., Friendly, M., Kindt, R., Legendre, P., McGlinn, D., et al. (2015). *Vegan: Community Ecology Package. R Package Version 2.4-2*.
- Øvreås, L., Forney, L., Daae, F. L., and Torsvik, V. (1997). Distribution of bacterioplankton in meromictic lake Saelenvannet, as determined by denaturing gradient gel electrophoresis of PCR-amplified gene fragments coding for 16S rRNA. *Appl. Environ. Microbiol.* 63, 3367–3373. doi: 10.1128/aem.63.9.3367-3373.1997
- Pallara, G., Todeschini, V., Lingua, G., Camussi, A., and Racchi, M. L. (2013). Transcript analysis of stress defence genes in a white poplar clone inoculated with the arbuscular mycorrhizal fungus *Glomus mosseae* and grown on a polluted soil. *Plant Physiol. Biochem.* 63, 131–139. doi: 10.1016/j.plaphy.2012.11.016
- Panchenko, L., Muratova, A., and Turkovskaya, O. (2017). Comparison of the phytoremediation potentials of *Medicago falcata* L. and *Medicago sativa* L. in aged oil-sludge-contaminated soil. *Environ. Sci. Pollut. Res.* 24, 3117–3130. doi: 10.1007/s11356-016-8025-y
- Perry, K. I., Hoekstra, N. C., Culman, S. W., and Gardiner, M. M. (2020). Vacant lot soil degradation and mowing frequency shape communities of belowground invertebrates and urban spontaneous vegetation. *Urban Ecosyst.* doi: 10.1007/s11252-020-01069-0
- Phillips, L. A., Greer, C. W., Farrell, R. E., and Germida, J. J. (2009). Field-scale assessment of weathered hydrocarbon degradation by mixed and single plant treatments. *Appl. Soil Ecol.* 42, 9–17. doi: 10.1016/j.apsoil.2009.01.002
- Quoreshi, A. M., and Khata, D. P. (2008). Effectiveness of mycorrhizal inoculation in the nursery on root colonization, growth, and nutrient uptake of aspen and balsam poplar. *Biomass Bioenergy* 32, 381–391. doi: 10.1016/j.biombioe.2007.10.010
- Shakya, M., Götzel, N., Castro, H., Yang, Z. K., Gunter, L., Labbé, J., et al. (2013). A multifactor analysis of fungal and bacterial community structure in the root microbiome of mature *Populus deltoides* trees. *PLoS One* 8:E76382. doi: 10.1371/journal.pone.0076382
- Steinauer, K., Chatzinotas, A., and Eisenhauer, N. (2016). Root exudate cocktails: the link between plant diversity and soil microorganisms? *Ecol. Evol.* 6, 7387–7396. doi: 10.1002/ece3.2454
- Szuba, A. (2015). Ectomycorrhiza of populus. *For. Ecol. Manage.* 347, 156–169. doi: 10.1016/j.foreco.2015.03.012
- Taghiyari, H. R., and Sisi, D. E. (2012). “The effects of tree-alfalfa intercropped systems on wood quality in temperate regions,” in *Agroforestry for Biodiversity and Ecosystem Services-Science and Practice*, ed. M. Kaonga (London: IntechOpen), 65–86.
- Teng, Y., Shen, Y., Luo, Y., Sun, X., Sun, M., Fu, D., et al. (2011). Influence of *Rhizobium meliloti* on phytoremediation of polycyclic aromatic hydrocarbons by alfalfa in an aged contaminated soil. *J. Hazard. Mater.* 186, 1271–1276. doi: 10.1016/j.jhazmat.2010.11.126
- ter Braak, C. J. F., and Smilauer, P. (2012). *Canoco Reference Manual and User's Guide: Software for Ordination, Version 5.0*. Ithaca, NY: Microcomputer Power.
- Thompson, P. L., and Polebitski, A. S. (2010). Effect of mycorrhizal fungi on the phytoremediation of hexahydro-1,3,5-trinitro-1,3,5-triazine (RDX). *Environ. Sci. Technol.* 44, 1112–1115.
- Thompson, P. L., Ramer, L. A., and Schnoor, J. L. (1998). Uptake and transformation of TNT by hybrid poplar trees. *Environ. Sci. Technol.* 32, 975–980.
- Tisdale, L. S., and Nelson, L. W. (1974). *Soil Fertility & Fertilizers*, 3rd Edn. London: Collier Macmillan.

- Tognetti, R., Coccozza, C., and Marchetti, M. (2013). Shaping the multifunctional tree: the use of *Salicaceae* in environmental restoration. *IForest* 6, 37–47. doi: 10.3832/for0920-006
- U.S. Environmental Protection Agency (US-EPA) (1996). *Method 3050B: Acid digestion of sediments, sludges, and soils*, Vol. 18. Washington, DC: U.S. Environmental Protection Agency, 723. doi: 10.18907/jjsre.18.7_723_2
- Vangronsveld, J., Herzig, R., Weyens, N., Boulet, J., Adriaensen, K., Ruttens, A., et al. (2009). Phytoremediation of contaminated soils and groundwater: lessons from the field. *Environ. Sci. Pollut. Res.* 16, 765–794. doi: 10.1007/s11356-009-0213-6
- Wellstein, C., Poschlod, P., Gohlke, A., Chelli, S., Campetella, G., Rosbakh, S., et al. (2017). Effects of extreme drought on specific leaf area of grassland species: a meta-analysis of experimental studies in temperate and sub-Mediterranean systems. *Glob. Chang. Biol.* 23, 2473–2481. doi: 10.1111/gcb.13662
- Yadav, A. N., Verma, P., Kumar, S., Kumar, V., Kumar, M., Kumari Sugitha, T. C., et al. (2018). “Actinobacteria from rhizosphere: molecular diversity, distributions, and potential biotechnological applications,” in *New and Future Developments in Microbial. Biotechnology and Bioengineering: Actinobacteria Diversity and Biotechnological Applications*, eds B. P. Singh, V. K. Gupta, and A. K. Passari (Amsterdam: Elsevier), 13–41. doi: 10.1016/B978-0-444-63994-3.00002-3
- Zeng, P., Guo, Z., Xiao, X., and Peng, C. (2019). Dynamic response of enzymatic activity and microbial community structure in metal(loid)-contaminated soil with tree-herb intercropping. *Geoderma* 345, 5–16. doi: 10.1016/j.geoderma.2019.03.013
- Zhang, H., Liu, T., Wang, Y., and Tang, M. (2019). Exogenous arbuscular mycorrhizal fungi increase soil organic carbon and change microbial community in poplar rhizosphere. *Plant Soil Environ.* 65, 152–158. doi: 10.17221/2/2019-PSE
- Zhang, M., Wang, J., Bai, S. H., Zhang, Y., Teng, Y., and Xu, Z. (2019). Assisted phytoremediation of a co-contaminated soil with biochar amendment: contaminant removals and bacterial community properties. *Geoderma* 348, 115–123. doi: 10.1016/j.geoderma.2019.04.031
- Zheng, J., Chen, J., Pan, G., Wang, G., Liu, X., Zhang, X., et al. (2017). A long-term hybrid poplar plantation on cropland reduces soil organic carbon mineralization and shifts microbial community abundance and composition. *Appl. Soil Ecol.* 111, 94–104. doi: 10.1016/j.apsoil.2016.11.017

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.

Copyright © 2021 Gómez-Sagasti, Garbisu, Urra, Míguez, Artetxe, Hernández, Vilela, Alkorta and Becerril. This is an open-access article distributed under the terms of the Creative Commons Attribution License (CC BY). The use, distribution or reproduction in other forums is permitted, provided the original author(s) and the copyright owner(s) are credited and that the original publication in this journal is cited, in accordance with accepted academic practice. No use, distribution or reproduction is permitted which does not comply with these terms.



Characterization of Arsenic-Resistant Endophytic Bacteria From Alfalfa and Chickpea Plants

Hazhir Tashan¹, Behrouz Harighi^{2*}, Jalal Rostamzadeh³ and Abdolbaset Azizi²

¹ Department of Life Science Engineering, Faculty of New Sciences and Technologies, University of Tehran, Tehran, Iran,

² Department of Plant Protection, Faculty of Agriculture, University of Kurdistan, Sanandaj, Iran, ³ Department of Animal Sciences, Faculty of Agriculture, University of Kurdistan, Sanandaj, Iran

OPEN ACCESS

Edited by:

Maite Lacuesta,
University of the Basque
Country, Spain

Reviewed by:

Sudisha Jogaiah,
Karnatak University, India
Maria Teresa Gomez-Sagasti,
University of the Basque
Country, Spain

*Correspondence:

Behrouz Harighi
bharighi@uok.ac.ir

Specialty section:

This article was submitted to
Plant Abiotic Stress,
a section of the journal
Frontiers in Plant Science

Received: 17 April 2021

Accepted: 24 June 2021

Published: 22 July 2021

Citation:

Tashan H, Harighi B, Rostamzadeh J
and Azizi A (2021) Characterization of
Arsenic-Resistant Endophytic Bacteria
From Alfalfa and Chickpea Plants.
Front. Plant Sci. 12:696750.
doi: 10.3389/fpls.2021.696750

The present investigation was carried out to isolate arsenic (As)-resistant endophytic bacteria from the roots of alfalfa and chickpea plants grown in arsenic-contamination soil, characterize their As tolerance ability, plant growth-promoting characteristics, and their role to induce As resistance by the plant. A total of four root endophytic bacteria were isolated from plants grown in As-contaminated soil (160–260-mg As kg⁻¹ of soil). These isolates were studied for plant growth-promoting (PGP) characteristics through siderophore, phosphate solubilization, nitrogen fixation, protease, and lipase production, and the presence of the arsenate reductase (*arsC*) gene. Based on 16S rDNA sequence analysis, these isolates belong to the genera *Acinetobacter*, *Pseudomonas*, and *Rahnella*. All isolates were found As tolerant, of which one isolate, *Pseudomonas* sp. QNC1, showed the highest tolerance up to 350-mM concentration in the LB medium. All isolates exhibited phosphate solubilization activity. Siderophore production activity was shown by only *Pseudomonas* sp. QNC1, while nitrogen fixation activity was shown by only *Rahnella* sp. QNC2 isolate. *Acinetobacter* sp. QNA1, QNA2, and *Rahnella* sp. QNC2 exhibited lipase production, while only *Pseudomonas* sp. QNC1 was able to produce protease. The presence of the *arsC* gene was detected in all isolates. The effect of endophytic bacteria on biomass production of alfalfa and chickpea in five levels of arsenic concentrations (0-, 10-, 50-, 75-, and 100-mg kg⁻¹ soil) was evaluated. The fresh and dry weights of roots of alfalfa and chickpea plants were decreased as the arsenic concentration of the soil was increased. Results indicate that the fresh and dry root weights of alfalfa and chickpea plants were significantly higher in endophytic bacteria-treated plants compared with non-treated plants. Inoculation of chickpea plants with *Pseudomonas* sp. QNC1 and *Rahnella* sp. QNC2 induced lower *NPR3* gene expression in chickpea roots grown in soil with the final concentration of 100-mg kg⁻¹ sodium arsenate compared with the non-endophyte-treated control. The same results were obtained in *Acinetobacter* sp. QNA2-treated alfalfa plants grown in the

soil plus 50-mg kg⁻¹ sodium arsenate. These results demonstrated that arsenic-resistant endophytic bacteria are potential candidates to enhance plant-growth promotion in As contamination soils. Characterization of bacterial endophytes with plant growth potential can help us apply them to improve plant yield under stress conditions.

Keywords: alfalfa, arsenic resistance, chickpea, endophytic bacteria, plant growth-promoting

INTRODUCTION

Plants are constantly affected by abiotic stress excreted by the environment. Heavy metals can reduce plant development and crop productivity when their concentration rises beyond supraoptimal values for plant normal functioning (Tiware and Lata, 2018). As a consequence of industrialization during the past or from geogenic sources, the heavy metal concentration of soils has been increased worldwide (Adriano, 2001). Arsenic (As) is a heavy metal present in various soil and water ecosystem (Chowdhury et al., 1999; Abernathy et al., 2003). Arsenic contamination of natural environments is a major problem and has been reported in more than 20 countries (Jack et al., 2003). The contamination of as in groundwater used for irrigation not only affects plant growth but also accumulates in different plant tissues and contaminates the food chain (Tiware and Lata, 2018). A high concentration of arsenic in water, plants, and soils has been reported from the south-eastern part of Kurdistan province, Iran (Mosaferi et al., 2003; Zandsalimi et al., 2011). The concentration of arsenic in these sites ranged from 100–300 mg kg⁻¹ of soil. Heavy contamination of this trace element has adversely affected human health. Uptake and accumulation of arsenic in crops grown in polluted soils lead to poor growth and subsequent yield loss. Besides, arsenic can enter into edible parts of the plant, such as seeds and fruit, making them dangerous for consumers.

Excessive release of heavy metals into the environment results in plants to develop different mechanisms to cope with their negative effects (Franco-Franklin et al., 2021). A study of plant responses and tolerance revealed that several genes are induced under heavy metal stress. Heavy metals activate different signaling pathways in plants, such as calcium-dependent signaling, mitogen-activated protein kinase (MAPK) signaling, reactive oxygen species (ROS) signaling, and phytohormonal response (Dutta et al., 2018).

Plants are naturally associated with diverse microorganisms in various ways. Among them, endophytic bacteria can be defined as those bacteria that colonize the internal tissue of the plant, showing no external sign of infection or negative effect on their host (Schulz and Boyle, 2006). Endophytic bacteria can promote plant growth by several mechanisms that include indole acetic acid (IAA) synthesis, phosphate solubilization activity, production of siderophore under Fe-limiting conditions, improving mineral nutrient uptake by plants, and nitrogen fixation activity (Ryan et al., 2008). Endophytic bacteria can promote the growth of the plant hosts, even under abiotic stress conditions (Franco-Franklin et al., 2021). Several reports also

indicate the induction of genes in plants that are central to heavy metal stress signaling in the presence of beneficial bacteria, such as *Pseudomonas* and *Bacillus* (Tiware and Lata, 2018).

It is well-known that bacteria isolated from polluted habitats are tolerant to higher levels of heavy-metal concentration (Sessitsch and Puschenreiter, 2008). Arsenic-tolerant bacteria were described more than a century ago (Green, 1918; Green and Kestell, 1920). They play an important role in the transformation of arsenic, thus affecting its bioavailability and toxicity (Oremland and Stolz, 2005). Recently, Satapute et al. (2019) have reported *Pseudomonas taiwanensis* strain resistant to arsenic. Genes responsible for arsenic tolerance have been identified in many bacteria and organized in the *ars* cluster (Ben Fekih et al., 2018). Recent research has shown that endophytic bacteria play important roles in plant response to abiotic stresses, helping the plant host to adapt to the ecological niche occupied (Sim et al., 2019). Indeed, some of the endophytic bacteria could alleviate plant hosts from heavy metals toxicity (Román-Ponce et al., 2018). Several mechanisms in the arsenic-resistant bacteria have been characterized that enable them to affect the arsenic mobility, solubility, and toxicity in the contaminated environments (Gadd, 2010). Several studies have shown that several endophytic bacteria are suitable candidates for reducing the arsenic stress to the host plants (Nie et al., 2002; Wang et al., 2011; Babu et al., 2013; Mesa et al., 2017).

Chickpea (*Cicer arietinum* L.) and alfalfa (*Medicago sativa* L.) are rich sources of seed proteins and primarily grown for use in animal feed, respectively, and consumed worldwide. In Iran, chickpea and alfalfa plants, in ~450 and 500,000 hectares of land, are planted. Total production is estimated at over 200,000 and 4 million tons, respectively, and they are among the top five most important crops growing in various parts of Iran (Anonymous, 2019). Both plants are also being grown in arsenic-contaminated areas in Iran. Despite its economical importance, not much study exists on the negative effects of As on these plants. Previous study reported As toxicity on chickpea plants grown in As-contaminated situations (Malik et al., 2011). The previous studies on alfalfa and chickpea plants grown in arsenic-contaminated conditions showed this heavy metal to be reduced plant biomass production (Saadati et al., 2017). Srivastava and Singh (2014) reported arsenic-tolerant chickpea growth-promoting bacteria isolated from As-contaminated sites. Although the use of endophytic bacteria to promote the growth of plant hosts had been reported, their influence on increased resistant to high concentration of As is still not well-understood. Therefore, this study aimed to identify endophytic bacteria resistance to the high concentrations of arsenic isolated from contaminated areas, examined the effects of arsenic on biomass

production of alfalfa and chickpea, inoculated with these strains, and evaluated the effects of these bacteria on the induction of systemic resistance in plants grown in an As-polluted soil.

MATERIALS AND METHODS

Isolation and Identification of Endophytic Bacteria

The sampling sites are located in Neyband in the Kurdistan province. Chickpea (*Cicer arietinum* var. Kabuli ILC482) and alfalfa (*Medicago sativa* var. Hamedani) plants were collected from arable land sampling site 1 (35° 12' N, 48° 04' E) and sampling site 2 (35° 12' N, 48° 7' E), with the altitude of about 2,040 and 2,021 m, respectively, during June–July 2019. The two sampling sites have a distance of about 4.5 km. The total arsenic concentrations of about 260- and 160-mg kg⁻¹ soil have been detected in the sampling sites 1 and 2, respectively. In total, eight samples from each chickpea and alfalfa plants were collected during the vegetative growth stage.

For isolation of endophytic bacteria, 1 g of roots was washed with tap water, surface sterilized with 0.5% sodium hypochlorite solution for 2–3 min, and rinsed three times in sterile distilled water. Afterward, they were crushed in 5-ml sterile distilled water and cultured on yeast extract mannitol agar (YMA) or nutrient agar (NA) media. To confirm that the disinfection process was successful, the aliquots of the sterile distilled water used in the final rinse were cultured on an NA medium. Plates were incubated at 26–28°C for 14 h, and single colonies with different morphologies were purified onto the same medium. Bacteria were grown in the Luria-Bertani (LB) medium at 26–28°C for 24–48 h; sterile glycerol was added to a final concentration of 20% and stored at –70°C.

Genomic DNA of endophytic bacteria was extracted from each isolate. Overnight-grown culture of bacteria in 5 ml of LB medium was treated by adding SDS/lysozyme, followed by three-time DNA extraction with phenol/chloroform/isoamyl alcohol (25/24/1 volume) and once with chloroform/isoamyl alcohol (24/1). DNA was precipitated with 1-volume 3-M sodium acetate (pH: 4.8) and two volumes of absolute ethanol overnight at –20°C. Finally, the suspension was centrifuged at 14,000 rpm for 5 min; DNA was washed with 70% ethanol, dried, and resuspended in 50 µl of TE (Tris-Cl 0.1M, EDTA 0.01M, pH:8) buffer. Through PCR reactions, a DNA fragment corresponding to 16S rRNA gene was amplified by using two universal primers, fD2 (5'-AGA GTT TGA TCA TGG CTC AG-3', position 8-27) and rP1 [5'-ACG GTT ACC TTG TTA CGA CTT-3', position 1,512-1,492 (*Escherichia coli*)], following the method described by Weisburg et al. (1991). The purified amplicons were sequenced, using an ABI3730XL DNA sequencer (Applied Biosystems, Foster City, CA, USA). The 16S rRNA sequences were manually edited with BioEdit Sequence Alignment Editor 7.0.9.0 software (Hall, 2011). The phylogenetic tree was constructed by the neighbor-joining method with Kimura's two-parameter calculation model in

MEGA version 6.0 (Tamura et al., 2013). The reliability of the phylogenetic analysis was evaluated by bootstrap analysis with 1,000 replicates, using the same program. The sequences obtained were deposited in the GenBank under accession numbers MN658698 to MN658701.

Characterization of Isolated Endophytic Bacteria

The presence of the *arsC* gene was targeted to detect arsenic resistance in isolated endophytes. PCR was performed, using the degenerate primers *arsCF* (5'-ATGACCGTCACCATTAYCAYAAAYC-3') and *arsCR* (5'-ACGACCGCCTCGCCRTCYTCYTT-3') located 1–25 and 393–415 bp, respectively, under the following conditions: 1 cycle at 95°C for 3 min; 35 cycles at 95°C for 1 min, 48°C for 1 min; and 72°C for 45 s; and a final extension cycle at 72°C for 10 min (Sa-Pereira et al., 2007). The PCR products were sequenced as described above. The sequences were used for a Blast search in the NCBI GenBank database to verify the gene identity. The sequences obtained were deposited in the GenBank under accession numbers MN782313 and MN782314.

Phosphate solubilization activity was assayed on Pikovskaya agar medium according to Naik et al. (2008). Siderophore production was measured on chrome azurol-S (CAS) medium, following the method previously described (Schwyn and Neilands, 1987). The ability of isolates to fix nitrogen on nitrogen-free NFB medium was assessed, using methods previously described by Döbereiner et al. (1995).

The production of protease and lipase was screened for each bacterial isolate. Protease production was determined according to the method previously described (Sgroy et al., 2009), with some modifications. Plates containing skim milk agar (SMA) medium (g⁻¹ of distilled water: a pancreatic digest of casein, 5; yeast extract, 2.5; glucose, 1; 7% skim milk and agar, 15) were inoculated with 10 µl of freshly prepared bacterial isolates, incubated at 28°C for up to 4 days and observed for the formation of the halo zone around the colonies. Lipase production by endophytic bacteria was tested based on the method described by Sierra (1957). A nutrient agar medium was prepared, and tween 80 was added to a final concentration of about 1%. The bacterial suspension was spot inoculated on media; the plates were incubated at 26–28°C and observed for the production of an opaque halo around the colonies.

To investigate the arsenic tolerance property, bacterial strains were grown in the LB liquid medium at 26–28°C for 24 h. Cultures were centrifuged (5 min, 7,000 rpm), and the pellet was suspended in sterile-distilled water to a concentration of ~1 × 10⁷ CFU ml⁻¹ (OD_{600nm} = 0.1). About 10 µl of suspension was inoculated into culture tubes of 10-ml LB media, containing sodium arsenate (Na₂HAsO₄·7H₂O) at final concentrations of 150, 200, 250, 300, 350, and 400 mM. Inoculated media were incubated at 26–28°C with shaking at 150 rpm. The minimum inhibitory concentration (MIC) at which bacterial strains failed to grow was monitored by measuring OD_{600 nm} after 72 h.

TABLE 1 | Primer sequences used for RT-PCR analysis of a defense-related gene.

Primer	Sequence (5'→3')	Length	Tm	GC%	Self complementarity	Self 3' complementarity
<i>EF1α</i> Forward	TCTGTTGTTGTAACAAGATGGATGC	25	60.05	40.00	8.00	2.00
<i>EF1α</i> Reverse	AAGGTTGGTGGACCTCTCAATC	22	59.96	50.00	5.00	5.00
<i>NPR3</i> Forward	GTACCTTGAGAACAGAGTGGCA	22	59.96	50.00	4.00	3.00
<i>NPR3</i> Reverse	GAAAGACCAGCAAACCTCGGATG	22	59.84	50.00	3.00	1.00

Inoculated media without sodium arsenate was used as a control. Assays were conducted with three replications.

Effect of Endophytic Bacteria on Biomass Production

Pots containing steam-sterilized soil (As: 62.933 ppb; Silica: 28.44%; Clay: 14.44%; Sand: 57.12%; EC: 2.7 ms cm⁻¹; pH: 7.24) plus 10-, 50-, 75-, 100-mg kg⁻¹ soil of sodium arsenate or without As (control) were prepared and incubated for 4 weeks to allow the As to equilibrate. After that, seeds of alfalfa (var. Hamedani) and chickpea (Kabuli ILC482) were sterilized and potted. After adding 5 ml from 1 × 10¹⁰ CFU ml⁻¹ of each bacterial strain, pots were placed in the greenhouse (28–30°C; 16:8 day/night photoperiod) and watered every 2 days. Eight weeks after inoculation, the plants were harvested, the roots with nodules excised, and the number of nodules per plant was counted. Fresh roots of each plant were weighted and rinsed three times in distilled water before being dried for 48 h at 70°C, and dry roots were weighted. The experiments were carried out with three replications, each pot containing five plants, and the results are the mean of at least three harvested plant samples.

Plant Colonization

Ten days after inoculation of alfalfa and chickpea plants with endophytic bacteria, 200-mg samples of roots, stems, and leaves were selected, surface sterilized with 0.8% sodium hypochlorite for 5 min, and washed several times with sterile water. Plant tissues were then homogenized aseptically with a pestle and mortar in 1-ml distilled water. Aliquots of 30 µl were spread onto nutrient agar media, incubated at 26–28°C, and, after 48 h, the colonies were counted as CFU g⁻¹ of plant tissues. All the experiments were repeated three times with three replicates, using control healthy plants inoculated with a PBS buffer.

$$\text{Fold change due to treatment} = 2^{-\Delta\Delta C} \\ = 2^{-[\text{CT of the target gene} - \text{CT of the reference gene}] \text{ sample A} - [\text{CT of the target gene} - \text{CT of the reference gene}] \text{ sample B.}}$$

NPR3 Gene Expression Analysis

Roots of endophytic bacteria-treated plants grown in soil containing 50 and/or 100 mg kg⁻¹ of sodium arsenate (sample A) and untreated plants grown in soil without sodium arsenate (sample B) were collected after 10 days, grounded in liquid nitrogen, and stored in a sterile microtube at -70°C until used. For total RNA extraction, 50 mg of frozen powdered tissues were homogenized in 1 ml of TRIzol reagent and incubated for 5 min at room temperature. Then, 0.2 ml of chloroform was added to

the sample, vortexed vigorously for 15 s, and incubated at room temperature for 2–3 min. Samples were centrifuged (12,000 × g, 10 min, 4°C), and the upper aqueous phase was transferred into a fresh tube. For RNA precipitation, the aqueous phase was mixed with an equal volume of isopropyl alcohol; the sample was incubated at 15 to 30°C for 10 min and centrifuged (12,000 × g, 10 min, 4°C). The supernatant was removed completely, an RNA pellet was washed with 70% ethanol and centrifuged at 7,000 × g for 10 min at 4°C. The above washing procedure was repeated one time and RNA pellet was air-dried for 5–10 min. RNA was dissolved in 50-µl DEPC-treated water and stored at -70°C until used. The concentration and the purity of RNA were determined by measuring the ratio of OD at 260/280 nm, using Multidrop™ Pico 1 Digital Dispenser (Thermo Fisher Scientific, USA).

cDNA was amplified from 200 ng of total RNA, using the cDNA synthesis Kit (Yekta Tajhiz Azma, Iran) according to the instructions of the manufacturer. The expression of the targeted gene, including the *NPR3*, was checked in roots 10 days after treated by endophytic bacteria. Real-time PCR (RT-PCR) was performed with SYBR Green qPCR Master Mix (Yekta Tajhiz Azma, Iran), using an AB Applied Biosystem StepOne thermal cycler. PCR reactions were carried out in a 15-µl final volume, containing 5 µl of SYBR Green Master Mix, 10 µM forward and reverse primers (Table 1), and 1 µl of 10-fold diluted cDNA. Cycling conditions were 1 min at 95°C, followed by 40 cycles of 20 s at 95°C; 30 s, 58°C; and 30 s at 72°C. Traditional reference gene *EF1α*, with stable expression, was used as internal controls (a housekeeping gene) for normalization. Several studies have shown the stability of the *EF1α* gene for RT-PCR in alfalfa and chickpea plants (Garg et al., 2010; Wang et al., 2015). Before RT-PCR, the specificity of each primer was checked, using heat dissociation at 60°C. Relative gene expression was calculated with the following formula (Schmittgen and Livak, 2008):

CT values were the means of three biological replications and three technical replications.

Statistical Analysis

The experiments were carried out with a factorial arrangement in a completely randomized design with three replications, two levels of inoculation, and five levels of sodium arsenate concentration (0-, 10-, 50-, 75-, and 100-mg kg⁻¹ soil). Data analysis was performed, using the SAS software program, and

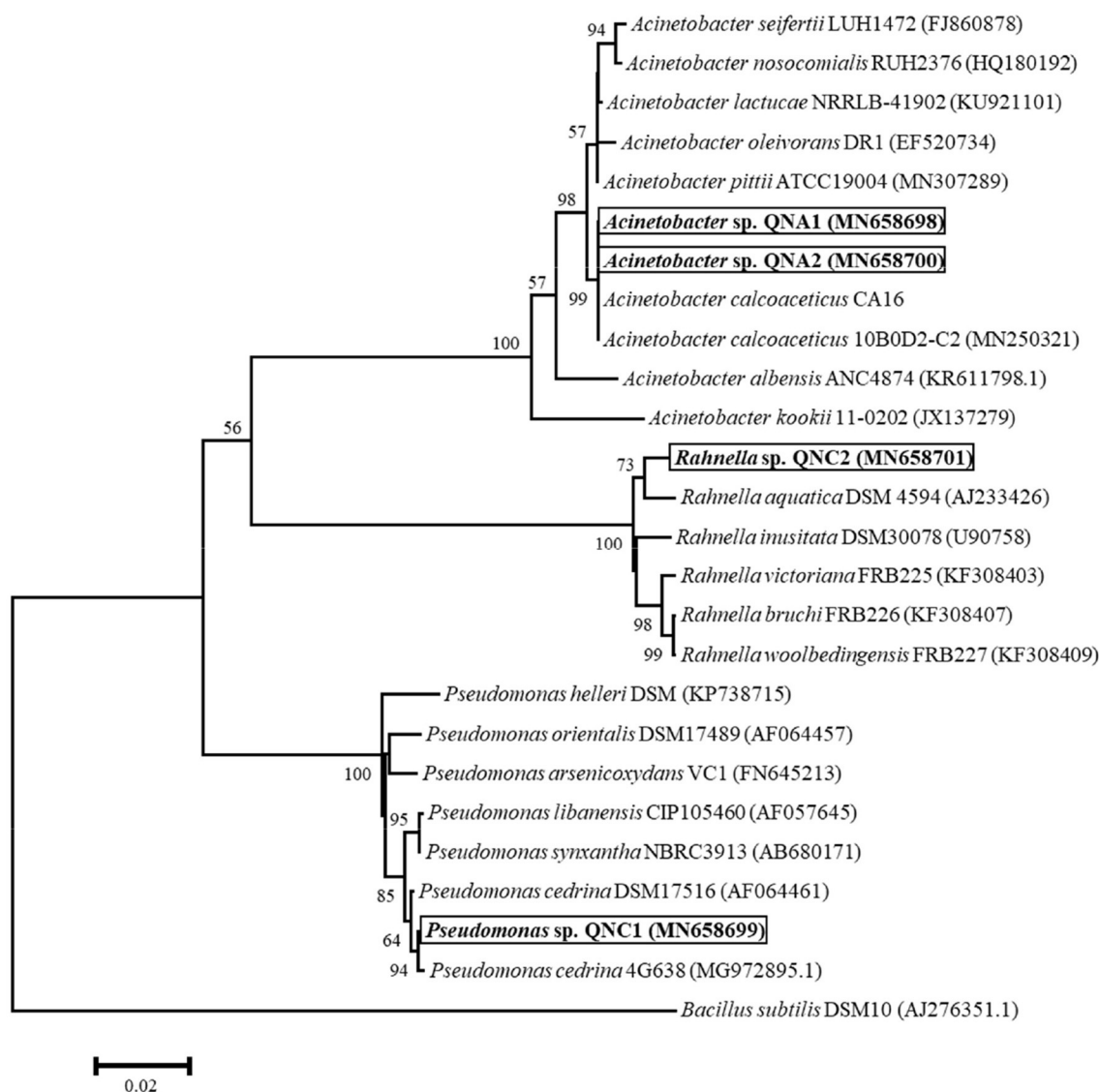


FIGURE 1 | The phylogenetic tree of partial 16S rRNA gene sequences, showing the position of *Pseudomonas* sp. QNC1, *Rahnella* sp. QNC2, and *Acinetobacter* sp. QNA1 and QNA2 strains in addition to taxonomically closest reference strains. The analysis was conducted by the neighbor-joining method with Kimura's two-parameter calculation model in MEGA version 6.0. The scale bar represents the number of substitutions per site. Numbers at branching points indicate bootstrap value derived from 1,000 replicates.

a comparison of means was done with Fisher's least significant difference (LSD) test at a 5% level. Normality of data was verified, using the Shapiro-Wilk test. The graphs were plotted with Excel software.

RESULTS

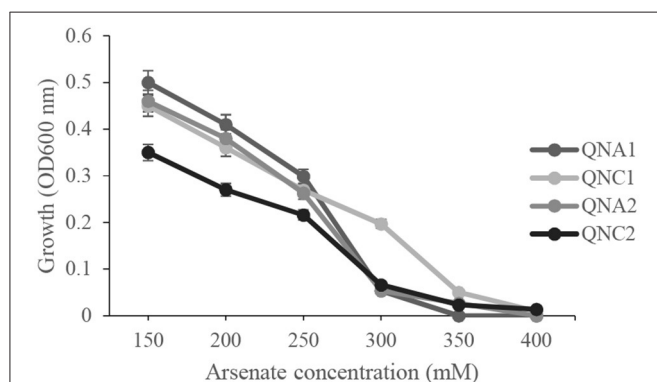
Identification of Endophytic Bacteria

A total of four cultivable endophytic bacteria were isolated from the roots, including two from alfalfa (QNA1 and QNA2) and two from chickpea (QNC1 and QNC2). Based on the 16S rRNA gene sequencing results, a phylogenetic tree was constructed, showing the position of strains within the type strains of

the species of the genera *Pseudomonas*, *Acinetobacter*, and *Rahnella* (Figure 1). The phylogenetic analysis illustrated that two strains, namely QNA1 (MN658698) and QNA2 (MN658700) were grouped among species of the genus *Acinetobacter* and showing the closest 100% similarity with *A. calcoaceticus* CA16, a bacterium isolated from Canadian soil with bioremediation potential (Ho et al., 2020). QNA1 and QNA2 strains showed 100% similarity; therefore, QNA2 was selected for further analysis. Strain QNC1 (MN658699) formed a subclade and showing 99% similarity with *Pseudomonas cedrina* DSM17516 within the genus *Pseudomonas*. Also, QNC2 (MN658701) formed a subclade and showed the closest 99% similarity with *Rahnella aquatica* DSM4594.

TABLE 2 | Plant growth potential properties of endophytic bacterial strains isolated from alfalfa and chickpea plants.

Strain	Phosphate solubilization	Siderophore production	Nitrogen fixation activity	Protease production	Lipase production
QNA2	+	–	–	–	+
QNC1	+	+	–	+	–
QNC2	+	–	+	–	+

**FIGURE 2** | Effect of different concentrations of sodium arsenate on the growth of *Pseudomonas* sp. QNC1, *Rahnella* sp. QNC2, *Acinetobacter* sp. QNA1 and *Acinetobacter* sp. QNA2.

Molecular Characterization of the Arsenic Resistant Isolate

The arsenic resistance gene, namely, *arsC*, reported for the biosynthesis of arsenate reductase in bacteria was targeted to detect the isolated endophytic bacteria. PCR amplification, using specific primers, results in the production of an expected DNA fragment of about 400 bp in QNA2, QNC1, and, QNC2 strains. The resulting PCR product sequences obtained from QNA2 and QNC2 showed the closest homology with arsenate reductase gene sequences previously reported in *Acinetobacter calcoaceticus* str. 2117 and *Rahnella aquatilis* HX2, respectively.

Plant Growth-Promoting and Enzymatic Characteristics

Endophytic bacterial strains were assayed for several traits that are important for plant growth, and the results are summarized in Table 2. *Acinetobacter* sp. QNA2, *Rahnella* sp. QNC2 and *Pseudomonas* sp. QNC1 strains were able to solubilize phosphate. Among the endophytic bacteria tested, siderophore production and nitrogen fixation activity were found in *Pseudomonas* sp. QNC1 and *Rahnella* sp. QNC2, respectively. The strain *Pseudomonas* sp. QNC1 showed positive for protease production, while *Acinetobacter* sp. QNA2 and *Rahnella* sp. QNC2 were able to produce lipase.

Arsenic Tolerance

The isolated bacterial endophytes were further tested for arsenic tolerance by determining their growth in liquid media, containing 150–400 mM sodium arsenate. The minimal

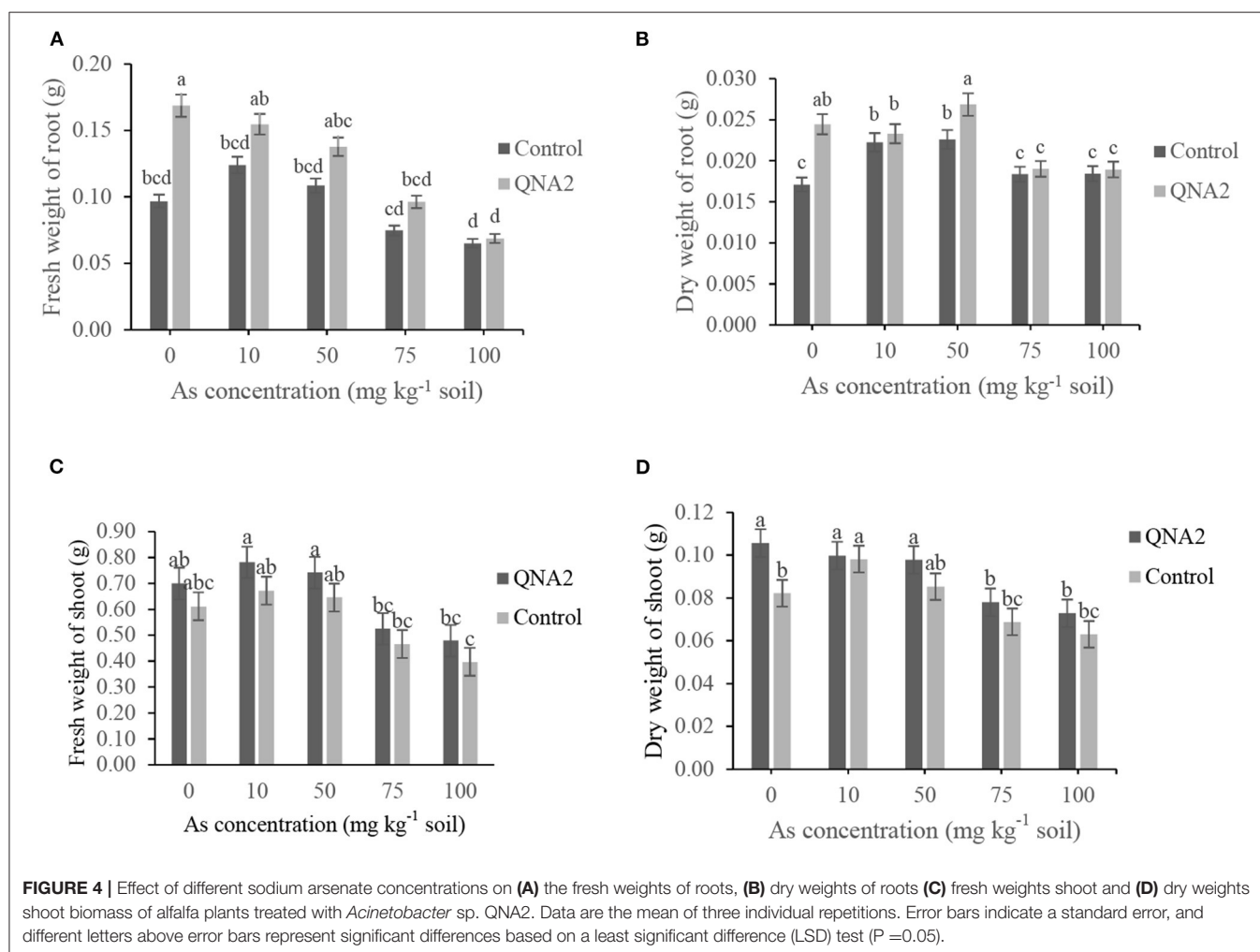
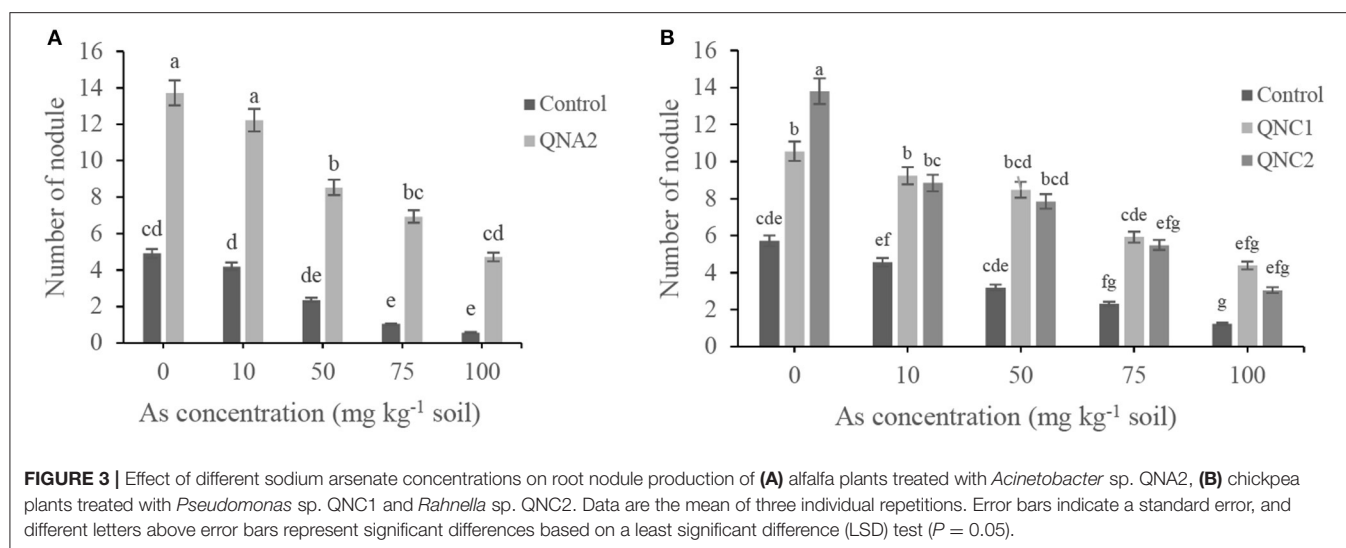
inhibitory concentration of arsenate against *Rahnella* sp. QNC2 and *Acinetobacter* sp. QNA1 and QNA2 was 300 mM, while only strain *Pseudomonas* sp. QNC1 showed tolerance to 350-mM sodium arsenate concentration (Figure 2).

Plant Biomass Production

Endophytic bacterial strains were screened for their plant growth-promoting attributes in soil supplemented with sodium arsenate. The number of nodules and root biomass of treated plants with endophytic bacteria was increased compared with non-treated plants. The number of nodules of alfalfa and chickpea plants was decreased as the As concentration was increased. For all of the traits, alfalfa plants treated with QNA2 showed significantly increased 2.8-, 2.9-, 3.6-, 6.9-, and 8.2-fold nodule numbers in plants grown in the presence of As (0-, 10-, 50-, 75-, and 100-mg kg⁻¹ soil), respectively, than non-treated plants (Figure 3A). Chickpea plants treated with QNC1 showed significantly increased 1.8-, 2.0-, 2.6-, 2.5-, and 2.4-fold nodule numbers in plants grown in the presence of As (0-, 10-, 50-, 75-, and 100-mg kg⁻¹ soil), respectively, than non-treated plants. In comparison, the endophytic strains QNC2 significantly increased nodule numbers by 2.4-, 1.9-, 2.4-, 2.3-, and 2.4-fold in plants grown in the presence of As (0-, 10-, 50-, 75-, and 100-mg kg⁻¹ soil), respectively, than non-treated plants. However, the number of nodules of plants treated with QNC1 did not significantly differ from that of those treated with QNC2 (Figure 3B).

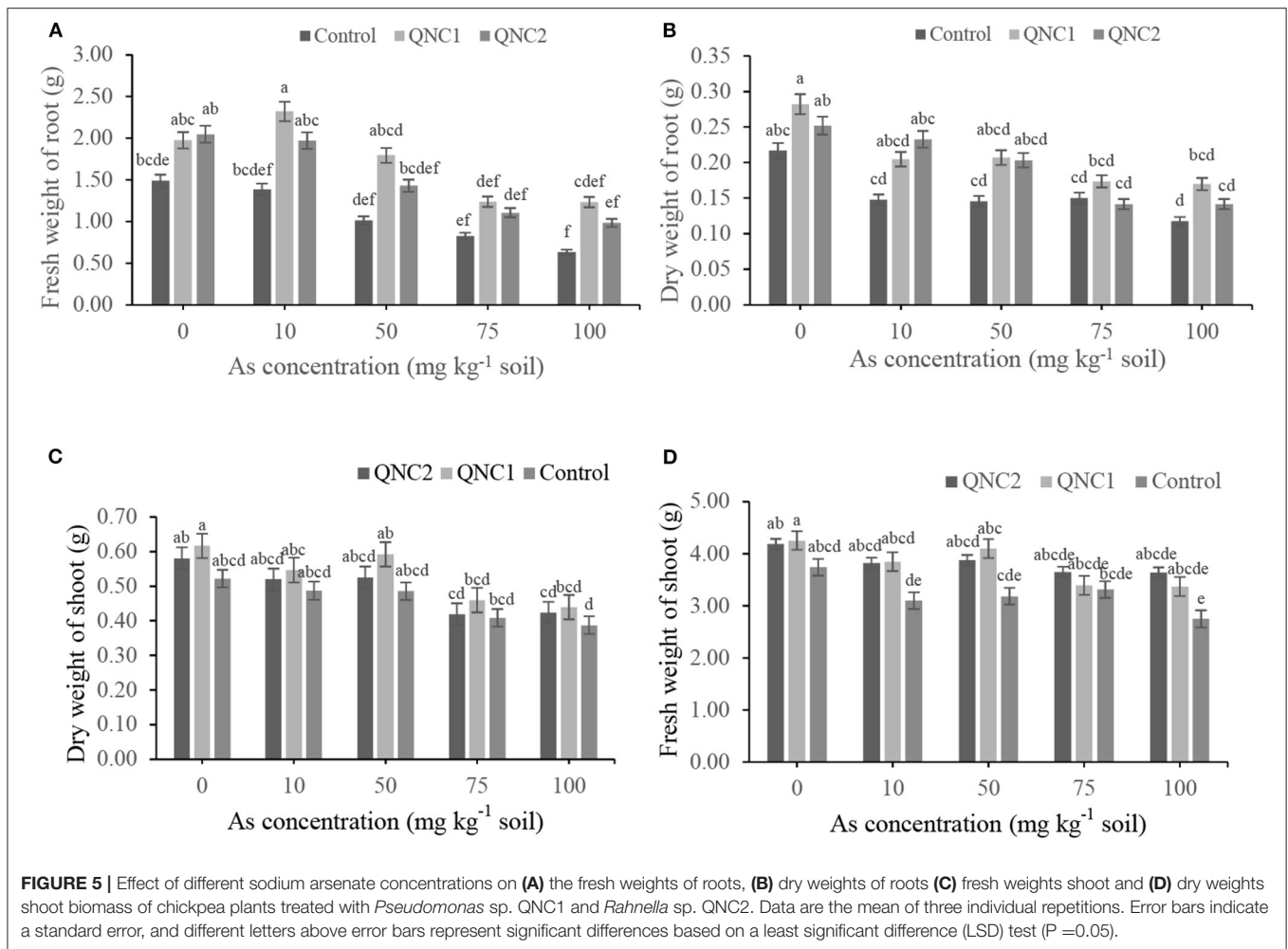
The fresh and dry weights of alfalfa roots were decreased as the As concentration of the soil was increased, both in the treated plants with QNA2 strain and the non-treated control (Figures 4A,B). In comparison with non-treated alfalfa, the endophytic strain QNA2 increased the root fresh weights by 74, 24, 26, 28, and 5% and root dry weights by 42, 4, 18, 3, and 2% in plants grown in the presence of As (0-, 10-, 50-, 75-, and 100-mg kg⁻¹ soil), respectively. In the presence of As (50-mg kg⁻¹ soil), significantly higher root biomass was observed in the plants treated with QNA2 than in the control. Similarly, the fresh and dry weights of alfalfa shoots were decreased as the As concentration of the soil was increased, both in the treated plants with QNA2 strain and the non-treated control (Figures 4C,D). The endophytic strain QNA2 increased the shoot fresh weights by 14, 16, 14, 13, 20% and shoot dry weights by 28, 1, 15, 13, and 14% in plants grown in the presence of As (0-, 10-, 50-, 75-, and 100-mg kg⁻¹ soil), respectively, compared with non-treated alfalfa plants.

The fresh and dry weights of chickpea roots were decreased as the As concentration of the soil was increased, both in the treated plants with QNC1 and QNC2 strains and the non-treated control (Figures 5A,B). In comparison with non-treated chickpea, the



endophytic strain QNC1 increased the root fresh weights by 32, 67, 77, 50, 94% and root dry weights by 30, 38, 42, 15, and 44% in plants grown in the presence of As (0-, 10-, 50-, 75-, and

100-mg kg⁻¹ soil), respectively. Chickpea plants treated with QNC2 increased the root fresh weights by 37, 42, 41, 34, 55% and root dry weight by 16, 57, 39, and 20% in plants grown in the



presence of As (0, 10, 50, 75, and 100-mg kg⁻¹ soil), respectively, than the non-treated plants. Although the plants treated with QNC1 and QNC2 always presented significantly higher fresh and dry weight of roots compared with control, no significant differences occurred between the two strains. Compared with non-treated chickpea, QNC1 strain significantly increased the shoot fresh weights by 13, 24, 26, 2, 22%, and shoot dry weights by 18, 12, 21, 12, and 9% in plants grown in the presence of As (0-, 10-, 50-, 75-, and 100-mg kg⁻¹ soil), respectively. Chickpea plants treated with QNC2 increased the shoot fresh weights by 11, 23, 21, 10, and 32% and shoot dry weight by 11, 6, 8, 2, and 9% in plants grown in the presence of As (0-, 10-, 50-, 75-, and 100-mg kg⁻¹ soil), respectively. In the majority of treatments, QNC1 and QNC2 strains showed increasing effects on fresh and dry shoot weights compared with control; no significant differences occurred between the two strains.

Colonization of Plants by Endophytic Bacteria

Ten days after treatment of alfalfa and chickpea plants with bacterial endophytes, shoot and root colonization was assessed quantitatively (CFU g⁻¹ of plant tissues). Colonies similar to

QNA2, QNC1, and QNC2 strains were recovered from both the surface-sterilized shoots and roots of plants suggesting that these bacteria were able to colonize and/or spread throughout the internal tissues of plants. No bacteria were recovered from the control plants. In all treatments, the number of bacteria was decreased as the As concentration of the soil was increased. For all of the traits, the number of bacteria recovered from the roots was higher than that recovered from the shoots (Table 3).

Gene Expression

qRT-PCR was conducted to examine the relative expression levels of the *NPR3* gene in roots of alfalfa plants treated by QNA2 or treated-chickpea plants by QNC1 and QNC2. The control treatment (plants grown in soil without As) was defined as calibrator and is expressed in the graphs with the value 1, and all the treatments were compared with the control. The results revealed that the expression of the *NPR3* gene in the roots of alfalfa plants treated with QNA2 grown in soil without sodium arsenate was 5.5-fold higher than the control. Surprisingly, the expression level of the *NPR3* gene in QNA2-treated alfalfa plants was 10-fold lower than control when the soil was supplemented with 50-mg/kg of sodium arsenate

TABLE 3 | The population of *Acinetobacter* sp. QNA2 in shoots and roots of alfalfa plants, *Pseudomonas* sp. QNC1 and *Rahnella* sp. QNC2 in chickpea plants grown in soils, containing 0-, 10-, 50-, 75- and 100-mg kg⁻¹ sodium arsenate 10 days after treatment.

Bacterial strain	Sodium arsenate concentration (mg kg ⁻¹ soil)	CFU g ⁻¹ of Alfalfa		CFU g ⁻¹ of Chickpea	
		Fresh shoot	Fresh root	Fresh shoot	Fresh root
<i>Acinetobacter</i> sp. QNA2	0	5.6 × 10 ³	1.1 × 10 ⁴	–	–
	10	5.2 × 10 ³	5.3 × 10 ³	–	–
	50	1.8 × 10 ³	3.2 × 10 ³	–	–
	75	1.5 × 10 ³	3.7 × 10 ³	–	–
	100	6.6 × 10 ²	1.6 × 10 ³	–	–
<i>Pseudomonas</i> sp. QNC1	0	–	–	6.5 × 10 ³	1.3 × 10 ⁴
	10	–	–	4.3 × 10 ³	7.3 × 10 ³
	50	–	–	4.5 × 10 ³	4.5 × 10 ³
	75	–	–	3.0 × 10 ³	4.0 × 10 ³
	100	–	–	2.0 × 10 ³	2.2 × 10 ³
<i>Rahnella</i> sp. QNC2	0	–	–	5.5 × 10 ³	8.7 × 10 ³
	10	–	–	4.7 × 10 ³	5.3 × 10 ³
	50	–	–	2.5 × 10 ³	4.2 × 10 ³
	75	–	–	1.2 × 10 ³	2.8 × 10 ³
	100	–	–	3.3 × 10 ²	2.5 × 10 ³

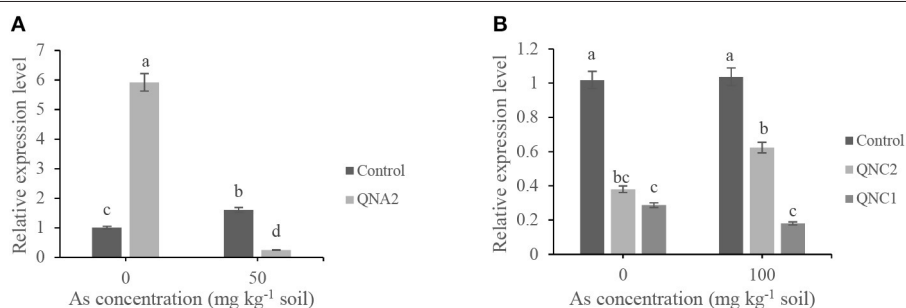


FIGURE 6 | Relative expression levels of the *NPR3* gene in the roots of (A) non-treated alfalfa plants (control) and plants treated by *Acinetobacter* sp. QNA2, (B) non-treated chickpea plants (control), and plants treated by *Pseudomonas* sp. QNC1 and *Rahnella* sp. QNC2. The results represent the means of three replicates. Vertical bars indicate standard errors (SE), and different letters indicate statistically significant differences between treatments at probability levels of 5%.

(Figure 6A). Significant differences were observed between the expression level of the *NPR3* gene in the roots of chickpea plants treated by QNC1 and QNC2 strains compared with the control (Figure 6B). The relative expression level of the *NPR3* gene was significantly decreased in chickpea plants treated by both strains in soil without or with 100 mg kg⁻¹ of sodium arsenate compared with the control.

DISCUSSION

Arsenic is one of the most hazardous environmental pollutants worldwide. It is present in high concentrations in some areas of Kurdistan province and has direct effects on human health. Although both organic arsenic and inorganic arsenic are toxic to organisms, some microorganisms are resistant to this heavy metal due to its genetic makeup. It is well-documented that endophytic bacteria can confer heavy metal resistance to plants due to their metal resistance capacity (Ma et al., 2016). Previous reports indicated that bacteria resistant

to arsenic mostly belong to phyla *Proteobacteria*, *firmicutes*, and *Actinobacteria* (Sheik et al., 2012). Several genera like *Bacillus*, *Paenibacillus*, *Staphylococcus*, *Kocuria*, *Sphigomonas*, *Arthrobacter*, *Brevibacterium*, *Pseudomonas*, *Microbacterium*, *Enterobacter*, *Stenotrophomonas*, and *Rhizobium* have been reported as arsenic-resistant endophytic bacteria (Zhu et al., 2014; Román-Ponce et al., 2016, 2018; Tiwari et al., 2016).

Our finding in the present study revealed the characterization of endophytic bacteria belonged to the genera *Pseudomonas*, *Acinetobacter*, and *Rahnella*. *Pseudomonas* is one of the most versatile bacteria with a dynamic distribution of species that adapted to different ecological environments. *Pseudomonas* species were previously reported as As-resistant endophytic bacteria in contaminated areas (Gu et al., 2018). Moreover, the existence of species from *Acinetobacter* and *Rahnella* genera as endophytic bacteria with plant growth promotion activity has also been reported by many researchers (He et al., 2013; Suzuki et al., 2014). All identified strains could grow in media, containing a high concentration of As, which displayed the

minimum inhibitory concentration of 300–350 mM for sodium arsenate under our experimental conditions. The ability of bacterial cells to tolerate high concentrations of As contributes to their ecological fitness. This finding is in agreement with previously reported the As-resistant properties of bacterial strains belonging to the same genera (Anderson and Cook, 2004; Valverde et al., 2011; Marwa et al., 2018). Our results suggest that adaptation of such endophytic bacteria to contaminated sites is a result of long-term exposure to high As concentrations (Cai et al., 2009). The resistance property was confirmed by PCR, using primers derived from the *arsC* gene. The *arsC* gene encodes a cytoplasmic arsenate reductase and is highly conserved among microorganisms (Mukhopadhyay et al., 2002). This gene has an essential function in the arsenic resistance mechanism. The presence of the *arsC* gene in strains identified in this study confirms the resistance to arsenic based on the *ars* operon mechanism. In a previous study, As-resistant rhizospheric bacteria were also isolated and characterized from plants (Saadati et al., 2017). The results presented in this study revealed that the resistance of endophytic bacterial strains to As is moderately lower than that of rhizospheric bacteria. It is well-known that, compared with endophytic bacteria, rhizospheric bacteria are more resistant to environmental stress because they are in direct contact with the outside environment (Abedinzadeh et al., 2019).

All endophytic bacteria strains reported in the current study were able to colonize and/or spread throughout the internal tissues of plant hosts. Plant colonization is an essential factor in the efficacy of bacteria to further stimulate plant growth (Silva et al., 2003). All strains exhibited at least one hydrolytic enzyme activity, thus demonstrating that such enzymatic activities may aid in the colonization of the plant hosts (Wang and Dai, 2010). Endophytic bacteria have several competitive advantages over other bacteria, because of their close and continued contact with plants. There is increasing interest in finding beneficial endophytic bacteria and modulating their potential application for improving plant growth under stress conditions. Bacterial strains characterized in this study showed multiple plant growth promotion traits. This indicates that As contamination has not been able to prevent the production of such properties.

There are many endophytic bacteria reported to be directly or indirectly involved in plant growth promotion by siderophore production, nitrogen fixation, and solubilization of phosphate (Compant et al., 2010). Siderophores are organic molecules with a high affinity for Fe (III), thus participating in iron availability for plants, even under limited essential metal availability or in polluted soil (Rajkumar et al., 2010, 2012; Schalk et al., 2011). The previous report indicated that siderophore forms a stable complex with As; thus, the application of bacteria with siderophore production activity proves beneficial for As remediation (Jeong et al., 2014). Several reports have indicated that different endophytic bacterial species can solubilize insoluble inorganic phosphate compounds. These bacteria make available the soluble phosphates to the plant hosts (Oteino et al., 2015). In addition, arsenic is an analog of phosphorus that enters through phosphate transporters into the roots (Pinter et al., 2017). In

addition to As-resistance, detection of some of the plant growth promotion properties among the strains in the present study indicates that these endophytic bacteria may help their plant host to resist in the arsenic-contaminated areas, although their ability to express such properties under high As concentration needs to be investigated. Because these bacteria were isolated from arsenic-contaminated sites, they have the advantage of being adapted to such stressful situations.

In the present study, the high As concentration added to the soil negatively affected plant growth. Usually, a high concentration of As in the soil interferes with the normal absorption of nutrients like P and Fe, which results in inhibiting plant growth. In this context, inoculation with endophytic bacteria showed an improved positive effect on the number of nodules, shoot and root biomass compared with the non-treated plants. This effect can occur due to their ability to solubilize phosphate, siderophore production, and/or fix nitrogen to provide a suitable form of P, Fe, and N, resulting in stimulating plant growth. It is well-documented that many non-rhizobial bacteria are nitrogen fixers and induce nitrogen-fixing nodules on legume roots (Martinez-Hidalgo and Hirsch, 2017). Results presented in this study indicated that inoculation of plants with endophytic bacteria showed increasing effects on the number of nodules. Although confirming the direct role of these bacteria in inducing nodule formation needs more experiments. Based on the results obtained, all bacterial strains tested could colonize roots and shoots of inoculated alfalfa and chickpea plants grown in soil supplemented with different concentrations of As. It was previously reported that the ability of bacteria to colonize plant tissues made them more effective for enhancing beneficial effects (Mallick et al., 2014).

Induction of systemic resistance is the immunity response mechanism in plants that can be triggered by endophytic bacteria during abiotic stress conditions, including heavy metal (Ghosh et al., 2020). The relative expression level of the *NPR3* gene in alfalfa and chickpea plants treated with endophytic bacteria in soil supplemented with 50- and 100-mg kg⁻¹ concentrations of As, respectively, was analyzed. As shown in **Figure 4**, in most experiments, higher significant differences were obtained between QNA2-treated alfalfa plants and non-treated control under 50 mg kg⁻¹ of sodium arsenate. Similarly, based on the results presented in **Figure 5**, in most experiments, higher significant differences were obtained between QNC1, QNC2-treated chickpea plants, and non-treated control under 100 mg kg⁻¹ of sodium arsenate. Therefore, we have determined the relative expression levels of the *NPR3* gene in alfalfa and chickpea plants grown under 50 and 100 mg kg⁻¹ of sodium arsenate, respectively. Before RT-PCR, analysis of primers specificity was conducted by the heat dissociation test. The corresponding dissociation curve shows primers specificity, and the heat dissociation curve was formed at 60°C. It is well-documented that salicylic acid (SA) ameliorates As toxicity in plants. Salicylic acid is reported to eventually trigger the NPR1 (Non-expressor of Pathogenesis-Related genes1) mediated pathway for systemic acquired resistance (SAR) in plants under stress (Klessig et al.,

2000). In addition, the NPR1 paralogues NPR3 and NPR4 are SA receptors that mediate NPR1 degradation in an SA-regulated manner (Fu et al., 2012). The results presented in this study indicated that the expression level of the *NPR3* gene in the roots of alfalfa and chickpea plants induced a low level or remained unchanged, respectively, when As was added to the final concentration of 50 and 100 mg kg⁻¹ of soil. These results suggest that As cannot induce systemic-acquired resistance *via* an NPR-dependent pathway. In contrast, upon an endophytic bacteria challenge, treated plants demonstrated a lower abundance of *NPR3* gene expression compared with the non-treated plants, which indicates activated NPR-dependent defense pathways.

In conclusion, we isolated and characterized several arsenic-resistant endophytic bacteria from the root of leguminous plants growing in arsenic-contaminated areas in Kurdistan province, Iran for the first time. All bacterial strains showed a high level of tolerance to As. This finding suggests that the acquisition of tolerance to As may play an essential role in an adaptation of bacteria to As. All these bacteria studied had a beneficial effect on the plants and promoted growth in soil supplemented with As. The As-resistance and plant growth promotion properties of these bacteria demonstrate their

potentiality for the establishment of plants in As-contaminated environments. Further studies will be done on understanding the mechanisms involved in the interactions between plant hosts and such endophytic bacteria finding applications as a bioremediation tool with great ecological importance.

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.

AUTHOR CONTRIBUTIONS

HT conducted all the experiments and analyzed all the data but was assisted by JR, BH, and AA performed the gene expression quantification experiments. BH wrote the manuscript. All the authors read and approved the manuscript.

FUNDING

This research work was supported by the University of Kurdistan, Iran.

REFERENCES

- Abedinzadeh, M., Etesami, H., and Alikhani, H. A. (2019). Characterization of rhizosphere and endophytic bacteria from roots of maize (*Zea mays* L.) plant irrigated with wastewater with biotechnological potential in agriculture. *Biotechnol. rep.* 21:e00305. doi: 10.1016/j.btre.2019.e00305
- Abernathy, C. O., Thomas, D. J., Calderon, R. L. (2003). Health effects and risk assessment of arsenic. *J. Nutr.* 133, 1536–1538. doi: 10.1093/jn/133.5.1536S
- Adriano, D. C. (2001). *Trace Elements in Terrestrial Environments: Biogeochemistry, Bioavailability and Risk of Metals*. New York, NY: Springer, New York.
- Anderson, C. R., and Cook, G. M. (2004). Isolation and characterization of arsenate-reducing bacteria from arsenic-contaminated sites in New Zealand. *Curr. Microbiol.* 48, 341–347. doi: 10.1007/s00284-003-4205-3
- Anonymous (2019). FAOSTAT, *Statistics, Crops Production*. Available online at: <http://www.fao.org/faostat/en/#data/QC/visualize>.
- Babu, A.G., Kim, J. D., and Oh, B. T. (2013). Enhancement of heavy metal phytoremediation by *Alnus firma* with endophytic *Bacillus thuringiensis* GDB-1. *J. Hazard. Mater.* 250, 477–483. doi: 10.1016/j.jhazmat.2013.02.014
- Ben Fekih, I., Zhang, C., Li, Y. P., Zhao, Y., Alwathnani, H. A., Saquib, Q., et al. (2018). Distribution of arsenic resistance genes in prokaryotes. *Front. Microbiol.* 9:2473. doi: 10.3389/fmicb.2018.02473
- Cai, L., Liu, G. H., Rensing, C., and Wang, G. J. (2009). Genes involved in arsenic transformation and resistance associated with different levels of arsenic-contaminated soils. *BMC Microbiol.* 9:4. doi: 10.1186/1471-2180-9-4
- Chowdhury, T. R., Basu, G. K., Mandal, B. K., Biswas, B. K., Samanta, G., Chowdhury, U. K., et al. (1999). Arsenic poisoning in Ganges delta. *Nature* 401, 545–546. doi: 10.1038/44056
- Compant, S., Christophe 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
- Döbereiner, J., Baldani, V., and Baldani, J. (1995). Como isolar e identificar bacterias diazotroficas de plantas nao-leguminosas. *Brasilia:EMBRAPA-SPI: Itagui:EMBRAPA-CNPAB*, 19–25.
- Dutta, S., Mitra, M., Agarwal, P., Mahapatra, K., De, S., Sett, U., et al. (2018). Oxidative and genotoxic damages in plants in response to heavy metal stress and maintenance of genome stability. *Plant Signal. Behav.* 13:e1460048. doi: 10.1080/15592324.2018.1460048
- Franco-Franklin, V., Moreno-Riascos, S., and Ghneim-Herrera, T. (2021). Are endophytic bacteria an option for increasing heavy metal tolerance of plants? A meta-analysis of the effect size. *Front. Environ. Sci.* 8:294. doi: 10.3389/fenvs.2020.603668
- Fu, Z.Q., Yan, S., Saleh, A., Wang, W., Ruble, J., Oka, N., et al. (2012). NPR3 and NPR4 are receptors for the immune signal salicylic acid in plants. *Nature* 486, 228–232. doi: 10.1038/nature11162
- Gadd, G. M. (2010). Metals, minerals and microbes: geomicrobiology and bioremediation. *Microbiology* 156, 609–643. doi: 10.1099/mic.0.037143-0
- Garg, R., Sahoo, A., Tyagi, A. K., and Jain, M. (2010). Validation of internal control genes for quantitative gene expression studies in chickpea (*Cicer arietinum* L.). *Biochem. Biophys. Res. Commun.* 396, 283–288. doi: 10.1016/j.bbrc.2010.04.079
- Ghosh, S., Bhagwat, T., and Webster, T. J. (2020). Endophytic Microbiomes and their plant growth-promoting attributes for plant health. *Curr. Trends Microbial Biotech. Sustain. Agric.* 245–278. doi: 10.1007/978-981-15-6949-4_11
- Green, H. H. (1918). “Description of a bacterium isolated from a cattle dipping tank, which reduces arsenate to arsenite,” in *Union of South Africa, of Agriculture, 5th and 6th Reports of the Director of Veterinary Research*, 613–624.
- Green, H. H., and Kestell, N. H. (1920). “Behaviour of bacteria towards arsenic,” in *Union of South Africa, of Agriculture, 5th and 6th Reports of the Director of Veterinary Research*, 701–706.
- Gu, Y., Wang, Y., Sun, Y., Zhao, K., Xiang, Q., Yu, X., et al. (2018). Genetic diversity and characterization of arsenic-resistant endophytic bacteria isolated from *Pteris vittata*, an arsenic hyperaccumulator. *BMC Microbiol.* 18:42. doi: 10.1186/s12866-018-1184-x
- Hall, T. (2011). BioEdit: an important software for molecular biology. *GERF Bull. Biosci.* 2, 60–61.
- He, H., Ye, Z., Yang, D., Yan, J., Xiao, L., Zhong, T., et al. (2013). Characterization of endophytic *Rahnella* sp. JN6 from *Polygonum pubescens* and its potential in promoting growth and Cd, Pb, Zn uptake by *Brassica napus*. *Chemosphere* 90, 1960–1965. doi: 10.1016/j.chemosphere.2012.10.057
- Ho, M.T., Li, M.S.M., McDowell, T., MacDonald, J., and Yuan, Z.C. (2020). Characterization and genomic analysis of a diesel-degrading bacterium,

- Acinetobacter calcoaceticus* CA16, isolated from Canadian soil. *BMC Biotechnol.* 20:39. doi: 10.1186/s12896-020-00632-z
- Jack, C. N., Wang, J., and Shraim, A. A. (2003). Global health problem caused by arsenic from natural sources. *Chemosphere* 52, 1353–1359. doi: 10.1016/S0045-6535(03)00470-3
- Jeong, S., Moon, H. S., and Nam, K. (2014). Enhanced uptake and translocation of arsenic in Cretan brake fern (*Pteris cretica* L.) through siderophore arsenic complex formation with an aid of rhizospheric bacterial activity. *J. Hazard. Mater.* 280, 536–543. doi: 10.1016/j.jhazmat.2014.08.057
- Klessig, D. F., Durner, J., Noad, R., Navarre, D. A., Wendehenne, D., Kumar, D., et al. (2000). Nitric oxide and salicylic acid signaling in plant defense. *Proc. Natl. Acad. Sci.* 97, 8849–8855. doi: 10.1073/pnas.97.16.8849
- Ma, Y., Rajkumar, M., Zhang, C., and Freitas, H. (2016). Beneficial role of bacterial endophytes in heavy metal phytoremediation. *J. Environ. Manag.* 174, 14–25. doi: 10.1016/j.jenvman.2016.02.047
- Malik, J. A., Goel, S., Sandhir, R., and Nayyar, H. (2011). Uptake and distribution of arsenic in chickpea: effects on seed yield and seed composition. *Commun. Soil Sci. Plant Anal.* 42, 1728–1738. doi: 10.1080/00103624.2011.584593
- Mallick, I., Hossain, S. T., Sinha, S., and Mukherjee, S. K. (2014). *Brevibacillus* sp. KUMAs2, a bacterial isolate for possible bioremediation of arsenic in rhizosphere. *Ecotoxicol. Environ. Saf.* 107, 236–244. doi: 10.1016/j.ecoenv.2014.06.007
- Martinez-Hidalgo, P., and Hirsch, A. M. (2017). The nodule microbiome: N₂-fixing rhizobia do not live alone. *Phytobiomes* 1, 70–82. doi: 10.1094/PBIOMES-12-16-0019-RVW
- Marwa, N., Singh, N., Srivastava, S., Saxena, G., Pandey, V., and Singh, N. (2018). Characterizing the hypertolerance potential of two indigenous bacterial strains (*Bacillus flexus* and *Acinetobacter junii*) and their efficacy in arsenic bioremediation. *J. Appl. Microbiol.* 126, 1117–1127. doi: 10.1111/jam.14179
- Mesa, V., Navas, A., González-Gil, R., Gonzalez, A., Weyens, N., Lauga, B., et al. (2017). Use of endophytic and rhizosphere bacteria to improve phytoremediation of As-contaminated industrial soils by autochthonous *Betula celti-berica*. *Appl. Environ. Microbiol.* 83, 03411–03416. doi: 10.1128/AEM.03411-16
- Mosaferi, M., Yunesian, M., Mesdaghinia, A. R., Nadim, A., Naseri, S., and Mahvi, A. H. (2003). "Occurrence of arsenic in Kurdistan province of Iran," in *BUET-UNU International Symposium, International Training Network Centre*. Bangladesh, Tokyo: Dhaka.
- Mukhopadhyay, R., Rosen, B. P., Phung, L. T., and Silver, S. (2002). Microbial arsenic: from geocycles to genes and enzymes. *FEMS Microbiol. Rev.* 26, 311–325. doi: 10.1111/j.1574-6976.2002.tb00617.x
- Naik, P. R., Raman, G., Narayanan, K. B., and Sakthivel, N. (2008). Assessment of genetic and functional diversity of phosphate solubilizing fluorescent pseudomonads isolated from rhizospheric soil. *BMC Microbiol.* 8:230. doi: 10.1186/1471-2180-8-230
- Nie, L., Shah, S., Rashid, A., Burd, G. I., Dixon, D. G., and Glick, B. R. (2002). Phytoremediation of arsenate contaminated soil by transgenic canola and the plant growth-promoting bacterium *Enterobacter cloacae* CAL2. *Plant Physiol. Biochem.* 40, 355–361. doi: 10.1016/S0981-9428(02)01375-X
- Oremland, R. S., and Stolz, J. F. (2005). Arsenic, microbes and contaminated aquifers. *Trends Microbiol.* 13, 45–49. doi: 10.1016/j.tim.2004.12.002
- Oteino, N., Lally, R. D., Kiwanuka, S., Lloyd, A., Ryan, D., Germaine, K. J., and Dowling, D. N. (2015). Plant growth promotion induced by phosphate solubilizing endophytic *Pseudomonas* isolates. *Front. Microbiol.* 6:745. doi: 10.3389/fmicb.2015.00745
- Pinter, I. F., Salomon, M. V., Berli, F., Bottini, R. and Piccoli, P. (2017). Characterization of the As (III) tolerance conferred growth promoting rhizobacteria to in vitro grown grapevine. *Appl. Soil Ecol.* 109, 60–68. doi: 10.1016/j.apsoil.2016.10.003
- Rajkumar, M., Ae, N., Prasad, M. N., and Freitas, H. (2010). Potential of siderophore-producing bacteria for improving heavy metal phytoextraction. *Trends Biotechnol.* 28, 142–149. doi: 10.1016/j.tibtech.2009.12.002
- Rajkumar, M., Sandhya, S., Prasad, M. N. V., and Freitas, H. (2012). Perspectives of plant-associated microbes in heavy metal phytoremediation. *Biotechnol. Adv.* 30, 1562–1574. doi: 10.1016/j.biotechadv.2012.04.011
- Román-Ponce, B., Ramos-Garza, J., Arroyo-Herrera, I., Maldonado-Hernández, J., Bahena-Osorio, Y., Vásquez-Murrieta, M. S., et al. (2018). Mechanism of arsenic resistance in endophytic bacteria isolated from endemic plant of mine tailings and their arsenophore production. *Arch. Microbiol.* 200, 883–895. doi: 10.1007/s00203-018-1495-1
- Román-Ponce, B., Ramos-Garza, J., Vásquez-Murrieta, M.S., et al. (2016). Cultivable endophytic bacteria from heavy metal (loid)-tolerant plants. *Arch. Microbiol.* 198, 941–956. doi: 10.1007/s00203-016-1252-2
- 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
- Saadati, R., Bahramnejad, B., and Harighi, B. (2017). Characterization of rhizobial bacteria isolated from arsenic contaminated site in south-eastern Kurdistan province and their influence on plant growth. *J. Soil Biol.* 5, 15–27. doi: 10.22092/sbj.2017.113117
- Sa-Pereira, P., Rodrigues, M., Videira e Casatro, I., and Simoes, F. (2007). Identification of an arsenic resistance in mechanism rhizobial strains. *World J. Microbiol. Biotech.* 23, 1351–1356. doi: 10.1007/s11274-007-9370-2
- Satapute, P., Paidi, M. K., Kurjogi, M., and Jogaiah, S. (2019). Physiological adaptation and spectral annotation of Arsenic and Cadmium heavy metal-resistant and susceptible strain *Pseudomonas taiwanensis*. *Environ. Pollut.* 251, 555–563. doi: 10.1016/j.envpol.2019.05.054
- 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
- Schmittgen, T., and Livak, K. (2008). Analyzing real-time PCR data by the comparative C_T method. *Nat. Protoc.* 3, 1101–1108. doi: 10.1038/nprot.2008.73
- Schulz, B., and Boyle, C. (2006). "What are endophytes?" in *Microbial Root Endophytes*, eds B. J. E. Schulz, C. J. C. Boyle, T. N. Sieber (Springer-Verlag, Berlin), 1–13
- Schwyn, B., and Neilands, J. B. (1987). Universal chemical assay for the detection and determination of siderophores. *Anal. Biochem.* 160, 47–56. doi: 10.1016/0003-2697(87)90612-9
- Sessitsch, A., and Puschenreiter, M. (2008). "Endophytes and rhizosphere bacteria of plants growing in heavy metal-containing soils," in *Microbiology of Extreme Soils. Soil Biology*, eds P. Dion, C. S. Nautiyal (New York, NY: Springer).
- Sgroi, V., Cassan, F., Masciarelli, O., Del Papa, M. F., Lagares, A., and Luna, V. (2009). Isolation and characterization of endophytic plant growth promoting (PGPB) or stress homeostasis-regulating (PSHB) bacteria associated to the halophyte *Prosopis strombulifera*. *Appl. Microbiol. Biotech.* 85, 371–381. doi: 10.1007/s00253-009-2116-3
- Sheik, C. S., Mitchell, T. W., Rizvi, F. Z., Rehman, Y., Faisal, M., Hasmain, S., et al. (2012). Exposure of soil microbial communities to chromium and arsenic alters their diversity and structure. *PLoS ONE* 7:e40059. doi: 10.1371/journal.pone.0040059
- Sierra, G. A. (1957). A simple method for the detection of lipolytic activity of micro-organisms and some observations on the influence of the contact between cells and fatty substrates. *Antonie van Leeuwenhoek* 23, 15–22. doi: 10.1007/BF02545855
- Silva, H. S. A., Romeiro, R. S., and Munteer, A. (2003). Development of a root colonization bioassay for rapid screening of Rhizobacteria for potential biocontrol agents. *J. Phytopathol.* 151, 42–46. doi: 10.1046/j.1439-0434.2003.00678.x
- Sim, C. S. F., Chen, S. H., and Ting, A. S. Y. (2019). "Endophytes: emerging tools for the bioremediation of pollutants," in *Emerging and Eco-Friendly Approaches for Waste Management*, (Singapore, Springer), 189–217.
- Srivastava, S., and Singh, N. (2014). Mitigation approach of arsenic toxicity in chickpea grown in arsenic amended soil with arsenic tolerant plant growth promoting *Acinetobacter* sp., *Ecol. Engin* 70, 146–153. doi: 10.1016/j.ecoleng.2014.05.008
- Suzuki, W., Sugawara, M., Miwa, K., and Morikawa, M. (2014). Plant growth-promoting bacterium *Acinetobacter calcoaceticus* P23 increases the chlorophyll content of the monocot *Lemna minor* (duckweed) and the dicot *Lactuca sativa* (lettuce). *J. Biosci. Bioeng.* 118, 41–44. doi: 10.1016/j.jbiosc.2013.12.007
- Tamura, K., Stecher, G., Peterson, D., Filipowski, A., and Kumar, S. (2013). MEGA6: molecular evolutionary genetics analysis version 6.0. *Mol. Biol. Evol.* 30, 2725–2729. doi: 10.1093/molbev/mst197

- Tiwari, S., and Lata, C. (2018). Heavy metal stress, signaling, and tolerance due to plant-associated microbes: an overview. *Front. Plant Sci.* 9:452. doi: 10.3389/fpls.2018.00452
- Tiwari, S., Sarangi, B. K., and Thul, S. T. (2016). Identification of arsenic resistant endophytic bacteria from *Pteris vittata* roots and characterization for arsenic remediation application. *J. Environ. Manag.* 180, 359–365. doi: 10.1016/j.jenvman.2016.05.029
- Valverde, A., González-Tirante, M., Medina-Sierra, M., Santa-Regina, I., García-Sánchez, A., and Igual, J. M. (2011). Diversity and community structure of culturable arsenic-resistant bacteria across a soil arsenic gradient at an abandoned tungsten–tin mining area. *Chemosphere* 85, 129–134. doi: 10.1016/j.chemosphere.2011.06.025
- Wang, Q., Xiong, D., Zhao, P. et al. (2011). Effect of applying an arsenic-resistant and plant growth-promoting rhizobacterium to enhance soil arsenic phytoremediation by *Populus deltoides* LH05-17. *J. Appl. Microbiol.* 111, 1065–1074. doi: 10.1111/j.1365-2672.2011.05142.x
- Wang, X., Fu, Y., Ban, L., Wang, Z., Feng, G., Li, J., et al. (2015). Selection of reliable reference genes for quantitative real-time RT-PCR in alfalfa. *Genes Genet. Syst.* 90, 175–180. doi: 10.1266/ggs.90.175
- Wang, Y., and Dai, C. C. (2010). Endophytes: a potential resource for biosynthesis, biotransformation, and biodegradation. *Ann. Microbiol.* 61, 207–215. doi: 10.1007/s13213-010-0120-6
- Weisburg, W. G., Barns, S. M., Pelletier, D. A., and Lane, D. J. (1991). 16S ribosomal DNA amplification for phylogenetic study. *J. Bacteriol.* 173, 697–703. doi: 10.1128/jb.173.2.697-703.1991
- Zandsalimi, S., Karimi, N., and Kohandel, A. (2011). Arsenic in soil, vegetation and water of a contaminated region. *Int. J. Environ. Sci. Technol.* 8, 331–333. doi: 10.1007/BF03326220
- Zhu, L. J., Guan, D. X., Luo, J., Rathinasabapathi, B., and Ma, L. Q. (2014). Characterization of arsenic-resistant endophytic bacteria from hyperaccumulators *Pteris vittata* and *Pteris multifida*. *Chemosphere* 113, 9–16. doi: 10.1016/j.chemosphere.2014.03.081

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.

Copyright © 2021 Tashan, Harighi, Rostamzadeh and Azizi. This is an open-access article distributed under the terms of the Creative Commons Attribution License (CC BY). The use, distribution or reproduction in other forums is permitted, provided the original author(s) and the copyright owner(s) are credited and that the original publication in this journal is cited, in accordance with accepted academic practice. No use, distribution or reproduction is permitted which does not comply with these terms.

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