ARBUSCULAR MYCORRHIZAL FUNGI: THE BRIDGE BETWEEN PLANTS, SOILS, AND HUMANS

EDITED BY: Sergio Saia and Jan Jansa

PUBLISHED IN: Frontiers in Plant Science

frontiers Research Topics

ISSN 1664-8714
ISBN 978-2-88974-499-2
DOI 10.3389/978-2-88974-499-2

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

ARBUSCULAR MYCORRHIZAL FUNGI: THE BRIDGE BETWEEN PLANTS, SOILS, AND HUMANS

Topic Editors:
Sergio Saia, University of Pisa, Italy
Jan Jansa, Institute of Microbiology, Academy of Sciences of the Czech Republic (ASCR), Czechia

Citation: Saia, S., Jansa, J., eds. (2022). Arbuscular Mycorrhizal Fungi: The Bridge Between Plants, Soils, and Humans. Lausanne: Frontiers Media SA.
doi: 10.3389/978-2-88974-499-2

Table of Contents

frontiers | Frontiers in Plant Science

EDITORIAL
published: 04 April 2022
doi: 10.3389/fpls.2022.875958

Editorial: Arbuscular Mycorrhizal Fungi: The Bridge Between Plants, Soils, and Humans

Sergio Saia[1]* *and Jan Jansa*[2]

[1] Department of Veterinary Science, University of Pisa, Pisa, Italy, [2] Czech Academy of Sciences, Institute of Microbiology, Prague, Czechia

Keywords: beneficial microbes, nutrient limitation, human needs, soil health, plant stress, pathogens, sustainable agro-ecosystem, sustainability

Editorial on the Research Topic

Arbuscular Mycorrhizal Fungi: The Bridge Between Plants, Soils, and Humans

It is assumed that arbuscular mycorrhizal (AM) symbiosis is established between the roots/rhizoids of ca 70% of all plant species, including some of the most important crops, and specialized soil fungi (Brundrett and Tedersoo, 2018). The AM fungi provide a direct interconnection between roots and soil as well as between root systems of different plant individuals belonging to the same or different plant species (Walder et al., 2012; Weremijewicz et al., 2016; Rezáčová et al., 2018b). The AM fungi exert several direct (e.g., enhanced nutrient acquisition, pollutant immobilization/detoxification, plant carbon reallocation, induced pathogen tolerance, signal transfer), and indirect (e.g., photosynthesis stimulation, drought tolerance, soil physical, and microbial conditioning) effects on the plants, with possible consequences to yield and agricultural product quality, multitrophic interaction networks, and soil quality (Kaschuk et al., 2009; Johnson, 2010; Smith et al., 2010; Garg and Chandel, 2011; Rezáčová et al., 2018a). Besides having a finely tuned molecular dialogue with their plant hosts, the fungi also interact with soil microbes fulfilling important ecosystem functions, such as organic nutrient mineralization or stabilization of soil organic matter (Verbruggen et al., 2016; Jiang F. et al., 2021; Rozmoš et al., 2021; Sánchez et al., 2021), and these interactions are further modulated by environmental (soil, climatic, ecosystem management) contexts (Frey-Klett et al., 2007; Hoeksema et al., 2010).

The goal of this Research Topic was to provide an illustration of the range of different views on the functioning of AM symbiosis in natural and anthropogenic systems, to promote mechanistic understanding of the formation, extent, and dynamics of the AM fungal hyphal networks and associated microbes in the soil and to add to the mounting knowledge on the feedbacks between human activities and ecosystem functioning, with a particular focus on the role of AM fungal symbiosis across various plant species/genotypes, soil types, and climatic regions.

We received 30 manuscripts dedicated to the various aspects of mycorrhizal symbioses specified above, out of which 11 were eventually accepted for including in this collection. These publications deal with plant-AM fungi interactions (and occasionally also with other mycorrhizal types, **Table 1**) and their role in satisfying various human needs. They include "review/meta-analyses" (three articles), "genotype, genetic, and omics aspects" (five articles), "abiotic stress and plant nutrition aspects" (nine articles); "monocots" (two articles); "tree species" (three articles); and works performed under "field conditions" (eight articles). Particularly, we are happy to cover the latter aspect quite broadly, of which the studies are usually underrepresented in the literature compared to the research carried out under controlled conditions (Lekberg and Koide, 2005; Kaschuk et al., 2010; Pellegrino et al., 2015; Zhang et al., 2019; Jiang S. et al., 2021; Qiu et al., 2022; Salomon et al., 2022).

OPEN ACCESS

Edited and reviewed by:
Raffaella Balestrini, Institute for Sustainable Plant Protection (CNR), Italy

***Correspondence:**
Sergio Saia
sergio.saia@unipi.it

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

Received: *14 February 2022*
Accepted: *14 March 2022*
Published: *04 April 2022*

Citation:
Saia S and Jansa J (2022) Editorial: Arbuscular Mycorrhizal Fungi: The Bridge Between Plants, Soils, and Humans. Front. Plant Sci. 13:875958. doi: 10.3389/fpls.2022.875958

TABLE 1 | Synopsis of the articles published in the collections and number of topics covered.

Title (Citation)	Review or metanalysis	Genotype, genetic, and omic	Abiotic stress and nutrition	Monocots	Trees	Field conditions
Understanding Multilevel Selection May Facilitate Management of Arbuscular Mycorrhizae in Sustainable Agroecosystems (Johnson and Gibson)	1					1
Nitrogen fertilization increases specific root respiration in ectomycorrhizal but not in arbuscular mycorrhizal plants: a meta-analysis (Bicharanloo et al.)	1		1			1
Potential effects of microplastic on arbuscular mycorrhizal fungi (Leifheit et al.)	1		1			1
Arbuscular mycrorrhizal fungi inoculation and applied water amounts modulate the response of young grapevines to mild water stress in a hyper-arid season (Torres et al.)	0	0	1	0	1	1
Production of organic acids by arbuscular mycorrhizal fungi and their contribution in the mobilization of phosphorus bound to iron oxides (Andrino et al.)	0	0	1	0		0
The phosphate inhibition paradigm: host and fungal genotypes determine arbuscular mycorrhizal fungal colonization and responsiveness to inoculation in cassava with increasing phosphorus supply (Peña Venegas et al.)	0	1	1	0		1
Arbuscular mycorrhizal fungi (*Rhizophagus clarus*) and rhizobacteria (*Bacillus subtilis*) can improve the clonal propagation and development of teak for commercial plantings (Alexandre et al.)	0	0	0	0	1	1
Effects of mycorrhizal colonization on transcriptional expression of the responsive factor JERF3 and stress-responsive genes in banana plantlets in response to combined biotic and abiotic stresses (Rashad et al.)	0	1	2	1	0	0
Stoichiometry of carbon, nitrogen and phosphorus in shrub organs linked closely with mycorrhizal strategy in Northern China (Yang et al.)	0	1	1	0	1	1
Similar arbuscular mycorrhizal fungal communities in 31 durum wheat cultivars (*Triticum turgidum* L. var. durum) under field conditions in Eastern Canada (Stefani et al.)	0	1	0	1	0	1
Arbuscular mycorrhizal fungi improve tolerance of the medicinal plant *Eclipta prostrata* (L.) and induce major changes in polyphenol profiles under salt stresses (Duc et al.)	0	1	1	0	0	0

The results of the currently presented research thus contribute in a relevant way to the long ongoing debate on the use and usefulness of the mycorrhizal technology in agriculture and its prospective as an agronomic strategy for the future (Frossard et al., 2009; Gianinazzi et al., 2010; Ryan and Graham, 2018; Rillig et al., 2019; Ryan et al., 2019; Benami et al., 2020; Brito et al., 2021). We believe that such a multifaceted debate, also happening in other aspects of the crops and/or environmental management (Sadras et al., 2020), is much needed and to a large extent still not reaching a broad consensus, mainly due to the lack of sufficient data and generally valid concepts. Indeed, more detailed information seems to be needed on how different agronomic practices affect indigenous AM fungi in the different soils, how this links to human needs, and how this all is affected by changing environmental context. Besides, novel concepts and demonstrations of their validity are needed in terms of potential manipulation of the systems for the good of the humans and the ecosystems. This collection contributes to reducing such knowledge gaps.

In particular, two excellent perspectives cover the establishment and functions of the AMF symbiosis in sustainable agroecosystems: Johnson and Gibson pointed out that the mycorrhizal role and benefit should be studied and understood while considering a multilevel selection resulting in a local adaption. Along similar lines, Bicharanloo et al. clarified, through a meta-analytical approach, that specific root respiration (SRR), which could be regarded as a proxy for mycorrhizal costs, did not vary among the AM and non-mycorrhizal (NM) plants subjected to different N fertilization levels, in contrast to ectomycorrhizal (ECM) plants. These results are complementary to insightful comparisons between the two different types of mycorrhizal symbiosis across gradients of environmental stoichiometry provided by Yang et al., who found a higher nitrogen concentration in AM-associated compared to the ECM fungi-associated shrub plants, and who also observed opposite trends for phosphorus concentrations, thus suggesting that under C non-limited conditions, such as in the shrub plants, the N demand by AM fungi can stimulate plant growth benefits particularly under higher N availability. Results by Bicharanloo et al. may have thus depended the variability in the C-sink limitations among the plant species included (Gamper et al., 2004). Nonetheless, the results also provide indirect evidence that management of AM fungal activity (either through inoculation or conservation of the indigenous AM fungi) should not be ignored even under intensively fertilized systems, particularly if underrepresented plant/crop species are considered.

Leifheit et al. tackled the possible implication for the AM fungi of one of the understudied factor of global change, namely the soil pollution with microplastics. After reviewing the current knowledge, they elaborate on the priorities for future experimental work focused on understanding the importance of such emerging thread as the microplastics for ecosystem functioning in general and the AM symbiosis in particular.

Other articles included in this collection cover a wealth of aspects relevant to mycorrhizal functioning under various agroecosystems and environmental settings. Stefani et al. studied the recruitment of the native AM fungal communities in contrasting genotypes of durum wheat in Canada, showing that the main environmental filter was actually the plant genotype identity and the traits independent of the root colonization rate. These results are not only demonstrating that different genotypes recruit quite different AM fungi from the same soil pool. Together with previous research (e.g., Jansa et al., 2008; Lendenmann et al., 2011; Veiga et al., 2011; Thonar et al., 2013; Knegt et al., 2016), these results allow speculations about consequences of changing crop genotype (or crop mixture composition) to derive different mycorrhizal benefits in terms of plant nutrition and/or growth, as well as co-existence of the crops with weeds.

Peña Venegas et al. showed that cassava farming in equatorial Africa could benefit from the inoculation with specific genotypes of AM fungi even under high P supply, which has previously been considered detrimental for the development and/or functioning of the AM fungi. These results align nicely with the elegant experiment of Andrino et al., who showed that in tomato, the AM fungi could release low-molecular-weight organic acids that facilitate P uptake from highly P-fixing soil along with other elements, such as Fe. Andrino et al. also pointed that membrane fluidity could vary in the AM fungi supplied with various P sources and this can further affect their P uptake from the soil and delivery to their host plants.

Alexandre et al. worked on teak (*Tectona grandis* L.f.), an important timber species in Brazil, and confirmed that the amendment of clonally propagated plantlets with AM fungi and plant growth-promoting rhizobacteria could significantly improve plant growth and nutrition, thus fostering their field growth between 4.75 and 11.04%. Similarly, Rashad et al. demonstrated mycorrhizal benefits and underlying metabolic pathways in banana plantlets subjected to different abiotic (salinity) and biotic (pathogen) stresses. Along similar lines, mycorrhizal benefits were also elaborated on the medicinal plant *Eclipta prostrata* (Duc et al.) subjected to salinity stress, which seem to have been coupled with modulation of the polyphenol production. Thus, the present results generally confirm previous findings on the ability of the AM fungi to modulate secondary metabolism (Zeng et al., 2013; Lazzara et al., 2017; Saia et al., 2019) of a range of medicinal and other plants, despite the remaining uncertainty in the mycorrhizal effects on the volatile compounds composition (Saia et al., 2021).

Somewhat contrasting with the previous research, Torres et al. found that in grapevines, the AM fungi affected more the secondary compound production under contrasting water availability regimes than the leaf mineral content (indicative of direct nutritional effects). These differences between the results of Torres et al. and the previously cited works could possibly be explained by differential nutrient reserves accumulated in the plants before onset of the stresses, which could potentially buffer the stress impact on plants and promote apparition of other than nutritional mycorrhizal benefit.

The majority of the studies thus aimed at elucidating physiological mechanism of the abiotic stress responses in AM plants. All the studies contributed to the debate on

greater utilization of mycorrhizal symbiosis in agronomy and forestry practices, either as planned interventions or as a collateral effect. Importantly, 8 out of the 11 studies has been conducted, or dealing with, field conditions. We believe the integration of these results contributed to create useful resource for our peers and students. We would like to thank all contributors for their work and patience in these uneasy COVID times and to the Frontiers staff for their relentless support and assistance for making this to happen.

AUTHOR CONTRIBUTIONS

Both authors equally contributed to this editorial and the special issue management. All authors contributed to the article and approved the submitted version.

ACKNOWLEDGMENTS

The guest editors are heartfully thanks the reviewers in their aid helping this special issue.

REFERENCES

Benami, M., Isack, Y., Grotsky, D., Levy, D., and Kofman, Y. (2020). "The economic potential of arbuscular mycorrhizal fungi in agriculture," in *Grand Challenges in Fungal Biotechnology: Grand Challenges in Biology and Biotechnology*, ed H. Nevalainen (Cham: Springer), 239–279. doi: 10.1007/978-3-030-29541-7_9

Brito, I., Carvalho, M., and Goss, M. J. (2021). Managing the functional diversity of arbuscular mycorrhizal fungi for the sustainable intensification of crop production. *Plants People Planet* 3, 491–505. doi: 10.1002/ppp3.10212

Brundrett, M. C., and Tedersoo, L. (2018). Evolutionary history of mycorrhizal symbioses and global host plant diversity. *New Phytol.* 220, 1108–1115. doi: 10.1111/nph.14976

Frey-Klett, P., Garbaye, J., and Tarkka, M. (2007). The mycorrhiza helper bacteria revisited. *New Phytol.* 176, 22–36. doi: 10.1111/j.1469-8137.2007.02191.x

Frossard, E., Bünemann, E., Jansa, J., Oberson, A., and Feller, C. (2009). Concepts and practices of nutrient management in agro-ecosystems: can we draw lessons from history to design future sustainable agricultural production systems? *Bodenkultur* 60, 43–60. Available online at: https://diebodenkultur.boku.ac.at/volltexte/band-60/heft-1/frossard.pdf

Gamper, H., Peter, M., Jansa, J., Luscher, A., Hartwig, U., a., et al. (2004). Arbuscular mycorrhizal fungi benefit from 7 years of free air CO2 enrichment in well-fertilized grass and legume monocultures. *Glob. Chang. Biol.* 10, 189–199. doi: 10.1111/j.1529-8817.2003.00734.x

Garg, N., and Chandel, S. (2011). "Arbuscular mycorrhizal networks: process and functions," in *Sustainable Agriculture*, Vol. 2, eds E. Lichtfouse, M. Hamelin, M. Navarrete, and P. Debaeke (Dordrecht: Springer Netherlands), 907–930. doi: 10.1007/978-94-007-0394-0_40

Gianinazzi, S., Gollotte, A., Binet, M.-N. N., van Tuinen, D., Redecker, D., and Wipf, D. (2010). Agroecology: the key role of arbuscular mycorrhizas in ecosystem services. *Mycorrhiza* 20, 519–530. doi: 10.1007/s00572-010-0333-3

Hoeksema, J. D., Chaudhary, V. B., Gehring, C. A., Johnson, N. C., Karst, J., Koide, R. T., et al. (2010). A meta-analysis of context-dependency in plant response to inoculation with mycorrhizal fungi. *Ecol. Lett.* 13, 394–407. doi: 10.1111/j.1461-0248.2009.01430.x

Jansa, J., Smith, F., and Smith, S. E. (2008). Are there benefits of simultaneous root colonization by diferent arbuscular mycorrhizal fungi? *New Phytol.* 177, 779–789. doi: 10.1111/j.1469-8137.2007.02294.x

Jiang, F., Zhang, L., Zhou, J., George, T. S., and Feng, G. (2021). Arbuscular mycorrhizal fungi enhance mineralisation of organic phosphorus by carrying bacteria along their extraradical hyphae. *New Phytol.* 230, 304–315. doi: 10.1111/nph.17081

Jiang, S., An, X., Shao, Y., Kang, Y., Chen, T., Mei, X., et al. (2021). Responses of arbuscular mycorrhizal fungi occurrence to organic fertilizer: a meta-analysis of field studies. *Plant Soil* 469, 89–105. doi: 10.1007/s11104-021-05153-y

Johnson, N. C. (2010). Resource stoichiometry elucidates the structure and function of arbuscular mycorrhizas across scales. *New Phytol.* 185, 631–647. doi: 10.1111/j.1469-8137.2009.03110.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

Kaschuk, G., Leffelaar, P., Giller, K. E., Alberton, O., Hungria, M., and Kuyper, T. W. (2010). Responses of legumes to rhizobia and arbuscular mycorrhizal fungi: a meta-analysis of potential photosynthate limitation of symbioses. *Soil Biol. Biochem.* 42, 125–127. doi: 10.1016/j.soilbio.2009.10.017

Knegt, B., Jansa, J., Franken, O., Engelmoer, D. J. P., Werner, G. D. A., Bücking, H., et al. (2016). Host plant quality mediates competition between arbuscular mycorrhizal fungi. *Fungal Ecol.* 20, 233–240. doi: 10.1016/j.funeco.2014.09.011

Lazzara, S., Militello, M., Carrubba, A., Napoli, E., and Saia, S. (2017). Arbuscular mycorrhizal fungi altered the hypericin, pseudohypericin, and hyperforin content in flowers of Hypericum perforatum grown under contrasting P availability in a highly organic substrate. *Mycorrhiza* 27, 345–354. doi: 10.1007/s00572-016-0756-6

Lekberg, Y., and Koide, R. T. (2005). Is plant performance limited by abundance of arbuscular mycorrhizal fungi? A meta-analysis of studies published between 1988 and 2003. *New Phytol.* 168, 189–204. doi: 10.1111/j.1469-8137.2005.01490.x

Lendenmann, M., Thonar, C., Barnard, R. L., Salmon, Y., Werner, R. A., Frossard, E., et al. (2011). Symbiont identity matters: carbon and phosphorus fluxes between *Medicago truncatula* and different arbuscular mycorrhizal fungi. *Mycorrhiza* 21, 689–702. doi: 10.1007/s00572-011-0371-5

Pellegrino, E., Öpik, M., Bonari, E., and Ercoli, L. (2015). Responses of wheat to arbuscular mycorrhizal fungi: a meta-analysis of field studies from 1975 to 2013. *Soil Biol. Biochem.* 84, 210–217. doi: 10.1016/j.soilbio.2015.02.020

Qiu, Q., Bender, S. F., Mgelwa, A. S., and Hu, Y. (2022). Arbuscular mycorrhizal fungi mitigate soil nitrogen and phosphorus losses: a meta-analysis. *Sci. Total Environ.* 807:150857. doi: 10.1016/j.scitotenv.2021.150857

Rezáčová, V., Slavíková, R., Zemková, L., Konvalinková, T., Procházková, V., Štovíček, V., et al. (2018a). Mycorrhizal symbiosis induces plant carbon reallocation differently in C3 and C4 Panicum grasses. *Plant Soil* 425, 441–456. doi: 10.1007/s11104-018-3606-9

Rezáčová, V., Zemková, L., Beskid, O., Püschel, D., and Konvalinkov,á, T., Hujslová, M., et al. (2018b). Little cross-feeding of the mycorrhizal networks shared between C3-panicum bisulcatum and C4-panicum maximum under different temperature regimes. *Front. Plant Sci.* 9:449. doi: 10.3389/fpls.2018.00449

Rillig, M. C., Aguilar-Trigueros, C. A., Camenzind, T., Cavagnaro, T. R., Degrune, F., Hohmann, P., et al. (2019). Why farmers should manage the arbuscular mycorrhizal symbiosis. *New Phytol.* 222, 1171–1175. doi: 10.1111/nph.15602

Rozmoš, M., Bukovská, P., Hršelová, H., Kotianová, M., Dudáš, M., Gančarčíková, K., et al. (2021). Organic nitrogen utilisation by an arbuscular mycorrhizal fungus is mediated by specific soil bacteria and a protist. *ISME J.* 16, 676–685. doi: 10.1038/s41396-021-01112-8

Ryan, M. H., and Graham, J. H. (2018). Little evidence that farmers should consider abundance or diversity of arbuscular mycorrhizal fungi when managing crops. *New Phytol.* 220, 1092–1107. doi: 10.1111/nph.15308

Ryan, M. H., Graham, J. H., Morton, J. B., and Kirkegaard, J. A. (2019). Research must use a systems agronomy approach if management of the arbuscular mycorrhizal symbiosis is to contribute to sustainable intensification. *New Phytol.* 222, 1176–1178. doi: 10.1111/nph.15600

Sadras, V., Alston, J., Aphalo, P., Connor, D., Denison, R. F., Fischer, T., et al. (2020). "Making science more effective for agriculture," in *Advances in Agronomy*, ed D. L. Sparks (Academic Press), 153–177. doi: 10.1016/bs.agron.2020.05.003

Saia, S., Corrado, G., Vitaglione, P., Colla, G., Bonini, P., Giordano, M., et al. (2021). An endophytic fungi-based biostimulant modulates volatile and non-volatile secondary metabolites and yield of greenhouse basil (*Ocimum basilicum* L.) through variable mechanisms dependent on salinity stress level. *Pathogens* 10:797. doi: 10.3390/pathogens10070797

Saia, S., Fragasso, M., De Vita, P., and Beleggia, R. (2019). Metabolomics provides valuable insight for the study of durum wheat: a review. *J. Agric. Food Chem.* 67, 3069–3085. doi: 10.1021/acs.jafc.8b07097

Salomon, M. J., Demarmels, R., Watts-Williams, S. J., McLaughlin, M. J., Kafle, A., Ketelsen, C., et al. (2022). Global evaluation of commercial arbuscular mycorrhizal inoculants under greenhouse and field conditions. *Appl. Soil Ecol.* 169:104225. doi: 10.1016/j.apsoil.2021.104225

Sánchez, M. G., Saia, S., and Aranda, E. (2021). "The contribution of fungi and their lifestyle in the nitrogen cycle," in *Nitrogen Cycle 1st Edn,* eds J. Gonzalez-Lopez and A. Gonzalez-Martinez (Boca Raton, FL : CRC PRESS), 82–101. doi: 10.1201/9780429291180-5

Smith, S. E., Facelli, E., Pope, S., and Andrew Smith, F. (2010). Plant performance in stressful environments: interpreting new and established knowledge of the roles of arbuscular mycorrhizas. *Plant Soil* 326, 3–20. doi: 10.1007/s11104-009-9981-5

Thonar, C., Frossard, E., Šmilauer, P., and Jansa, J. (2013). Competition and facilitation in synthetic communities of arbuscular mycorrhizal fungi. *Mol. Ecol.* 23, 733–746. doi: 10.1111/mec.12625

Veiga, R. S. L., Jansa, J., Frossard, E., and Van Der Heijden, M. G. A. (2011). Can arbuscular mycorrhizal fungi reduce the growth of agricultural weeds? *PLoS ONE* 6:e27825. doi: 10.1371/journal.pone.0027825

Verbruggen, E., Jansa, J., Hammer, E. C., and Rillig, M. C. (2016). Do arbuscular mycorrhizal fungi stabilize litter-derived carbon in soil? *J. Ecol.* 104, 261–269. doi: 10.1111/1365-2745.12496

Walder, F., Niemann, H., Natarajan, M., Lehmann, M. F., Boller, T., and Wiemken, A. (2012). Mycorrhizal networks: common goods of plants shared under unequal terms of trade. *Plant Physiol.* 159, 789–797. doi: 10.1104/pp.112.195727

Weremijewicz, J., Sternberg, L., da, S. L. O., and Janos, D. P. (2016). Common mycorrhizal networks amplify competition by preferential mineral nutrient allocation to large host plants. *New Phytol.* 212, 461–471. doi: 10.1111/nph.14041

Zeng, Y., Guo, L.-P., Chen, B.-D., Hao, Z.-P., Wang, J.-Y., Huang, L.-Q., et al. (2013). Arbuscular mycorrhizal symbiosis and active ingredients of medicinal plants: current research status and prospectives. *Mycorrhiza* 23, 253–265. doi: 10.1007/s00572-013-0484-0

Zhang, S., Lehmann, A., Zheng, W., You, Z., and Rillig, M. C. (2019). Arbuscular mycorrhizal fungi increase grain yields: a meta-analysis. *New Phytol.* 222, 543–555. doi: 10.1111/nph.15570

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.

Publisher's Note: All claims expressed in this article are solely those of the authors and do not necessarily represent those of their affiliated organizations, or those of the publisher, the editors and the reviewers. Any product that may be evaluated in this article, or claim that may be made by its manufacturer, is not guaranteed or endorsed by the publisher.

frontiers in Plant Science

ORIGINAL RESEARCH
published: 11 August 2020
doi: 10.3389/fpls.2020.01206

Similar Arbuscular Mycorrhizal Fungal Communities in 31 Durum Wheat Cultivars (*Triticum turgidum* L. var. durum) Under Field Conditions in Eastern Canada

Franck Stefani[1], *Sarah Dupont*[2], *Mario Laterrière*[3], *Ron Knox*[4], *Yuefeng Ruan*[4], *Chantal Hamel*[3] *and Mohamed Hijri*[2,5]*

[1] Ottawa Research and Development Centre of Agriculture and Agri-Food Canada, Ottawa, ON, Canada, [2] Département de Sciences Biologiques, Institut de Recherche en Biologie Végétale, Université de Montréal, Montréal, QC, Canada, [3] Quebec Research and Development Centre of Agriculture and Agri-Food Canada, Quebec, QC, Canada, [4] Swift Current Research and Development Centre of Agriculture and Agri-Food Canada, Swift Current, SK, Canada, [5] AgroBioSciences, Mohammed VI Polytechnic University, Ben Guerir, Morocco

OPEN ACCESS

Edited by:
Sergio Saia,
Council for Agricultural and Economics Research (CREA), Italy

Reviewed by:
Valentina Fiorilli,
University of Turin, Italy
Manuela Giovannetti,
University of Pisa, Italy

***Correspondence:**
Mohamed Hijri
mohamed.hijri@umontreal.ca

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

Received: 18 June 2020
Accepted: 24 July 2020
Published: 11 August 2020

Citation:
Stefani F, Dupont S, Laterrière M, Knox R, Ruan Y, Hamel C and Hijri M (2020) Similar Arbuscular Mycorrhizal Fungal Communities in 31 Durum Wheat Cultivars (Triticum turgidum L. var. durum) Under Field Conditions in Eastern Canada. Front. Plant Sci. 11:1206. doi: 10.3389/fpls.2020.01206

Wheat is among the important crops harnessed by humans whose breeding efforts resulted in a diversity of genotypes with contrasting traits. The goal of this study was to determine whether different old and new cultivars of durum wheat (*Triticum turgidum* L. var. *durum*) recruit specific arbuscular mycorrhizal (AM) fungal communities from indigenous AM fungal populations of soil under field conditions. A historical set of five landraces and 26 durum wheat cultivars were field cultivated in a humid climate in Eastern Canada, under phosphorus-limiting conditions. To characterize the community of AMF inhabiting bulk soil, rhizosphere, and roots, MiSeq amplicon sequencing targeting the 18S rRNA gene (SSU) was performed on total DNAs using a nested PCR approach. Mycorrhizal colonization was estimated using root staining and microscope observations. A total of 317 amplicon sequence variants (ASVs) were identified as belonging to Glomeromycota. The core AM fungal community (i.e., ASVs present in > 50% of the samples) in the soil, rhizosphere, and root included 29, 30, and 29 ASVs, respectively. ASVs from the genera *Funneliformis*, *Claroideoglomus*, and *Rhizophagus* represented 37%, 18.6%, and 14.7% of the sequences recovered in the rarefied dataset, respectively. The two most abundant ASVs had sequence homology with the 18S sequences from well-identified herbarium cultures of *Funneliformis mosseae* BEG12 and *Rhizophagus irregularis* DAOM 197198, while the third most abundant ASV was assigned to the genus *Paraglomus*. Cultivars showed no significant difference of the percentage of root colonization ranging from 57.8% in Arnautka to 84.0% in AC Navigator. Cultivars were generally associated with similar soil, rhizosphere, and root communities, but the abundance of *F. mosseae*, *R. irregularis*, and *Claroideoglomus* sp. sequences varied in Eurostar, Golden Ball, and Wakooma. Although these results were obtained in one field trial using a non-restricted pool of durum wheat and at the time of sampling, that

may have filtered the community in biotopes. The low genetic variation between durum wheat cultivars for the diversity of AM symbiosis at the species level suggests breeding resources need not be committed to leveraging plant selective influence through the use of traditional methods for genotype development.

Keywords: arbuscular mycorrhizal fungal communities, durum wheat cultivars, plant breeding, symbiosis, *Triticum turgidum* var. *durum*

INTRODUCTION

Durum wheat (*Triticum turgidum* L. var. *durum* Desf.) is a major crop in Canada with an average annual production of 5.96 million tonnes from 2015 to 2019 (Statistics Canada, 2020), establishing Canada as the largest exporter of durum wheat in the world. The flour derived from durum grains is mostly used for the production of pasta, semolina bourghul, and breads. Durum wheat originates from the Fertile Crescent and was the major cultivated form of tetraploid wheat during the Hellenistic period ca. 2300 BP (Feldman and Kislev, 2008). It was introduced into Western Canada in the late 19th century where 80% of the production occurred in the Brown and Dark Brown soil zones (McCaig and Clarke, 1995). Farmers increased cultivation of durum wheat in Western Canada in the 1960s because it was less susceptible to stem rust compared to bread wheat varieties cultivated at that time. The first developed cultivar in Canada was Stewart 63 released in 1963. McCaig and Clarke (1995) estimated that the development of new cultivars through the Canadian durum breeding programs for the period 1960 to 1990 increased yields by about 25% compared to foreign cultivars available prior to Stewart 63. Gluten content, cadmium concentration, resistance to fungal pathogens (*Fusarium* head blight, leaf, and stem rust) and insect pests (wheat stem sawfly, wheat midge) were the main traits considered for developing new varieties (Dexter, 2008; Clarke et al., 2010).

The selection pressure applied in the 20th century on the new varieties of durum wheat was therefore driven by commercial purposes and high performance in high input systems (fertilizers, pesticides). For a long time, plants were considered as autonomous individuals and, as a consequence, breeding approaches completely overlook the complex microbial context of the soil environment in which crops grow. More specifically, breeding approaches do not take into account the performance of the newly developed varieties to recruit root mutualists.

The association of roots with microorganisms relies on intricate molecular crosstalk which results from long-term co-evolution between plant hosts and microbial partners (Lambers et al., 2009). Modification of the root exudates following domestication and breeding can shape different microbial communities. Selective breeding in wild emmer, domesticated emmer, and modern durum wheat has triggered changes in root exudate composition (Iannucci et al., 2017). Less is known on the effect of selective breeding on the microbial associations of durum than on common wheat (*Triticum aestivum* L.), its hexaploid relative. High-throughput sequencing of rhizospheric bacterial communities associated with different winter wheat cultivars showed a line effect on the structure of the bacterial communities, suggesting that these communities could be manipulated by wheat breeding (Donn et al., 2015; Mahoney et al., 2017).

Among beneficial root associates, arbuscular mycorrhizal fungi (phylum Glomeromycota) coevolve with plants since ~980 Ma – 600 Ma. Arbuscular mycorrhizal (AM) fungi colonize the root system of 72% of vascular plants (Brundrett and Tedersoo, 2018) where they form highly branched fungal structures called arbuscules (Smith and Read, 2010). Arbuscules are the sites for nutrient exchanges between both partners. AM symbionts obtain plant carbon and, in exchange, release mineral nutrients absorbed from the soil. Despite their limited species richness (334 species described so far, www.amf-phylogeny.com), AM fungi, together with their associated microbiota, provide a range of essential services, from drought stress mitigation and disease prevention, to plant nutrient and water uptake, and the maintenance of biological soil fertility (Gianinazzi et al., 2010; Turrini et al., 2018). Fine tuning the interaction between the naturally occurring AM fungal communities and crop plants through plant breeding and appropriate agronomy, could improve the sustainability of agroecosystems (Bakker et al., 2012; Gan et al., 2015; Hijri, 2016).

Wheat has long been recognized as a crop with mixed responses to AM fungi, from negative, neutral, to positive effects. Hetrick et al. (1993) found strong dependence of winter wheat on mycorrhiza in cultivars released prior to 1950, but more variable responses in recently released cultivars. The authors suggested that cultivars released after 1950 had reduced dependence on mycorrhizae due to breeding performed under high fertility conditions. The impact of breeding on the mycorrhiza of durum wheat is less clear. In a 'proof of concept' experiment conducted under greenhouse conditions by the Canadian Government durum wheat breeding program (Singh et al., 2012), plant growth response to the model AM fungus *R. irregularis* DAOM 197198 varied among five cultivars (AC Morse, Commander, DT 710, Strongfield, Mongibelllo). Then, a thorough examination of the AM symbiosis formed between *R. irregularis* DAOM 197198 and five landraces and 27 modern cultivars (Canadian historical set) revealed that breeding had inconsistent effects on mycorrhiza development in durum wheat under greenhouse conditions. It led to the identification of cultivars with unimproved patterns of regulation of symbiotic development (e.g.: Commander, AC Pathfinder), and in a few cases, to cultivars (e.g.: Hercules, Wascana, Eurostar) with crippled regulation and poor plant performance in soil with high fertility (Ellouze et al., 2016). In a study that investigated the

impact of these 32 cultivars on the structure of the AM fungal community in two field trials in the Canadian Prairies (Swift Current and Regina), Ellouze et al. (2018) reported significantly different relative abundance of the genus *Paraglomus* in the cultivars Ramsey (11%) and Strongfield (93.7%) and a significant effect of the cultivars on the structure of the AM fungal community in the rhizosphere, but not in the roots. However, this study was performed in the semiarid zone of Canadian prairie where moisture shortage could mask possible selective effects of cultivars on the AM fungal communities colonizing plant roots. Variation in soil moisture, the factor shaping the prairie ecosystems and more dramatically so the semiarid prairie (Hamel et al., 2006), was a confounding influence in this study. Moreover, the sequencing depth per sample was low with an average of 168 AM fungal pyrosequences per sample.

In order to overcome the abovementioned pitfalls and to discern possible differences in AM fungal community composition and root colonization percentages between genetically diverse durum wheat cultivars, a field trial seeded with five landraces and 26 cultivars released at different times in the history of durum wheat breeding was set up under a humid climate in Eastern Canada and the AM fungal community was thoroughly characterized by high-throughput sequencing. Based on the results from previous studies, we hypothesised that different field grown durum wheat cultivars associate with distinct AM fungal communities. The V3-V4 region of the nuclear 18S rRNA gene of AM fungi was sequenced to describe AM fungal communities located in bulk soil, rhizosphere soil, and roots, at anthesis.

MATERIALS AND METHODS

Experimental Field-Trial

The experimental field was set up in 2016 nearby the city of Lévis (Québec, Canada, GPS coordinates: 46°47′40′′N; 71°08′05′′W). The region is featured by a growing season of 140 to 150 days, a cool and humid climate with average temperatures of 12.5°C, 16.9°C, and 19.1°C in May, June, and July, respectively, according to the Environment Canada weather station (https://climate.weather.gc.ca) located at 3.5 km from the experimental field. These temperatures are similar to those recorded for the same months during the period 1981 to 2010 (11.0°C, 16.5°C, and 19.3°C).

The soil was a well-drained Saint-André gravelly loam (fragic, humo-ferric podzol or mixed, frigid typic dystrochrept, Soil Classification Working Group, 1998). Physical and chemical properties of soil are provided in **Table S1** as supplementary material. The field was previously used to grow switchgrass (*Panicum virgatum* L.) in 2014 to 2015. Glyphosate-Roundup® was applied, and the field was tilled in fall 2015. Harrowing and fertilization were carried out on May 11, 2016. The plots at time of sowing received 90 kg/ha of nitrogen (N) as calcium ammonium nitrate (27-0-0) and 37 kg/ha potassium (K) as potassium chloride (0-0-60). Phosphorous (P) fertilization was not applied in order to make P resource a limiting factor to favour the mycorrhizal association. Five landraces and 26 durum wheat cultivars (details about each cultivar is provided in **Table S2**) were seeded at a density of 118 seeds/m^2 using a 4-row cereal plot seeder on 12 May 2016. Seeds were obtained from the collection at Agriculture and Agri-Food Canada, Swift Current, SK. Each plot was 1 m × 1.7 m and four rows per plot were seeded with one out of the 31 cultivars. DyVel® herbicide (Dicamba) was applied at 1.25 L/ha on 7 June 2016 for weed control.

The field trial was arranged in a randomized complete block design, with four blocks, 31 cultivars per block, representing a total of 124 plots (**Figure S1**). Each block was layered in two rows of plots and a guard plot was seeded with cultivar AAC Cabri at both ends of each row.

Field Sampling

To characterize the AM fungal community associated with durum wheat, three biologically relevant compartments were sampled: bulk soil, rhizosphere, and roots. Sampling was performed at anthesis, on July 8 and 12, 2016, as follows: six plants were randomly selected from within rows 2 and 3 of each plot and dug out with a spade. The aboveground portion of each plant was cut off and discarded, and the root system was stored at 4°C in a cooler in the field and then at 4°C in a laboratory fridge until processing. Six soil cores were collected between rows 3 and 4 using a soil probe (2.5 cm in diameter, 15 cm long). For each plot, the six soil cores were sieved (mesh size 2 mm) and combined into a single composite sample. The root system of each of the six plants per plot was gently shaken to collect the rhizosphere soil. The six rhizosphere soil samples were sieved (mesh size 0.5 mm) and combined into a single composite sample. Finally, the root system of each of the six plants per plot was combined into a composite sample and rinsed, and the fine roots (≤ 1 mm thick) were cut into 1- to 2-cm-long fragments. Three subsamples were collected from each pool of root fragments: two subsamples were transferred into two plastic Shandon™ tissue cassettes (Thermo Scientific™) for root colonization analysis and one subsample was stored in 1.5 ml tubes. Composite samples of bulk soil, rhizosphere soil, and roots were stored at −80°C.

Root Colonization Analysis

The tissue cassettes containing the root fragments were stored in tap water acidified with a few drops of white vinegar at 4°C until all samples were processed. Root fragments were stained using the "ink and vinegar" technique (Vierheilig and Piché, 1998). Cassettes were boiled in 10% w/v KOH solution for 3 min, boiled in a 5% ink (Shaeffer black) and white vinegar solution for 3 min, soaked in acidified tap water for 20 min and stored in a 50% glycerol solution. Root samples were examined under a Zeiss Discovery V20 stereomicroscope coupled with an AxioCam ICC 5 camera (Carl Zeiss, Oberkochen, Germany). ZEN pro software v2012 (Carl Zeiss, Oberkochen, Germany) was used to digitize and visualize the root fragments. The percent of root length colonized was evaluated using the gridline intersection method (Giovannetti and Mosse, 1980). A total of 39,116 intersects were

recorded. Colonization percentage was calculated as the ratio of colonized intersects divided by the total number of intersects and multiplied by 100.

DNA Extraction

The UltraClean™ soil DNA Isolation Kit (MoBio, Laboratories, Carlsbad, CA) was initially used to isolate DNA from soil samples (48 out of 124). However, due to MoBio Laboratories not manufacturing anymore that kit during the wet lab stage of the study, the PowerSoil™ DNA Isolation Kit (Qiagen, Hilden, Germany) was then used to isolate DNA for the remaining soil samples and the rhizosphere samples. **Table S3** provides the list of the soil samples which were analysed with one or the other kit and the absence of difference between the AM fungal communities recovered with each kit is shown in supplementary material **Table S4**. The manufacturer's instructions for both kits were followed, except that soil and rhizosphere DNA was eluted in 50 µl for the PowerSoil kit. DNA extractions from soil and rhizosphere samples were performed in duplicate and the duplicates were pooled.

Root fragments were put in 2-ml tubes containing Tungsten Carbide Beads of 3 mm, cooled in liquid nitrogen and placed immediately in a TissueLyser II instrument (Qiagen) for crushing mechanically the roots. A DNeasy® Plant Mini Kit (Qiagen, Valencia, CA) was used following the manufacturer's instructions except that the final elution was done in 75 µl instead of 100 µl, and the flow-through from the first elution was reused for the second elution rather than using fresh elution buffer. The quantity and quality of the DNA extracts were first assessed on 1.5% agarose gel stained with GelRed® (1:10000, Biotium, USA), run at 70 V for 60 min, and visualized using the Gel-Doc system (Bio-Rad Laboratories, Mississauga, ON). The quantity and quality of the DNA extracts were also assessed by means of Qubit Fluorometer 2.0 (Life Technologies, Burlington, ON, Canada), using the Qubit dsDNA HS assay kit. DNA extracts were stored at –20°C until use.

DNA Amplification and Illumina Library Preparation

The AM fungal communities were characterized using the primer pair AML1/AML2 (Lee et al., 2008) which targets the V3-V4-V5 variable regions of the nuclear 18S small subunit (SSU) ribosomal RNA gene. The amplification was performed in 20 µl of reaction mix in triplicate as follows: 1 µl of gDNA, 200 µM of each dNTP, 2 mM of Mg^{2+}, 0.8 µM of each primer, and 2.5 U of Q5 HighFidelity DNA Polymerase (NEBNext® Q5 Hot Start HiFi PCR Master Mix). The thermocycling conditions were as follows: initial denaturation at 98°C for 30 s, 20 cycles at 98°C for 10 s, 64°C for 30 s, 65°C for 60 s, and final extension performed at 65°C for 5 min. The DNA was amplified in a Biometra TProfessional thermocycler (Biometra GmbH, Goettingen, Germany). The three amplicon replicates were pooled and purified using the QIAquick PCR Purification Kit (Qiagen, Valencia, CA) and eluted in 50 µl of elution buffer. This step is important to prevent interactions between the remaining primers during nested PCR. PCR products were visualized in a GelRed stained 1.5% agarose gel.

In order to comply with the sequencing length capacity of Illumina MiSeq® Reagent Kit v3 (2 × 300 bp), a new primer pair yielding a 490-bp-length amplicon (including primers) was designed to target the V3-V4 region of the nuclear 18S rRNA gene: nu-SSU-0450-5′ (5′- CGCAAATTACCCAATCCC-3′) and nu-SSU-0899-3′ (5′-ATAAATCCAAGAATTTCACCTC-3′). Primers were named according to the primer nomenclature system of Gargas and DePriest (1996). The number in the primer name refers to the 5′ end position of the primer on the 18S sequence standard of *Saccharomyces cerevisiae* (GenBank accession Z75578). Primers were designed based on the guidelines provided by Integrated DNA Technologies (IDT Inc., San Diego, CA USA). Thermodynamic features of each primer are provided as supplementary material (**Table S5**). Purified PCR products amplified with AML1/AML2 were used as templates for nested PCR. A one to three bp "heterogeneity spacer" was introduced between the 3′ end of the adapter and the 5′ end of the primer pair nu-SSU-0450-5′/nu-SSU-0899-3′ to dampen the effect of the low sequence diversity issue of the MiSeq platform (**Table S6**, Fadrosh et al., 2014). The recipe for the amplification reaction was similar to the first-round PCR, except for the primer concentration which was 0.5 µM. The thermocycling conditions were as for the first round PCR except for the number of cycles which was reduced to 15 and the annealing temperature which was 59°C. The nested PCR was performed in triplicate and verified by electrophoresis on a GelRed-stained 1.5% agarose gel. Replicates were pooled.

Library preparation followed the protocol described in Stefani et al. (2020). Briefly, the PCR products from the nested PCR were purified using Agencourt AMPure® XP beads (Beckman Coulter Inc., Indianapolis, IN, USA), normalized to 1 to 2 ng/µl with the SequalPrep™ Normalization Plate kit (ThermoFisher Scientific) and indexed using the Nextera index kit (Illumina, San Diego, CA, USA). Indexed amplicons were then purified and normalized. Purified indexed amplicons were quantified by qPCR using the LightCycler® 480 system (Roche Molecular Systems Inc., Branchburg, NJ, USA) with the KAPA library quantification kit for Illumina platforms (KAPA Biosystems, MA, USA) in order to determine the volume of each sample to make up a 1-nM amplicon pool for library preparation.

Paired-end sequencing (2 × 300 bp) was carried out using the Illumina MiSeq® sequencer for 500 cycles at the Molecular Technologies Laboratory of Agriculture and Agri-Food Canada Ottawa Research and Development Centre.

Bioinformatic Analyses

The bioinformatic workflow is illustrated in **Figure S2** and its impact on the sequence dataset is described in the supplementary material. The raw demultiplexed sequences were processed in QIIME 2 v2020.2.0 (Bolyen et al., 2019). Paired-end sequences were denoised, dereplicated, and filtered for chimeras using the DADA2 plugin (Callahan et al., 2016), as implemented in QIIME 2. Sequences were trimmed in order to include only bases with quality scores > 35. The first 18 and 22 nucleotides of the 5′ end of the forward and reverse sequences, respectively, were trimmed. The 3′ end of the forward and reverse sequences was truncated at positions 266 and 261, respectively. Reads with number of

expected errors higher than 1 were discarded. The number of sequences used to train the error model was set to 200,000. Amplicon sequence variants with a frequency of less than 0.1% of the mean sample depth were considered rare ASVs and removed. This threshold represents the MiSeq bleed-through between runs as reported by Illumina (https://github.com/LangilleLab/microbiome_helper/wiki/Amplicon-SOP-v2-(qiime2-2020.2)). De novo clustering using a threshold of 100% similarity was performed using *vsearch* (Rognes et al., 2016), as implemented in QIIME 2. This step was required because the use of degenerate fusion primers (**Table S6**) introduced one to three extra nucleotides in read length and the use of DADA2 (as implemented in QIIME 2) would have produced different ASVs for identical sequences of variable length. **Figure S3A** shows that the ASV richness in samples from each compartment was saturated with a sequencing depth of 5,000. **Figure S3B** showed that the number of analysed samples was appropriate to characterise the field resident AM fungal communities. Each sample was rarefied to 5,000 sequences which retained 1,680,000 (32.9%) sequences in 336 (90.3%) samples and 303 (95.6%) of the amplicon sequence variants (**Figure S4**). The taxonomic identification of each ASV was performed using a backbone phylogenetic tree as described in Stefani et al. (2020). The taxonomic assignment of each ASV is provided in **Table S7** and **Figure S5**.

Alpha-Diversity Analyses

AM fungal diversity was estimated *via* the number of ASVs as a proxy of species richness. Venn diagrams were produced using the package *venndiagram* v1.6.20 (Chen and Boutros, 2011; Chen, 2018). The matrix used to calculate the relative abundance of the main AM fungal clades per block and per compartment was obtained using the rarefied ASV table. The ASV table was converted into a "biom" file, and the taxonomic information was added using the command *biom add-metadata*. Then the command *collapse_samples.py* (QIIME 1 v1.9.1) was used to combine repetitions from each block per microbiome. The barplots were produced using the R package *ggplot2* v3.3.0 (Wickham, 2016). The core AM fungal community was calculated on the rarefied datasets (raw abundance) using the function *core* and *plot_core* from R package *microbiome* v1.8.0. (Lahti and Shetty, 2019). Detection and prevalence thresholds were set to 0 and 50, respectively. Within-sample (alpha) diversity was calculated using the sample size- and coverage-based rarefaction and extrapolation (R/E) of the Hill numbers of species, i.e., richness (q = 0), Shannon diversity (q = 1, the exponential of Shannon entropy), and Simpson diversity (q = 2, the inverse of Simpson concentration), as implemented in the R package *iNEXT* version 2.0.19 (Chao et al., 2014; Hsieh et al., 2016). The Faith's phylogenetic diversity index (Faith and Baker, 2006) was calculated using the QIIME 2 command *qiime diversity alpha-phylogenetic* on a RAxML phylogenetic tree that included 303 AM fungal ASVs as described in Stefani et al. (2020). Heatmaps showing the relative abundance of the core ASVs by cultivar for each compartment were produced using the R package *superheat* v0.1.0 (Barter and Yu, 2017). Principal component analysis was visualized using the function *fviz_pca_var* from the R package *factoextra* v1.0.7 (Kassambara and Mundt, 2020).

Statistical Analyses

Linear models were used to investigate the effects of durum wheat cultivar and compartment (i.e. bulk soil, rhizosphere soil, and root) on the structure of AM fungal community. In order to analyse data sharing a similar distribution, variables (ASVs) with > 50% of non-zero values (i.e. core AM fungal community represented by 29 ASVs, hereafter identified as category $ASVs_{50+}$), and variables with 10% to 50% of non-zero values (47 ASVs, category $ASVs_{10\text{-}50}$) were analysed separately. Variables with less than 10% of non-zero values (227 ASVs, category $ASVs_{10\text{-}}$) were ignored.

For the category $ASVs_{50+}$, a principal component analysis (PCA) was realized on the correlation matrix with a varimax rotation. The function *PCA* from the R package *FactoMineR* v2.3, (Le et al., 2008) was used, with data scale to unite variance and nine principal components (74.6% of cumulative variance) retained based on the Kaiser criterion (i.e. with an eigenvalue higher than one). In order to avoid running the linear mixed effects analysis on 29 ASVs, a single ASV per principal component was selected with a saturation coefficient close to +1 or −1 (**Figure S6**). The linear mixed effects analysis was performed to investigate the relationship between the abundance of sequences and durum wheat cultivars (31), compartments (3) and ASVs (9). Cultivars, compartments, and ASVs (with interaction terms) were set as fixed factors and block, block × cultivar, and block × cultivar × compartment were set as random factors. A squared root transformation was performed on the outcome value to respect the assumptions of the model. Means are presented on the original scale, while the *p* values come from the model on transformed data. For the category $ASVs_{10\text{-}50}$, ASVs were dichotomized as null or non-null values. Principal component analysis was realized on the tetrachoric correlation matrix. Three dimensions (58.9% of cumulative variance) were retained based on variance criteria (one dimension should explain > 10% variance), with eigenvalues ranking from 0.83 to 0.25. Again, a single ASV showing the highest positive or negative saturation coefficient was selected per dimension, leading to the selection of ASV021, ASV030 and ASV031. A generalized linear mixed model (GLMM), with a logit link, was then performed using the same technique as for the LMM. Finally, linear mixed models were also used to investigate the effects of cultivars and compartments on diversity indices (ASV richness, Shannon and Simpson diversity, Faith's phylogenetic diversity index), with random effects for block and block × cultivar. Heterogeneous variances were modelled for Shannon diversity values. Linear mixed models were also used to test the effects of cultivars on the colonisation rate, with a random effect for block. In all LMM and GLMM, multiple comparisons using Tukey adjustment were done for significant effects.

Statistical analyses were done using R v3.6.3 (R Core Team, 2020), and the following packages: *car v3.0-7* (Fox and Weisberg, 2019), *emmeans v1.4.6* (Lenth, 2020), *factoextra v1.0.7* (Kassambara and Mundt, 2020), *MASS v7.3-51.5* (Venables and Ripley, 2002), *mgcv v1.8-31* (Wood, 2004), *moments v0.14* (Komsta and Novomestky, 2015), *nlme v3.1-144* (Pinheiro et al., 2020), *psych v1.9.12.31* (Revelle, 2020), *reshape v0.8.8* (Wickham, 2007), and *sjmisc v2.8.4* (Lüdecke, 2018).

RESULTS

Characterization of the AM Fungal Community Associated With Durum Wheat

A total of 303 ASVs belonging to Glomeromycota were recovered in the rarefied dataset. About 50% of the 303 ASVs were shared between the bulk soil, rhizosphere soil, and root samples (**Figure 1A**). The number of ASVs recorded was relatively similar between soil (242 ASVs), rhizosphere (226 ASVs) and root (214 ASVs) compartments. The AM fungal community was dominated by the genera *Funneliformis, Claroideoglomus, Paraglomus*, and *Rhizophagus* (**Figure 1B**). Sequences from the genus *Funneliformis* were the most abundant (19 ASVs), with a relative abundance ranging from 30% in roots to 45% in soil. ASV001 was the most abundant (21.6% of sequences) and the sequences were homologous to the 18S sequence of *F. mosseae* BEG12. The clade Claroideoglomus-7 was the second most abundant in bulk soil (12%) and rhizosphere soil (22%). It included 9 ASVs with sequence similarities close to the species *C. claroideum, C. etunicatum, C. lamellosum*, and *C. luteum* (**Figure S5**). The clade Paraglomus-1 (8 ASVs) was the third most abundant in bulk soil (11%) and rhizosphere soil (11%). A BLAST search for these 8 ASVs showed that their sequences were closely related to 18S sequences assigned to *P. occultum* (MN793990) and *P. laccatum* (MN517120). However, these ASVs did not cluster with the 18S sequences from well identified herbarium cultures of *P. occultum* (HA771 and IA702, **Figure S5**). The clade Archaeospora-1 (14 ASVs) was well represented in rhizosphere soil with an average relative abundance of 10%. It is interesting to observe the increasing abundance of the clade Rhizophagus-1 (21 ASVs) from bulk soil (4.1%), to rhizosphere soil (9.5%), to roots (30.6%). The clade Rhizophagus-1 included sequences of well-identified herbarium cultures of species *R. irregularis*, *R. vesiculifer*, and *R. fasciculatum* (**Figure S5**).

The core AM fungal community (i.e. ASVs present in > 50% of the samples) included 29, 30 and 29 ASVs for the bulk soil, rhizosphere soil, and root compartments, respectively (**Figure 2**) and 29 ASVs (category $ASVs_{50+}$) when the three compartments were considered together. These ASVs were assigned to 8 genera (*Archaeospora, Claroideoglomus, Diversispora, Dominikia, Funneliformis, Glomus, Paraglomus, Rhizophagus*), and represented five families (Archaeosporaceae, Claroideoglomeraceae, Diversisporaceae, Glomeraceae, Paraglomeraceae), four orders (Archaeosporales, Diversisporales, Glomerales, Paraglomerales) and three classes (Archaeosporomycetes, Glomeromycetes, and Paraglomeromycetes).

Cultivar and Compartments Effects on AM Fungal Community

The lowest percentages of root colonisation were recorded in Arnautka (57.8% ± 14.8), Hercules (59.8% ± 6.7) and AC Pathfinder (62.8% ± 10.4) while the highest percentages were observed in roots from Transcend (77.3% ± 19), Enterprise (79.8% ± 7.3) and AC Navigator (84% ± 17.5, **Figure S7**).

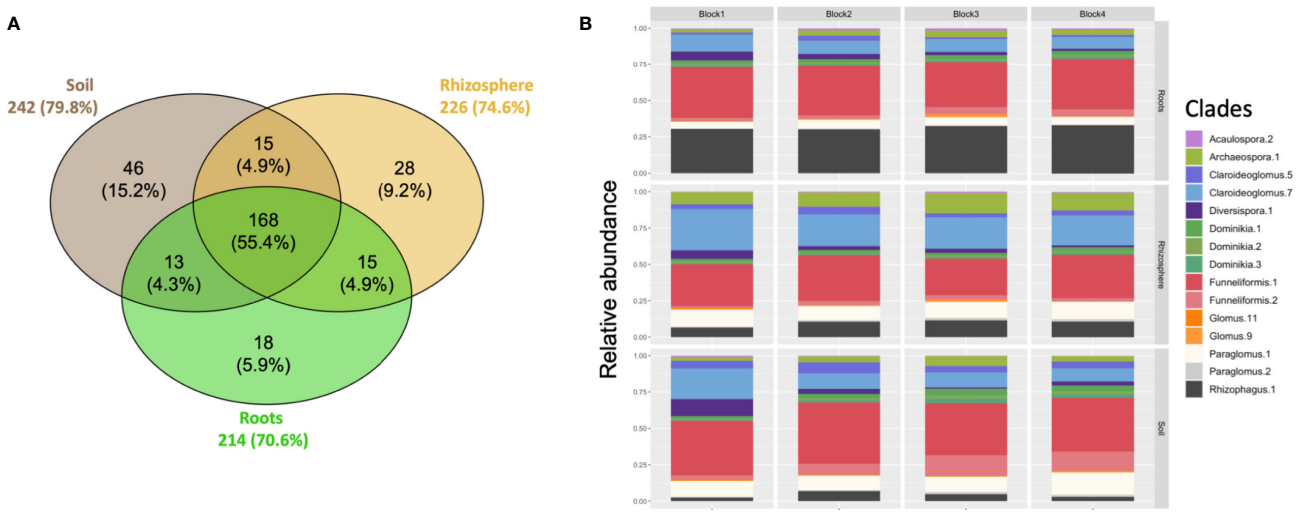

FIGURE 1 | (A) Venn diagram showing overlap of amplicon sequence variants (ASVs) between soil, rhizosphere, and root compartments. The size of the circles is proportional to the number of ASVs recorded in each compartment. Each sample was randomly subsampled to a common sequencing depth of 5000 sequences. **(B)** Taxonomic profile of AM fungi recovered in soil, rhizosphere and root compartments for each block. The clades with a relative abundance < 1% are not shown.

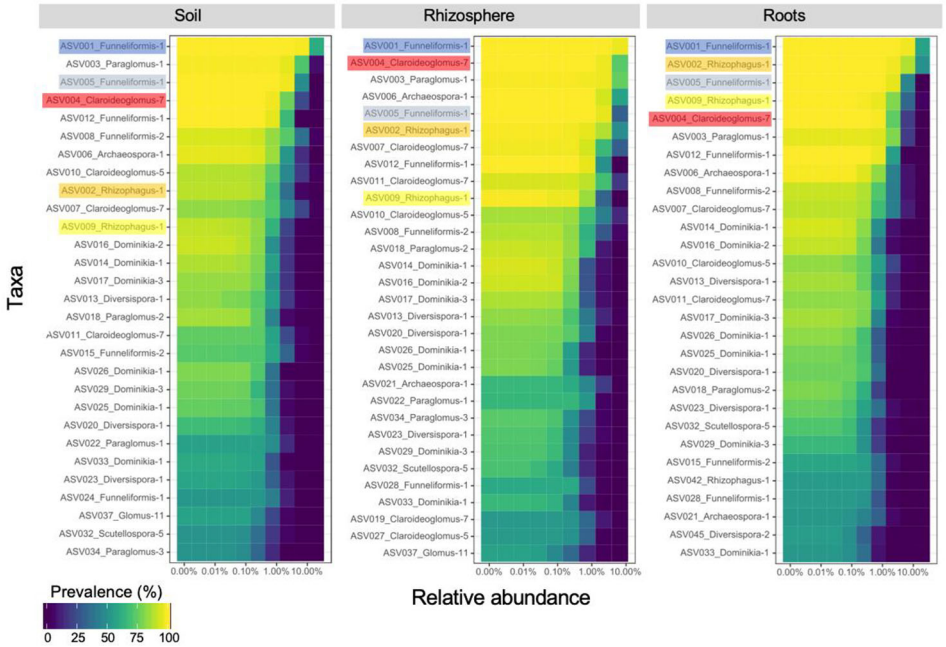

FIGURE 2 | Core community of arbuscular mycorrhizal fungi recovered in soil (29 ASVs), rhizosphere (30 ASVs) and root (29 ASVs) samples. The top five amplicon sequence variants recovered in root samples are highlighted to emphasize their ranking in rhizosphere and soil samples. ASVs are ranked on the ordinate axis from the most (top) to the least (bottom) prevalent in each compartment while they are ordered by ascending relative abundance on the abscissa axis.

However, the effect of cultivars on the percentages of root colonisation was not significant ($F_{30,90} = 1.02$, $p = 0.45$).

The average ASV richness per cultivar and compartment ranged between 50 and 100 (**Figure 3**). Only cultivar Quilafen consistently showed low average ASV richness for each compartment (57, 49 and 46 for bulk soil, rhizosphere soil, and roots, respectively). No clear relationship in the average ASVs richness between compartment was observed for the other cultivars. For example, 57 ASVs were recorded in bulk soil samples under Arnautka while 97 ASVs were recorded in its roots. High number of ASVs in root samples does not mean high percentage of root colonisation because the percentage of root colonization was lowest in Arnautka (57.8% ± 14.8). The opposite situation was observed in Quilafen which had the lowest average number of ASVs in roots, and the fifth highest average rate of root colonization (76% ± 7.8).

No significant effect of cultivars ($F_{30,90} = 1.23$, $p > 0.05$) and compartments ($F_{2,150} = 1.90$, $p > 0.05$) was observed on ASV richness (**Figure 3**). No significant effect of cultivars ($F_{30,90} = 1.15$, $p > 0.05$) and compartments ($F_{2,150} = 2.38$, $p > 0.05$) was observed on Faith's phylogenetic diversity index. A compartment effect was found significant on Shannon ($F_{2,150} = 17.7$, $p < 0.0001$) and Simpson diversity ($F_{2,150} = 34.2$, $p < 0.0001$). The Tukey *post hoc* tests showed that estimated marginal means of both indices were significantly lower ($p < 0.0001$) in roots than in bulk soil and rhizosphere soil samples (data not shown), which indicates a degree of dominance in the AM fungal community recorded in the root samples.

The heatmaps of the relative abundance of the 29 core ASVs by compartment and cultivar did not show major differences between cultivars for most of the ASVs (**Figure 4**). A clear shift in the relative abundance of ASV001 (Funneliformis-1) was visible in each compartment but it did not involve the same groups of cultivars, with exception of Eurostar. The heatmap based on the root compartment clearly showed an antagonist behavior between ASV001 and ASV002 (Rhizophagus-1) for two clusters of 9 and 22 cultivars. The dendrograms of core ASVs community profiles were statistically not similar, according to the Bray-Curtis distance (**Table S8**).

The PCA performed on the 29 core ASVs (category $ASVs_{50+}$) showed that ASVs from the genera *Dominikia* and *Funneliformis* were highly correlated with the first and second principal component, respectively (**Figures 5A** and **S6**). ASVs from the genera *Rhizophagus* (ASV002), *Claroideoglomus* (ASV010), *Scutellospora* (ASV32) and *Paraglomus* (ASV003) were correlated with the fourth, sixth, seventh, and eighth principal component, respectively (**Figure S6**). The linear mixed model analysis performed on one representative ASV per principal component showed that the two-way interactions cultivar × ASV ($F_{240,1944} = 1.4$, $p = 0.0003$) and compartment × ASV ($F_{16,1944} = 71.4$, $p < 0.0001$) were significant. The pairwise comparisons of estimated marginal means between cultivars were significantly different ($p < 0.05$) for ASV001 (Funneliformis-1), ASV002 (Rhizophagus-1), and ASV010 (Claroideoglomus-5), following Tukey's correction. For ASV001, the estimated marginal mean calculated for Eurostar

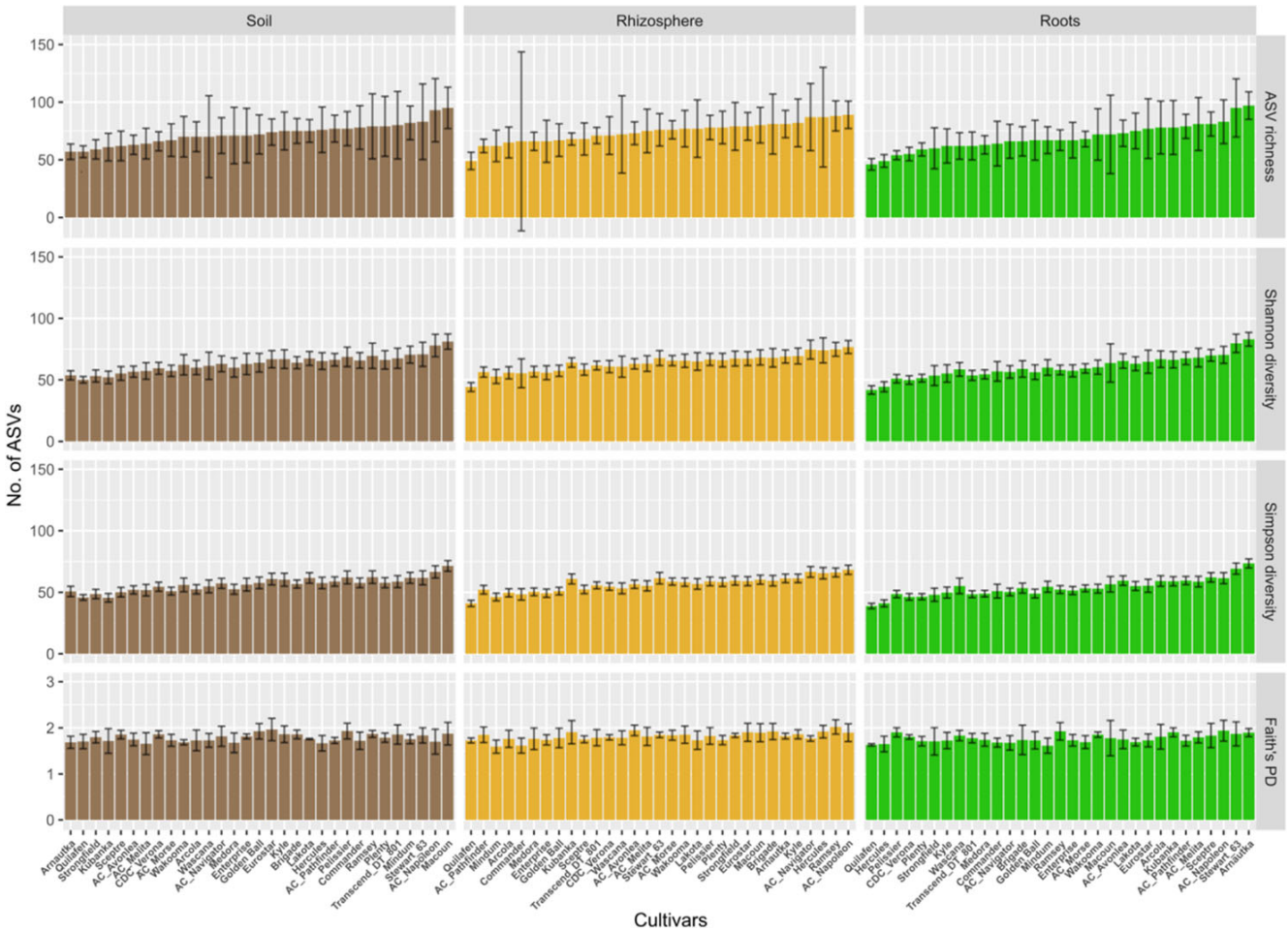

FIGURE 3 | Diversity measured in soil, rhizosphere and root samples for each of the five landraces and 26 durum wheat cultivars. The ordinate displays Hill numbers of three orders: ASV richness (q = 0), Shannon diversity (q = 1), and Simpson diversity (q = 2), and Faith's Phylogenetic diversity. Cultivars are sorted according to the mean ASV richness observed in root samples, from the lowest to the highest. Error bars represent estimated bootstrap standard error.

was significantly inferior to Commander, Macoun, and Plenty (**Figure 5B**). The same differences between these cultivars are expected for ASV005 and ASV012 since they are negatively correlated on principal component 2 (r = −0.925 and r = −0.917, respectively), similarly to ASV001 (r = −0.925, **Figure S6**). For ASV002, the estimated marginal means calculated for AC Napoleon, Lakota, and Mindum were significantly inferior to Wakooma. For ASV010, the estimated marginal means calculated for AC Avonlea, AC Pathfinder, CDC Verona, Eurostar, Golden Ball, Hercules, Macoun, Mindum, Strongfield were significantly inferior to cultivar Kubanka. Finally, the abundance recorded for six out of nine ASVs were significantly different between the compartments (**Figure 5C**). Only ASV002 (Rhizophagus-1) was significantly more abundant in roots than in rhizosphere and bulk soil. ASV003 (Paraglomus-1), ASV008 (Funneliformis-2), and ASV010 (Claroideoglomus-5) followed the opposite dynamic since their abundance were significantly lower in roots compared to rhizosphere and/or soil. ASV001 (Funnliformis-1) was significantly less abundant in rhizosphere compared to soil and roots while ASV004 (Claroideoglomus-7) showed the opposite pattern. Finally, the generalized linear mixed model analysis performed on the category $ASVs_{10\text{-}50}$ showed no significant difference (data not shown).

DISCUSSION

AM Fungal Communities Associated With Durum Wheat

Results from this study provide an in-depth overview of the AM fungal communities associating with wheat genotypes representative of Canadian durum, in a humid climate. The core AM fungal community associated with durum wheat recorded in Eastern Canada was relatively similar to that recorded in the Canadian prairie (Ellouze et al., 2018). Results from the current study and from Ellouze et al. (2018) showed that the genus *Funneliformis* was core for durum wheat. In the current study, a sequence homologous to that from the culture of

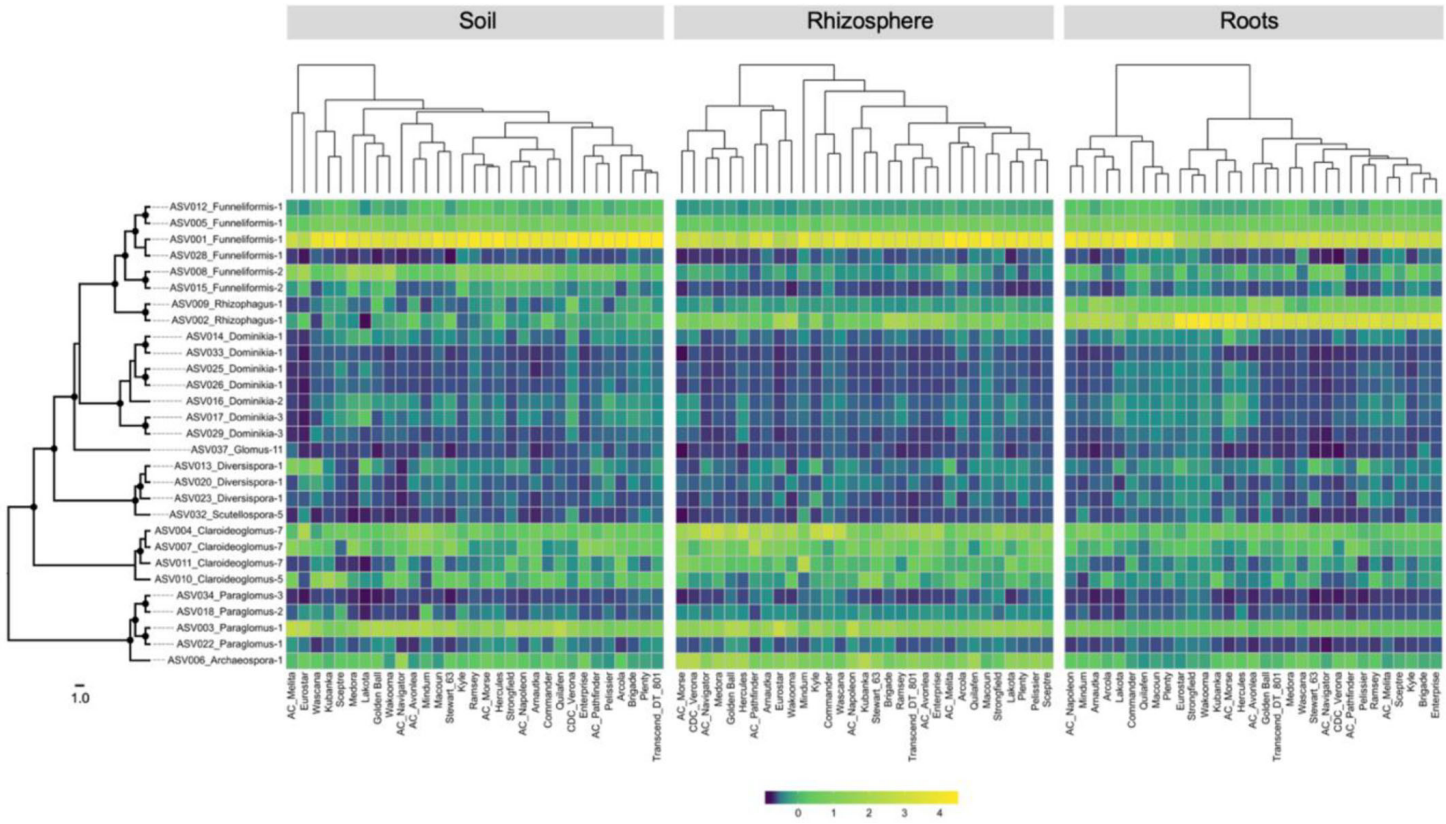

FIGURE 4 | Heatmaps showing the relative abundance of each of the 29-core amplicon sequence variants recorded in soil, rhizosphere, and root compartments for the five landraces and 26 durum wheat cultivars. Left: RAxML phylogeny of 29 core ASVs, black circles on the nodes represent bootstrap values > 70. The scale represents the branch length corresponding to expected substitutions per site. Top: cladogram showing the relationships between each cultivar based on the distance matrix calculated on ASV relative abundance.

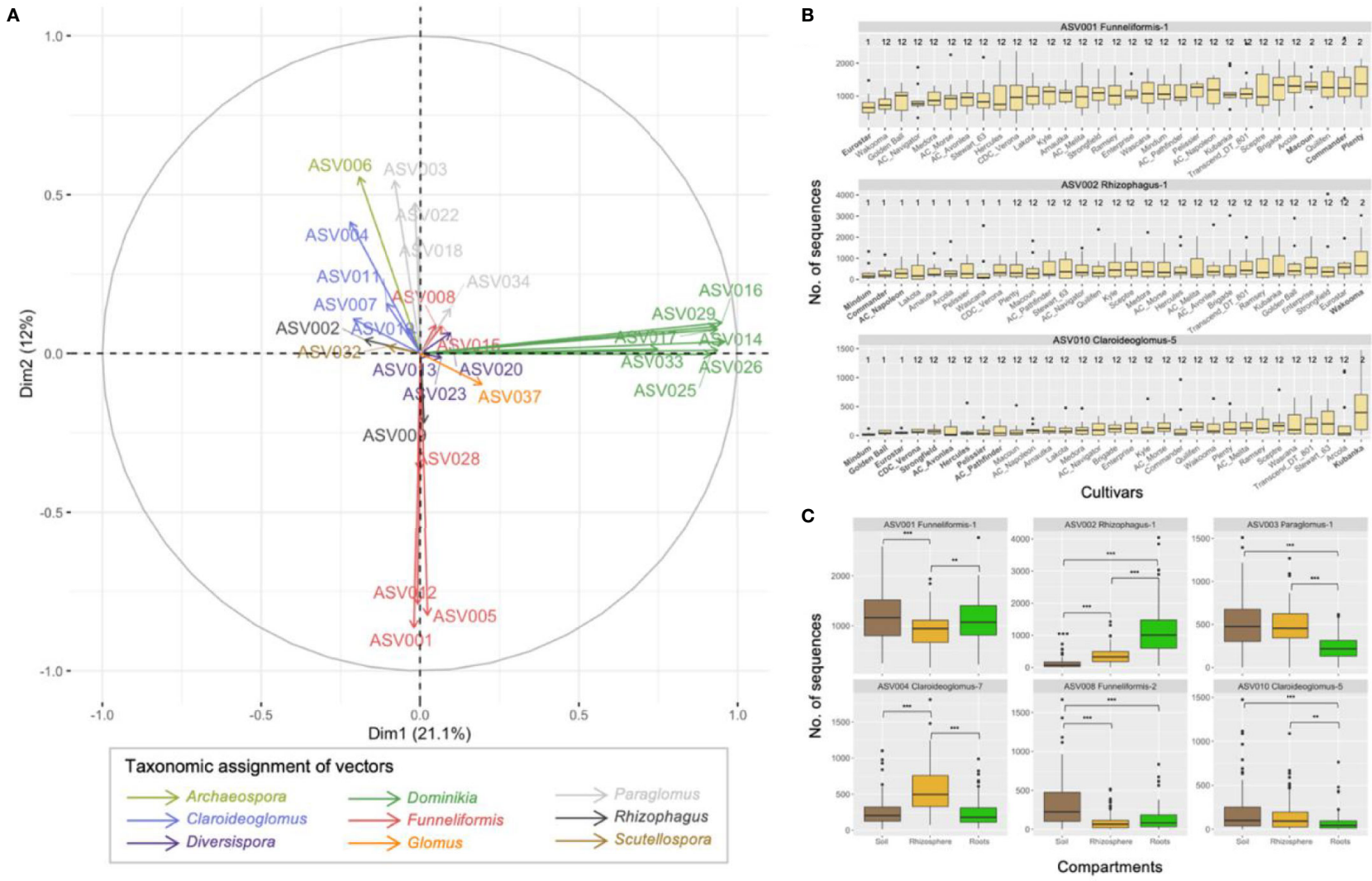

FIGURE 5 | (A) Principal component analyses on 29 ASVs with non-zero values in > 50% of the samples across the three compartments (category ASV_{50+}). The first two principal components explained 33.1% of variance. **(B)** Boxplots showing the abundance of the three ASVs for which significant differences were recorded between the pairwise comparisons of the 31 cultivars of durum wheat. Boxplots with a different number indicate significant differences between cultivars ($p < 0.05$). Boxplots were ordered by increasing mean abundance. **(C)** Boxplots showing the abundance of the six ASVs for which the significant differences were found between soil, rhizosphere and root compartments. Non-significant differences are not shown, ** and *** indicate p values < 0.01 and < 0.001, respectively.

Funneliformis mosseae BEG12 was the most abundant in soil, rhizosphere, and root compartments. In Ellouze et al. (2018), *Funneliformis* (50.3% of the reads), *Claroideoglomus* (10.7%) and *Dominikia* (2.9%) were the main genera recorded in soil and root samples associated with five landraces and 32 durum wheat cultivars grown on the Brown and Black soils in the Canadian prairie. In the current study, the genera *Claroideoglomus* and *Paraglomus* were also part of the five most abundant genera recorded in each compartment. The genus *Dominikia* was also included in the core AM fungal community with a relative abundance ranging from 4% to 6% across the three sampled compartments. A sequence from an unknown *Archaeospora* species (98.5% of pairwise similarity with the 18S sequence of the herbarium culture of *A. trappei* NB112) was part of the 10 most abundant sequences in bulk soil, rhizosphere soil, as well as in roots. Ellouze et al. (2018) reported the genus *Archaeospora* was abundant in root samples from the Brown soil site only. None of the ASVs were assigned to the genus *Gigaspora*. It has been previously observed that taxa from *Gigaspora* and *Scutellospora* tended to disappear in agricultural field under conventional management such as tillage (Hamel et al., 1994; Boddington and Dodd, 2000; Kabir, 2005). Here the experimental field was tilled in fall 2015 and harrowed in spring 2016. A total of 317 ASVs was recorded in the current study while 190 AM fungal OTUs were associated with durum wheat in the Prairies using a similarity threshold of 97% to cluster 18S AM fungal sequences. As discussed in Stefani et al. (2020) and in Schlaeppi et al. (2016), 18S sequences from different AM fungal species are lumped together at a similarity threshold of 99%, making the ASVs approach the least inaccurate as a proxy for species for datasets based on the 18S sequences of the nuclear ribosomal DNA. However, using ASVs based on 18S sequences as a proxy for AM fungal species could overestimate species richness. For instance, within the clade Rhizophagus-1, 10 ASVs were closely related to the sequences from cultures of *R. irregularis* (DAOM 197198, MUCL 43195, and W4533). The pairwise similarities between these 10 ASVs ranged from 98.9% (5 diverging nucleotides) to 99.8% (1 diverging nucleotide). Maeda et al. (2018) determined that the genome of *R. irregularis* DAOM 181602 was characterized by ten rDNA paralogs which the mean intra-genomic similarity was 99.91% (SD = 0.06) for the 18S gene. Therefore, it is possible that 490 bp long 18S fragments diverging by a single nucleotide could represent paralogs from the same species. Similarly, ten ASVs closely related to the sequences from well characterised cultures of *F. mosseae* (BEG12, FL126, DAOM 212595) had pairwise similarities ranging from 99.6% to 99.8%, i.e. two to one nucleotide of divergence, respectively. With exception to *R. irregularis*, the number of rDNA paralogs and their intra-genomic variability remain unknown in the other species of AM fungi. While it is undetermined if a unique similarity threshold can be applied across the phylum Glomeromycota to accurately recognise species, a threshold ranging from 99.5% to 100% seems to be safe for not lumping into the same contig 18S sequences from closely related species.

ASV002 (100% of pairwise similarity with *R. irregularis* DAOM 181602/DAOM 197198) was the second most abundant sequence recorded in the root samples, while it was ranked in 11th position in bulk soil. Its relative abundance in roots was significantly more important than in rhizosphere and bulk soil compartments. This clearly shows the preferential selection of the host plant for *R. irregularis*. Compared to *Glomus custos* and *G. aggregatum*, Kiers et al. (2011) showed that *R. irregularis* (identified in the publication as *G. intraradices*) was the most cooperative species as it provided to its host the best rate of nutrient exchange (more phosphorus for less carbon), resulting in a host preference for resources allocation to *R. irregularis*. This makes *R. irregularis* a strong competitor in *in vivo* or *in vitro* system (Engelmoer et al., 2014), but also under field conditions as suggested by the data from the current study. Among the five most abundant sequences recorded in roots, two were from the genus *Rhizophagus* and two from the genus *Funneliformis*.

ASVs from all four AM fungal orders were recovered, showing that the nested PCR approach based on the primer set AML1/AML2 and the new primer set nu-SSU-0450-5′/nu-SSU-0899-3′ is able to target all AM fungal taxa. Moreover, non-AM fungal sequences represented only 6.1% of the sequences obtained after the quality filtering. Berruti et al. (2017) used a similar approach with a nested PCR based on the primer set AML1/AML2 for the first round PCR and the primer set AMADf/AMDGR (Sato et al., 2005) for the second round PCR to characterize the AM fungal community in roots and soils of three mountain vineyards. Both primer sets nu-SSU-0450-5′/nu-SSU-0899-3′ and AMADf/AMDGR target the V3-V4 regions of the 18S and are able to recover all known AM fungal lineages when used in combination with the AM fungi specific primer set AML1/AML2 (Lee et al., 2008). The new primer set nu-SSU-0450-5′/nu-SSU-0899-3′ amplifies a fragment slightly longer (490 bp) than the primer set AMADf/AMDGR (423 bp). This length is compatible with 2 × 300 paired-end sequencing and allows a 37 bp of overlap between forward and reverse reads once they were truncated in 3′ position due to decrease in quality.

Cultivar Impact on AM Fungal Communities

To our knowledge, results from this study provide the most comprehensive characterisation of the AM fungal communities associated with durum wheat under field conditions. However, our results were obtained in one field trial using a non-restricted pool of durum wheat and at the time of sampling, that may have filtered the community in bulk soils, rhizosphere soils, and roots. A total of 317 ASVs were recorded representing the four AM fungal orders, thus a filtering effect on the AMF pool due to a single site and sampling time is unlikely. The levels of AM fungal diversity were similar between each cultivar, in all three compartments examined. However, the results clearly show a differential affinity of some cultivars for ASVs related to *F. mosseae* (ASV001, ASV005, and ASV010), *R. irregularis* (ASV002), and *Claroideoglomus* sp. (ASV010). ASV001 and

ASV002 were the most abundant in the whole dataset and were assigned to genera previously identified as predominant in the AM fungal community associated with durum wheat growing in the dry environment of the Canadian prairie (Ellouze et al., 2018). Cultivars with strong affinity for *F. mosseae* had less affinity for *R. irregularis* and vice and versa. Indeed, the abundance of ASV001 was the lowest in cultivars Eurostar, Wakooma, and Golden Ball while the abundance of ASV002 was among the highest for these cultivars. Moreover, the responsiveness of cultivars Eurostar and Golden Ball to ASV010 (*Claroideoglomus* sp.) was limited compared to the other cultivars. Overall results showed that the genotypic differences between the five landraces and 26 durum wheat cultivars had only a minor impact on the structure of the AM fungal community. This suggests that the symbiotic signalling system (Bonfante and Requena, 2011) and the molecules (i.e. flavonoids, strigolactones, Steinkellner et al., 2007) released by the durum wheat cultivars to initiate the mutualistic interaction with AM fungi are well conserved for each genotype and that the set of genes involved with the recognition of the Myc-factors (pre-physical contact stage) and with the establishment of the mycorrhization (post-physical contact stage) were only marginally altered through the breeding. Tian et al. (2019) showed that 2360 genes were differentially expressed in the roots of *Triticum aestivum* under the influence of the molecular signals produced by *R. irregularis*. Zhao et al. (2014) showed that the orchid mycorrhizae trigger in the host the induction of various genes involved with cell wall modification or defence-related phytohormone and phosphate transport. It is possible that the genotypic differences between durum wheat cultivars lead to slightly different molecular interactions with some AM taxa. This could result in less compatible partners featured by a less abundant fungal biomass, leading to less sequence count.

Despite Arnautka, Hercules, and AC Pathfinder cultivars were less colonized than Transcend, Enterprise, and AC Navigators ones, the analysis of the percentages of root colonisation showed non-significant variation within durum wheat cultivars. However, a spread of 26% between the cultivars showing the lowest and highest percentages of colonisation was observed. Because the phenotypic variation of these cultivars has not been characterized so far, one cannot exclude that some genotypes have had variable phenotypical traits (such as root branching, biomass, shoot branching) that could result in different level of root colonization. The non-significant differences observed in the percentages of root colonisation between cultivars grown under field conditions contrasts with experiments performed in greenhouse with commercial inoculum of *R. irregularis* DAOM 197198 (Singh et al., 2012; Ellouze et al., 2016). Singh et al. (2012) inoculated five cultivars of durum wheat under low and medium fertility conditions. The type of cultivar was identified as having a significant effect on the percentages of root colonization at both low and medium soil fertility, and the cultivars showing percentages of root colonisation significantly lower or higher varied according to the levels of fertility. At low and medium fertility, the cultivar "Commander" had the highest and lowest percentages of colonisation respectively. The same trend was observed with "Commander" and "Pathfinder" in Ellouze et al. (2016). In the current study, the cultivar "Commander" had the fifth lowest average colonisation percentage. In a pot trial carried out in greenhouse and involving a set of 94 bread wheat genotypes, Lehnert et al. (2017) reported significant genotypic differences with regard to root colonization with a blend of three AM species (*Rhizophagus intraradices, Claroideoglomus claroideum*, and *C. etunicatum*). The authors also identified 30 significant markers (representing six quantitative trait loci (QTL) regions) associated with root colonization, and they estimated that the heritability for root colonization was moderate. This suggests it is possible to improve root colonization by breeding. Similarly, De Vita et al. (2018) investigated the percentages of root colonisation and its genetic basis in the plant host by inoculating 108 durum wheat cultivars with *F. mosseae* and *R. irregularis*. They identified seven putative QTL associated with mycorrhizal susceptibility with each AM species and reported high variability in the percentage of root colonisation. These results suggest a complex genetic control of root colonisation.

Surprisingly, the percentages of root colonisation were very different between greenhouse experiments (Singh et al., 2012; Ellouze et al., 2016; De Vita et al., 2018) and the current field-based study while the same approach based on gridline intersect method (Giovannetti and Mosse, 1980) was used. Indeed, the average colonisation percentage recorded across all the cultivars was high (71%), with a spread of 26% between the cultivars showing the lowest (Arnautka) and highest (AC Navigator) percentages of colonisation. The percentages of root colonisation recorded in Singh et al. (2012); Ellouze et al. (2016) and De Vita et al. (2018) ranged between 5% and 45%. In their study on the effect of domestication on AM association at different fertility regimes, Martín-Robles et al. (2017) found better AM symbiotic development at low P fertility levels, in both domesticated crops and wild progenitors. However, P fertility was limited either in the greenhouse experiments as in the current field study. High percentages of root colonization here likely reflect other field conditions conducive to AM symbiotic development. The colonization percentages reported here are in line with what Graham and Abbott (2000) observed from "aggressive colonizers" at low P fertility (50–89% of root length colonization). In their case the aggressive colonizers included species such as *Scutellospora calospora, Glomus invermaium, Acaulospora laevis*, and *Gigaspora decipiens* inoculated onto specimens of Kulin wheat. These species triggered growth depression and reduced sucrose concentration in roots.

CONCLUSION

Using deep 18S rDNA sequencing, the AM communities associating with the historical set of durum wheat genotypes in the field under an humid climate were comprehensively characterised and allowed to detect minor impacts of the

cultivars on the structure of the AM fungal community. The hypothesis that different cultivars host distinct AM fungal communities is not supported in durum wheat, contrary to what some previous studies using other plant species have suggested. The genetic variation among durum wheat genotypes seems to be too narrow to select for specific plant-AM fungal associations from field resident AM fungal communities, using traditional breeding techniques. However, few cultivars had a differential responsiveness to *F. mosseae*, *R. irregularis*, and *Claroideoglomus* sp. Because the field trial was performed in a humid climate in Eastern Canada, results were not influenced by variation in soil moisture. This field trial along with the ones performed in the Canadian prairie examined the AM associations formed between durum wheat genotypes and resident AM fungi. In these three ecoregions, *F. mosseae* and *R. irregularis* were the main taxa recruited by durum wheat.

DATA AVAILABILITY STATEMENT

The Illumina data generated in this study were deposited in the NCBI Sequence Read Archive and are available under project number PRJNA645613.

AUTHOR CONTRIBUTIONS

CH designed the project and supervised field work. RK and YR designed the project and provided the biological material. SD performed the sampling, the DNA isolation and estimated the root length colonization. FS designed the primers nu-ssu-0450-5′/nu-ssu-0899-3′. FS and MH supervised laboratory work. FS and ML analyzed the data. ML performed data visualization. FS wrote the paper. MH and CH edited and provided critical review of the manuscript. All authors contributed to the article and approved the submitted version.

FUNDING

This work was supported by funding from the NSERC Discovery grant (RGPIN-2018-04178, MH) and from the Agriculture and Agri-Food Canada through the projects J-000617 (CH) and J-002272 (FS).

ACKNOWLEDGMENTS

The authors are grateful to Dr Pierre-Luc Chagnon and Dr Marc St-Arnaud for insightful comments on an early draft of the manuscript; Réjean Desgagnés and Benoît Bérubé for help in field work; David Gagné for bioinformatic support; Lisa Koziol and Julie Chapados from the Molecular Technology Laboratory (Ottawa-RDC, AAFC), for MiSeq library preparation and Illumina sequencing. The authors thank the two reviewers for their useful comments.

SUPPLEMENTARY MATERIAL

The Supplementary Material for this article can be found online at: https://www.frontiersin.org/articles/10.3389/fpls.2020.01206/full#supplementary-material

REFERENCES

Bakker, M. G., Manter, D. K., Sheflin, A. M., Weir, T. L., and Vivanco, J. M. (2012). Harnessing the rhizosphere microbiome through plant breeding and agricultural management. *Plant Soil* 360, 1–13. doi: 10.1007/s11104-012-1361-x

Barter, R., and Yu, B. (2017). *superheat: a graphical tool for exploring complex datasets using heatmaps. R package version 0.1.0*. https://CRAN.R-project.org/package=superheat

Berruti, A., Desirò, A., Visentin, S., Zecca, O., and Bonfante, P. (2017). ITS fungal barcoding primers versus 18S AMF specific primers reveal similar AMF based diversity patterns in roots and soils of three mountain vineyards. *Env. Microbiol. Rep.* 9, 658–667. doi: 10.1111/1758-2229.12574

Boddington, C. L., and Dodd, J. C. (2000). The effects of agricultural practices on the development of indigenous arbuscular mycorrhizal fungi. I. Field studies in an Indonesian ultisol. *Plant Soil* 218, 137–144. doi: 10.1023/A:1014966801446

Bolyen, E., Rideout, J., Dillon, M. R., Bokulich, N. A., Abnet, C. C., Al-Ghalith, G. A., et al. (2019). Reproducible, interactive, scalable and extensible microbiome data science using QIIME 2. *Nat. Biotech.* 37, 848–857. doi: 10.1038/s41587-019-0209-9

Bonfante, P., and Requena, N. (2011). Dating in the dark: how roots respond to fungal signals to establish arbuscular mycorrhizal symbiosis. *Curr. Opin. Plant Biol.* 14, 451–457. doi: 10.1016/j.pbi.2011.03.014

Brundrett, M. C., and Tedersoo, L. (2018). Evolutionary history of mycorrhizal symbioses and global host plant diversity. *New Phytol.* 220, 1108–1115. doi: 10.1111/nph.14976

Callahan, B. J., McMurdie, P. J., Rosen, M. J., Han, A. W., Johnson, A. A., and Holmes, S. P. (2016). DADA2: High-resolution sample inference from Illumina amplicon data. *Nat. Methods* 13, 581–587 nmeth.3869. doi: 10.1038/nmeth.3869

Chao, A., Gotelli, N. J., Hsieh, T., Sander, E. L., Ma, K., Colwell, R. K., et al. (2014). Rarefaction and extrapolation with Hill numbers: a framework for sampling and estimation in species diversity studies. *Ecol. Monogr.* 84, 45–67. doi: 10.1890/13-0133.1

Chen, H., and Boutros, P. C. (2011). VennDiagram: a package for the generation of highly-customizable Venn and Euler diagrams in R. *BMC Bioinf.* 12, 35. doi: 10.1186/1471-2105-12-35

Chen, H. (2018). *VennDiagram: Generate High-Resolution Venn and Euler Plots. R package version 1.6.20*. https://CRAN.R-project.org/package=VennDiagram

Clarke, J. M., Clarke, F. R., and Pozniak, C. J. (2010). Forty-six years of genetic improvement in Canadian durum wheat cultivars. *Can. J. Plant Sci.* 90, 791–801. doi: 10.4141/cjps10091

De Vita, P. D., Avio, L., Sbrana, C., Laidò, G., Marone, D., Mastrangelo, A. M., et al. (2018). Genetic markers associated to arbuscular mycorrhizal colonization in durum wheat. *Sci. Rep-UK* 8, 10612. doi: 10.1038/s41598-018-29020-6

Dexter, J. E. (2008). "The history of durum wheat breeding in Canada and summaries of recent research at the Canadian Grain Commission on factors associated with durum wheat processing," in *Bosphorus 2008 ICC (International Cereal Congress)*. (Istanbul).

Donn, S., Kirkegaard, J., Perera, G., Richardson, A., and Watt, M. (2015). Evolution of bacterial communities in the wheat crop rhizosphere:

rhizosphere bacteria in field-grown intensive wheat crops. *Environ. Microbiol.* 17, 610–621. doi: 10.1111/1462-2920.12452

Ellouze, W., Hamel, C., DePauw, R. M., Knox, R. E., Cuthbert, R. D., and Singh, A. K. (2016). Potential to breed for mycorrhizal association in durum wheat. *Can. J. Microbiol.* 62, 263–271. doi: 10.1139/cjm-2014-0598

Ellouze, W., Hamel, C., Singh, A. K., Mishra, V., DePauw, R. M., and Knox, R. E. (2018). Abundance of the arbuscular mycorrhizal fungal taxa associated with the roots and rhizosphere soil of different durum wheat cultivars in the Canadian prairies. *Can. J. Microbiol.* 64, 527–536. doi: 10.1139/cjm-2017-0637

Engelmoer, D. J. P., Behm, J. E., and Kiers, E. T. (2014). Intense competition between arbuscular mycorrhizal mutualists in an in vitro root microbiome negatively affects total fungal abundance. *Mol. Ecol.* 23, 1584–1593. doi: 10.1111/mec.12451

Fadrosh, D. W., Ma, B., Gajer, P., Sengamalay, N., Ott, S., Brotman, R. M., et al. (2014). An improved dual-indexing approach for multiplexed 16S rRNA gene sequencing on the Illumina MiSeq platform. *Microbiome* 2, 6. doi: 10.1186/2049-2618-2-6

Faith, D., and Baker, A. (2006). Phylogenetic diversity (PD) and biodiversity conservation: some bioinformatics challenges. *Evol. Bioinform.* 2, 121–128. doi: 10.1177/117693430600200007

Feldman, M., and Kislev, M. E. (2008). Domestication of emmer wheat and evolution of free-threshing tetraploid wheat. *Israel J. Plant Sci.* 55, 207–221. doi: 10.1560/ijps.55.3-4.207

Fox, F., and Weisberg, S. (2019). *An {R} companion to applied regression. 3rd ed.* (Thousand Oaks CA: Sage). https://socialsciences.mcmaster.ca/jfox/Books/Companion/

Gan, Y., Hamel, C., O'Donovan, J. T., Cutforth, H., Zentner, R. P., Campbell, C. A., et al. (2015). Diversifying crop rotations with pulses enhances system productivity. *Sci. Rep-UK* 5, 14625. doi: 10.1038/srep14625

Gargas, A., and DePriest, P. T. (1996). A nomenclature for fungal PCR primers with examples from intron-containing SSU rDNA. *Mycologia* 88, 745–748. doi: 10.1080/00275514.1996.12026712

Gianinazzi, S., Gollotte, A., Binet, M.-N., Tuinen, D., Redecker, D., and Wipf, D. (2010). Agroecology: the key role of arbuscular mycorrhizas in ecosystem services. *Mycorrhiza* 20, 519–530. doi: 10.1007/s00572-010-0333-3

Giovannetti, M., and Mosse, B. (1980). An evaluation of techniques for measuring vesicular arbuscular mycorrhizal infection in roots. *New Phytol.* 84, 489–500. doi: 10.1111/j.1469-8137.1980.tb04556.x

Graham, J. H., and Abbott, L. K. (2000). Wheat responses to aggressive and non-aggressive arbuscular mycorrhizal fungi. *Plant Soil* 220, 207–218. doi: 10.1023/a:1004709209009

Hamel, C., Dalpé, Y., Lapierre, C., Simard, R. R., and Smith, D. L. (1994). Composition of the vesicular-arbuscular fungi population in an old meadow as affected by pH, phosphate and soil disturbance. *Agric. Ecosyst. Environ.* 49, 223–231. doi: 10.1016/0167-8809(94)90051-5

Hamel, C., Hanson, K., Selles, F., Cruz, A. F., Lemke, R., McConkey, B., et al. (2006). Seasonal and long-term resource-related variations in soil microbial communities in wheat-based rotations of the Canadian prairie. *Soil Biol. Biochem.* 38, 2104–2116. doi: 10.1016/j.soilbio.2006.01.011

Hetrick, B. A. D., Wilson, G. W. T., and Cox, T. S. (1993). Mycorrhizal dependence of modern wheat cultivars and ancestors: a synthesis. *Can. J. Bot.* 71, 512–518. doi: 10.1139/b93-056

Hijri, M. (2016). Analysis of a large dataset of mycorrhiza inoculation field trials on potato shows highly significant increases in yield. *Mycorrhiza* 26, 209–214. doi: 10.1007/s00572-015-0661-4

Hsieh, T. C., Ma, K. H., and Chao, A. (2016). iNEXT: an R package for rarefaction and extrapolation of species diversity (Hill numbers). *Methods Ecol. Evol.* 7, 1451–1456. doi: 10.1111/2041-210x.12613

Iannucci, A., Fragasso, M., Beleggia, R., Nigro, F., and Papa, R. (2017). Evolution of the crop rhizosphere: impact of domestication on root exudates in tetraploid wheat (*Triticum turgidum* L.). *Front. Plant Sci.* 8, 2124. doi: 10.3389/fpls.2017.02124

Kabir, Z. (2005). Tillage or no-tillage: Impact on mycorrhizae. *Can. J. Plant Sc.* 85, 23–29. doi: 10.4141/p03-160

Kassambara, A., and Mundt, F. (2020). *factoextra: extract and visualize the results of multivariate data analyses. R package version 1.0.7.* https://CRAN.R-project.org/package=factoextra

Kiers, E. T., Duhamel, M., Beesetty, Y., Mensah, J. A., Franken, O., Verbruggen, E., et al. (2011). Reciprocal rewards stabilize cooperation in the mycorrhizal symbiosis. *Science* 333, 880–882. doi: 10.1126/science.1208473

Komsta, L., and Novomestky, F. (2015). *moments: moments, cumulants, skewness, kurtosis and related tests. R package version 0.14.* https://CRAN.R-project.org/package=moments

Lahti, L., and Shetty, S. (2019). *Microbiome R package.* http://microbiome.github.io

Lambers, H., Mougel, C., Jaillard, B., and Hinsinger, P. (2009). Plant-microbe-soil interactions in the rhizosphere: an evolutionary perspective. *Plant Soil* 321, 83–115. doi: 10.1007/s11104-009-0042-x

Le, S., Josse, J., and Husson, F. (2008). FactoMineR: an R package for multivariate analysis. *J. Stat. Software* 25, 1–18. doi: 10.18637/jss.v025.i01

Lee, J., Lee, S., and Young, J. P. W. (2008). Improved PCR primers for the detection and identification of arbuscular mycorrhizal fungi. *FEMS Microbiol. Ecol.* 65, 339–349. doi: 10.1111/j.1574-6941.2008.00531.x

Lehnert, H., Serfling, A., Enders, M., Friedt, W., and Ordon, F. (2017). Genetics of mycorrhizal symbiosis in winter wheat (*Triticum aestivum*). *New Phytol.* 215, 779–791. doi: 10.1111/nph.14595

Lenth, R. (2020). *emmeans: estimated marginal means, aka least-squares means. R package version 1.4.7.* https://CRAN.R-project.org/package=emmeans

Lüdecke, D. (2018). sjmisc: data and variable transformation functions. *J. Open Source Software* 3, 754. doi: 10.21105/joss.00754

Maeda, T., Kobayashi, Y., Kameoka, H., Okuma, N., Takeda, N., Yamaguchi, K., et al. (2018). Evidence of non-tandemly repeated rDNAs and their intragenomic heterogeneity in *Rhizophagus irregularis*. *Commun. Biol.* 1, 11841. doi: 10.1038/s42003-018-0094-7

Mahoney, A. K., Yin, C., and Hulbert, S. H. (2017). Community structure, species variation, and potential functions of rhizosphere-associated bacteria of different winter wheat (*Triticum aestivum*) cultivars. *Front. Plant Sci.* 8, 132. doi: 10.3389/fpls.2017.00132

Martín-Robles, N., Lehmann, A., Seco, E., Aroca, R., Rillig, M. C., and Milla, R. (2017). Impacts of domestication on the arbuscular mycorrhizal symbiosis of 27 crop species. *New Phytol.* 218, 322–334. doi: 10.1111/nph.14962

McCaig, T. N., and Clarke, J. M. (1995). Breeding durum wheat in western Canada: Historical trends in yield and related variables. *Can. J. Plant Sci.* 75, 55–60. doi: 10.4141/cjps95-009

Pinheiro, J., Bates, D., DebRoy, S., Sarkar, D.R Core Team (2020). *nlme: linear and nonlinear mixed effects models. R package version 3.1-148.* https://CRAN.R-project.org/package=nlme

R Core Team (2020). *R: A language and environment for statistical computing* (Vienna, Austria: R Foundation for Statistical Computing). https://www.R-project.org/

Revelle, W. (2020). *psych: procedures for personality and psychological research* (Evanston, Illinois, USA: Northwestern University). https://CRAN.R-project.org/package=psych Version = 1.9.12.

Rognes, T., Flouri, T., Nichols, B., Quince, C., and Mahé, F. (2016). VSEARCH: a versatile open source tool for metagenomics. *PeerJ* 4, e2584. doi: 10.7717/peerj.2584

Sato, K., Suyama, Y., Saito, M., and Sugawara, K. (2005). A new primer for discrimination of arbuscular mycorrhizal fungi with polymerase chain reaction-denature gradient gel electrophoresis. *Grassl. Sci.* 51, 179–181. doi: 10.1111/j.1744-697x.2005.00023.x

Schlaeppi, K., Bender, S. F., Mascher, F., Russo, G., Patrignani, A., Camenzind, T., et al. (2016). High-resolution community profiling of arbuscular mycorrhizal fungi. *New Phytol.* 212, 780–791. doi: 10.1111/nph.14070

Singh, A. K., Hamel, C., DePauw, R. M., and Knox, R. E. (2012). Genetic variability in arbuscular mycorrhizal fungi compatibility supports the selection of durum wheat genotypes for enhancing soil ecological services and cropping systems in Canada. *Can. J. Microbiol.* 58, 293–302. doi: 10.1139/w11-140

Smith, S., and Read, D. (2010). *Mycorrhizal symbiosis. 3rd ed.* (London: Academic Press).

Soil Classification Working Group. (1998). *The Canadian system of soil classification. 3rd ed.* (Agriculture and Agri-Food Canada Publication 1646), (Ottawa: NRC Research Press) 187 pp.

Statistics Canada (2020). *Estimated areas, yield, production, average farm price and total farm value of principal field crops, in metric and imperial units. Table 32-10-0359-01.* https://www150.statcan.gc.ca/t1/tbl1/en/tv.action?pid=3210035901

Stefani, F., Bencherif, K., Sabourin, S., Hadj-Sahraoui, A. L., Banchini, C., Séguin, S., et al. (2020). Taxonomic assignment of arbuscular mycorrhizal fungi in an

18S metagenomic dataset: a case study with saltcedar (*Tamarix aphylla*). *Mycorrhiza* 30, 243–255. doi: 10.1007/s00572-020-00946-y

Steinkellner, S., Lendzemo, V., Langer, I., Schweiger, P., Khaosaad, T., Toussaint, J., et al. (2007). Flavonoids and strigolactones in root exudates as signals in symbiotic and pathogenic plant-fungus interactions. *Molecules* 12, 1290–1306. doi: 10.3390/12071290

Tian, H., Wang, R., Li, M., Dang, H., and Solaiman, Z. M. (2019). Molecular signal communication during arbuscular mycorrhizal formation induces significant transcriptional reprogramming of wheat (*Triticum aestivum* L.) roots. *Ann. Bot-London* 124, 1109–1119. doi: 10.1093/aob/mcz119

Turrini, A., Avio, L., Giovannetti, M., and Agnolucci, M. (2018). Functional complementarity of arbuscular mycorrhizal fungi and associated microbiota: the challenge of translational research. *Front. Plant Sci.* 9, 1407. doi: 10.3389/fpls.2018.01407

Venables, W. N., and Ripley, B. D. (2002). *Modern Applied Statistics with S. 4th ed.* (New York: Springer), ISBN 0-387-95457-0.

Vierheilig, H., and Piché, Y. (1998). A modified procedure for staining arbuscular mycorrhizal fungi in roots. *Z. Pflanz. Bodenkunde* 161, 601–602. doi: 10.1002/jpln.1998.3581610515

Wickham, H. (2007). Reshaping data with the reshape package. *J. Stat. Software* 21, 1–20. doi: 10.18637/jss.v021.i12

Wickham, H. (2016). *ggplot2: elegant graphics for data analysis* (New York: Springer-Verlag).

Wood, S. N. (2004). Stable and efficient multiple smoothing parameter estimation for generalized additive models. *J. Am. Stat. Assoc.* 99, 673–686. doi: 10.1198/016214504000000980

Zhao, X., Zhang, J., Chen, C., Yang, J., Zhu, H., Liu, M., et al. (2014). Deep sequencing–based comparative transcriptional profiles of *Cymbidium hybridum* roots in response to mycorrhizal and non-mycorrhizal beneficial fungi. *BMC Genomics* 15, 747. doi: 10.1186/1471-2164-15-747

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

frontiers in Plant Science

ORIGINAL RESEARCH
published: 14 January 2021
doi: 10.3389/fpls.2020.622209

Arbuscular Mycrorrhizal Fungi Inoculation and Applied Water Amounts Modulate the Response of Young Grapevines to Mild Water Stress in a Hyper-Arid Season

Nazareth Torres†, Runze Yu and Sahap Kaan Kurtural†*

Department of Viticulture and Enology, University of California, Davis, Davis, CA, United States

OPEN ACCESS

Edited by:
Sergio Saia, University of Pisa, Italy

Reviewed by:
Paulo Emilio Lovato, Federal University of Santa Catarina, Brazil
Raffaella Balestrini, Institute for Sustainable Plant Protection, National Research Council (CNR), Italy

***Correspondence:**
Sahap Kaan Kurtural
skkurtural@ucdavis.edu

†ORCID:
Nazareth Torres
orcid.org/0000-0002-0597-4635
Sahap Kaan Kurtural
orcid.org/0000-0001-9578-831X

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

Received: *28 October 2020*
Accepted: *14 December 2020*
Published: *14 January 2021*

Citation:
Torres N, Yu R and Kurtural SK (2021) Arbuscular Mycrorrhizal Fungi Inoculation and Applied Water Amounts Modulate the Response of Young Grapevines to Mild Water Stress in a Hyper-Arid Season. Front. Plant Sci. 11:622209. doi: 10.3389/fpls.2020.622209

Several factors may affect the success of a replanting vineyard. Given the current environmental conditions, an optimized irrigation schedule would still be one of the most desirable tools to improve crop productivity and fruit quality. On the other hand, the symbiosis of grapevines with arbuscular mycorrhizal fungi (AMF) is a key component of the vineyard production systems improving the vine growth, nutrient uptake, and berry quality. The aim of this study was to characterize the response of Merlot grapevines to AMF inoculation and two different irrigation amounts in their first productive year. The experiment was conducted on 2-year Merlot grapevines inoculated with AMF (I) or not-inoculated (NI) and subjected to two irrigation amounts, full irrigated (FI), where the amount of water was enough to maintain expansive growth and half irrigated (HI) where plants received the half of the amount of water of FI plants. Water status, gas exchange parameters, growth, mineral content, berry composition, and mycorrhizal colonization were monitored through the season. AMF inoculation improved the grapevine vegetative growth, water status, and photosynthetic activity, especially when vines were subjected to HI irrigation; however, no effect was observed on the leaf mineral content, must pH, total soluble solids, or total acidity. The main effects were observed on the flavonoid composition of berry skins at harvest. Irrigation amounts and mycorrhizal inoculation modified cyanidin and peonidin derivatives whereas flavonol composition was mainly affected by irrigation treatments. A strong relationship between the mycorrhizal colonization rate of roots and total quercetins, cyanidins, and peonidins was found. Findings support the use of a mycorrhizal inoculum and a better water management in a hyper-arid growing season; however, these results may be affected by edaphoclimatic characteristics and living microbiota in vineyard soils, which should be taken into account before making the decision of inoculating the vineyard.

Keywords: climate change, water scarcity, grapevine physiology, berry metabolism, arbuscular mycorrhizal fungi, sustainable viticulture

INTRODUCTION

Grapevine is an economically important crop worldwide with a global surface area of 7.45 million ha, which is mainly cultivated for wine making. California stands out as the fourth leading wine producer in the world with 257,784 ha of wine grapes and 4.28 million tons of grapes harvested in 2018, leading to an annual economic impact of $57.6 billion (Wine Institute, 2020). Nevertheless, winegrowers face the challenge of replanting their vineyards when grapevines are not producing due to diseases such as grapevine red blotch virus, trunk diseases, or other viral diseases such as leaf roll disease, or because the plant material is producing substandard fruit and consequently compromising the wine quality. However, several factors need to be taken into account when replanting, as improper establishment during this stage causes considerable economic loss to the industry.

Arbuscular mycorrhizal fungi (AMF) are soil-borne fungi that form mutualistic relationships with 80% of the superior plants (Smith and Read, 2008). In viticultural regions, the AMF-grapevine symbiosis was pointed out as a key component of the vineyard system (Trouvelot et al., 2015). Recent research suggested the key role that this symbiosis might play in facing environmental constrains (Torres et al., 2018b). The application of mycorrhizal inocula has emerged as a reliable technique to enhance the agricultural productivity whereas reducing environmental costs (Berruti et al., 2016; Hamilton et al., 2016). Frequently, these commercial inoculants consist of a single or few AM fungal isolates grown in plant culture or greenhouse conditions with annual grasses or forbs (Gianinazzi and Vosátka, 2004), hence they might not establish on woody grapevines that have different ecosystem preferences (Holland et al., 2018). It is well established that under controlled conditions AMF inoculation of grapevines promotes increased growth (Linderman and Davis, 2001), drought tolerance (Nikolaou et al., 2003), and nutrient uptake (Karagiannidis et al., 2007). Moreover, AMF protect grapevines grown in controlled conditions against pathogens through stimulation of key genes of the phenylpropanoid biosynthesis in leaves (Bruisson et al., 2016) and inhibit their transmission by impairing the growth of nematode vectors in roots and their reproduction in soils (Hao et al., 2018). Although it is widely accepted that AMF-grapevine association improves grapevine growth and mineral uptake in vineyards (Trouvelot et al., 2015), contradictory results were recently reported when studying the protective role of the symbiosis against pathogens such as *Ilyonectria* (Holland et al., 2019). Similarly, AMF inoculation may affect berry primary and secondary metabolism in response to environmental stresses when grapevines were cultivated under controlled conditions (Torres et al., 2016, 2018c) but little is known about their effect under natural conditions. Additionally, rootstock genotype and type of inoculum could also influence the effectiveness of mycorrhizal inoculation and therefore the response of young vines to the environment (Holland et al., 2018).

On the other hand, most wine grape producing regions are subjected to seasonal drought, but based on the global climate models an increase in aridity is predicted in the future. Hence, an optimized irrigation schedule would still be one of the most desirable tools to improve crop productivity and quality in historically non-irrigated viticulture areas where irrigation is expanded fast to mitigate environmental stress (Costa et al., 2016; Resco et al., 2016). In addition, in warm and hot viticultural regions such as California that rely on irrigation for crop production, water resources, especially groundwater, are becoming scarce due to extended drought periods and overuse by irrigated agriculture (Wilson et al., 2020).

Currently, winegrowers are aware of the importance of a sustainable viticulture that ensures the profitability in the future, without compromising berry quality. However, to the best of our knowledge little is known about the contribution AMF inoculation may have for implementing the effects of different irrigation amounts on the performance and berry quality of young grapevines under field conditions. Therefore, the aim of this study was to characterize the response of young Merlot grapevines to AMF inoculation subjected to two different irrigation amounts in their first productive year.

MATERIALS AND METHODS

Plant Material and Experimental Design

This study was conducted in the Oakville Experimental Station (38.429°–122.410°). The vineyard was planted to Merlot clone 181 on 3,309 C rootstock in 2018 at 3 m × 2 m (row × vine) spacing in E–W orientation. The grapevines were spur pruned and trained to quadrilateral trellis system 1.38 m above vineyard floor with catch wires at 1.68 m. The experimental vineyard was drip-irrigated with one or two emitters spaced every 2 m along the drip line and with the capacity of deliver 3.8 L of water per hour. Natural vegetation was allowed to grow in alleys and mowed according to vineyard manager's discretion, with a no-till system in place.

The experiment consisted in a 2 × 2 factorial design (AMF inoculated or not-inoculated vines subjected to two irrigation amounts) with four replications of seven grapevine plots arranged in a split plot design. The commercial Myco Apply Endo Maxx inoculum (Mycorrhizal Applications LLC, OR, United States) consisted in a suspendable powder containing living propagules of *Rhizophagus intraradices* (basionym *Glomus intraradices*), *Funneliformis mosseae* (basionym *Glomus mosseae*), *Glomus aggregatum,* and *Glomus etunicatum* containing 5,625 propagules/g. The mycorrhizal inoculum was diluted in water to final concentration of 5.3 mg/L in order to achieve the manufacturer's recommended rate of 10 g each 1,000 plants. The diluted AMF inoculum was applied in-field drench during 50 s around the trunk of each vine at the beginning of the growing season (20 March) by using a 56 L spot sprayer. Although the inoculum manufacturer did not report other microorganisms accompanying AMF[1], it is known that commercial AMF inocula, obtained following industrial production processes, are home of a large and diverse community of bacteria with important functional plant promoting growth traits, that may act in

[1]www.valent.com/Products/1f42d59a-c1fd-4d1c-b6a2-8c4a6486cc81/mycoapply-endomaxx

synergy with AMF providing additional services and benefits (Agnolucci et al., 2019). Therefore, non-inoculated vines received the same amount of a filtrate inoculum with the objective of restoring rhizobacteria and other soil free-living microorganism accompanying AMF and that play an important role in the uptake of soil resources as well as in the infectivity and efficiency of AMF isolates (Agnolucci et al., 2015). The filtrate was obtained by passing diluted mycorrhizal inoculum through a Whatman filter paper Grade 5 with particle retention of 2.5 μm (Whatman 5; GE Healthcare, MA, United States). Phosphorus amounts in the vineyard soil was measured before the experiment and was low, thus, that phosphorus level (<10 mg/kg) was sufficient to ensure adequate development of non-inoculated plants, even under water deficit and not excessive enough to decrease the mycorrhizal diversity in the vineyard and thereby the root colonization (Van Geel et al., 2017). Irrigation treatments started at the beginning of summer (May 2020) until harvest (August 2020). Vineyard crop evapotranspiration (ET_c) was calculated by multiplying the reference evapotranspiration (ET_o, CIMIS #77) and the crop coefficient (K_c). Thus, half of the inoculated and non-inoculated vines were irrigated to ensure the full of expansive growth that corresponded with the amount of water needed to restore the 100% of the ET_c (Full irrigated, FI). The other half of inoculated and non-inoculated vines received half of the amount of water received by FI plants (half irrigated, HI). Irrigation was applied weekly. Each treatment had four replicates consisting in 7 grapevines, 3 of which were sampled and the 4 on distal ends were treated as border plants.

Weather Conditions

Weather data (**Figure 1**) were obtained from the California Irrigation Management Information Systems, CIMIS, station (#77, Oakville, California) located on site during the growing season covered by the trial and the reference period 2000 to 2020 (California Department of Water Resources, 2020).

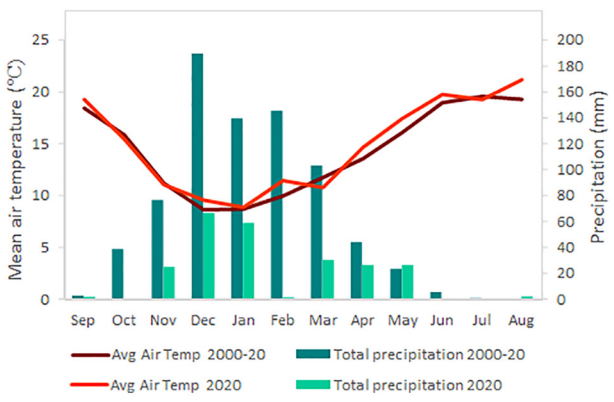

FIGURE 1 | Average air temperature (Avg air temp) and precipitation during the growing season 2019–2020 and the average for the same period in the last 20 years (2000–2020). Weather data were obtained from the CIMIS weather station #77 (Oakville, CA, United States) located at the research site.

Plant Water Status and Leaf Gas Exchange Parameters

Plant water status was measured as stem water potential (SWP) every 2 weeks during the growing season around the solar noon. A fully-expanded leaf per treatment-replicate exposed to sun and without signs of disease and/or damage was selected and covered 2 h before measurements with a foil-lined zip-top plastic bag in order to suppress transpiration. Then, the SWP was directly determined with a pressure chamber (Model 610 Pressure Chamber Instrument, PMS Instrument Co., Corvallis, OR, United States).

Coinciding with the main phenological events, leaf gas exchange was measured beginning at solar noon on one fully expanded leaf with a CIRAS-3 portable photosynthesis system (PP Systems, Amesbury, MA, United States) equipped with a leaf chamber with a 4.5 cm^2 window. The window of the chamber was oriented perpendicularly toward the sun to allow for saturation light conditions (1984 ± 52 μmol/m/s). Reference CO_2 was set to 390 μmol/mol CO_2 at a flow rate of 200 mL/min. Leaf gas exchange was performed, leaving the cuvette for 40–60 s until reaching a steady state and measurements were taken in triplicate.

AMF Colonization and Relative Mycorrhizal Dependency Index

Intraradical AMF colonization was estimated before treatment application (native colonization, 20 March), 3 months after treatment application (25 June), and at harvest (26 August). Root samples (mainly hair roots) from three grapevines per replicate were collected at a depth of 15 and 20 cm away from the vine trunk by using a fork, and stored in zip bags for further analysis. Then, each replicate root sample was washed with water in the sink, cleared, and stained according to methods described in Koske and Gemma (1989). AMF colonization was determined by examining 1-cm root segments (50 per treatment/replicate) under the microscope (**Supplementary Figure 1**). Then, intensity of the intraradical mycorrhizal colonization was calculated for each treatment/replicate as described previously by Torres et al. (2016). Briefly, the extension of mycorrhizal colonization was determined by estimating the product of the mycorrhizal colonization in width and length according to a scale range between 0 and 10 where 0 is complete absence of fungal structures. The extension of each treatment/replicate was calculated as the sum of the product of mycorrhizal colonization in width and length divided to the number of root segments. Then, the incidence of mycorrhizal colonization was estimated by dividing the number of root segments with presence of fungal structures and the total observed segments. Finally, the intensity of the colonization was calculated as the product between the extension and incidence, and the result was expressed as percentage of colonization.

Relative mycorrhizal dependency (RMD) index was calculated following Bagyaraj (1994): RMD = Leaf fresh weight of I vines × 100/Leaf fresh weight of NI vines. This index allows establishment of the crop dependency upon the mycorrhizal symbiosis for reaching its maximum growth for given environmental conditions.

Mineral Composition of Leaf Blades

During the growing season (05 June) two leaves per vine/replicate were collected, petioles were removed, and leaf blades were dried at 70°C in an oven. Then, mineral analysis was carried out by using couple plasma-mass spectrometry by Dellavalle, Inc. (Fresno, CA, United States). Nitrogen (N) was determined via automated combustion analysis (method B-2.20) while phosphorus (P), potassium (K), sodium (Na), calcium (Ca), magnesium (Mg), zinc (Zn), manganese (Mn), boron (B), iron (Fe), and cuprum (Cu) were analyzed via Nitric/Perchloric Acid Digestion (method B-4.20) as described by Gavlak et al. (1994).

Canopy Architecture, Grapevine Growth, and Yield Components

All the growth parameters were measured on the three middle vines in each replicate and the values were averaged for the replicate value. Green pruning was carried out before the cluster development (06 May) to avoid the excessive vegetative growth and ensure a good balance between the growth of vegetative and reproductive organs of the grapevines. Removed shoots from the three middle grapevines were weighed. Trunk diameter was measured with a carbon fiber composite digital caliper (Fisher Scientific, Waltham, MA, United States). At harvest, leaves were removed and leaf area was measured with a LI-3100 Area meter (LI-COR, Lincoln, NE, United States). Clusters were harvested and weighted to obtain the yield per vine. Measurements were performed on the three middle grapevines within each replicate and averaged.

Berry Size and Composition

Thirty berries were randomly collected from the middle vines within each replicate and immediately processed. Berries were weighed and gently pressed by hand to squeeze the juice. Total soluble solids (TSS) were determined using a temperature-compensating digital refractometer (Atago PR-32, Bellevue, WA, United States). Must pH and titratable acidity (TA) were determined with an autotritrator (Metrohm 862 Compact Titrosampler, Herisau, Switzerland). TA was estimated by titration with 0.1 N sodium hydroxide to an end point of 8.3 pH and reported as g/L of tartaric acid.

Berry Skin Flavonoid Composition

For flavonoid analysis 20 berries were randomly collected from each treatment-replicate and after gently peeling, skins were freeze-dried (Cold Trap 7385020, Labconco, Kansas City, MO, United States). Dried tissues were ground with a tissue lyser (MM400, Retsch, Germany). Fifty mg of the resultant powder was extracted in methanol: water: 7 M hydrochloric acid (70:29:1, V:V:V) to simultaneously determine flavonol and anthocyanin concentration and profile as previously described by Martínez-Lüscher et al. (2019). Briefly, extracts were filtered (0.45 μm, Thermo Fisher Scientific, San Jose, CA, United States) and analyzed using an Agilent 1260 series reversed-phase high performance liquid chromatography (HPLC) system (Agilent 1260, Santa Clara, CA, United States) coupled to a diode array detector. Separation was performed on a reversed-phase C18 column LiChrospher® 100, 250 mm × 4 mm with a 5 μm particle size and a 4 mm guard column of the same material at 25°C with elution at 0.5 mL per minute. The mobile phase was designed to avoid co-elution of anthocyanins and flavonols (Martínez-Lüscher et al., 2019) and consisted in a constant 5% of acetic acid and the following gradient (v/v) of acetonitrile in water: 0 min 8%, at 25 min 12.2%, at 35 min 16.9%, at 70 min 35.7%, 65% between 70–75 min, and 8% between 80–90 min. The identification of flavonoid compounds was conducted by determining the peak area of the absorbance at 280, 365, and 520 nm for flavan-3-ols, flavonols, and anthocyanins, respectively. Identification of individual flavan-3-ols, anthocyanins, and flavonols were made by comparison of the commercial standard retention times found in the literature. Commercial standards of epicatechin, malvidin-3-*O*-glucoside, and quercetin-3-*O*-glucoside (Sigma-Aldrich, St. Louis, MO, United States) were used for the quantification of flavan-3-ols, anthocyanins, and flavonols, respectively.

Labor Operation Costs, Gross Income, and Water Footprint of Irrigation Systems and AMF Inoculation

Cost estimates on labor operations and gross income per hectare were calculated based on yield and net returns per hectare (Kurtural et al., 2020). Water footprint (WF) was calculated as described by Zotou and Tsihrintzis (2017). Briefly, for the green component of the WF (green WF), precipitation data during the growing season was obtained from the CIMIS Station (#77, Oakville, CA, United States) and estimated as m^3/ha to obtain the total green consumed water volume (green CWU). Then the value was divided by the yield expressed as ton/ha. The blue component of the WF (blue WF) was calculated with the total irrigation water amount that grapevines received per hectare, and this blue consumed water volume (blue CWU) value was divided by the yield (ton/ha). The gray component of the WF was not calculated given that our experimental conditions avoided the use of fertilizers. Then, the total WF was estimated as the sum of green WF and blue WF.

Statistical Analysis

Statistical analyses were performed in R-Studio version 3.6.1 (RStudio: Integrated Development for R., Boston, MA, United States) for Windows. All the monitored parameters were fit in linear mixed-effect models (LMEM) by using the lmer function from lme4 package (Bates et al., 2020) with AMF inoculation (M), irrigation treatment (I), and their combination (M × I) as fixed factors, and replicate as random factor (Bates et al., 2015). The significance of the models was tested with the lmerTest package (Kuznetsova et al., 2020). Then, pairwise contrasts were conducted with function lsmeans from lsmeans package (Lenth, 2018) using the Kenward–Roger method and Tukey adjustment for *p*-values. Previously, for gas exchange parameters, stem water potential, mycorrhizal colonization, and flavonoid contents a mixed-effect model including sampling date (T) as fixed factor was run (**Supplementary Tables 1–3**). However, as the treatment effect seemed to be independent in

TABLE 1 | Mycorrhizal colonization at the beginning of the season, 3 months post-treatment application, and at harvest and relative mycorrhizal dependency (RMD) for vegetative growth of Merlot/3309C grapevines subjected to different irrigation amount (FI, Full Irrigated; HI, Half Irrigated), AMF inoculation (I, inoculated; NI, non-inoculated), and their combinations.

	Mycorrhizal colonization (%)			RMD (%)
	Native	After 3 months	Harvest	
Treatments				
FINI	1.68 ± 1.04	4.19 ± 1.16 b	14.14 ± 4 ab	78.43 ± 10.64 b
FII	1.11 ± 0.45	21.16 ± 2.66 a	26.28 ± 3.47 a	
HINI	2.21 ± 0.67	7.91 ± 0.76 b	9.23 ± 1.99 b	116.52 ± 8.54 a
HII	2.78 ± 0.67	16.38 ± 5.17 a	24.09 ± 4.02 a	
LMEM				
Irrigation (I)	*	ns	ns	*
Mycorrhizal inoculation (M)	ns	**	**	
I × M	ns	ns	ns	

*Grapevines were measured in Oakville (California) during the 2020 growing season. Values represent means ± SE (n = 4) separated by Kenward–Roger method and Tukey's p-value adjustment (P ≤ 0.05). Different letters within column, indicate significant differences as affected by Irrigation amount, I, AMF inoculation, M, and their interaction (I × M). ns, * and ** indicate non-significance or significance at 5%, and 1% probability levels, respectively. LMEM, linear mixed-effect model.*

the sampling date (with the exception of WUE and quercetin-3-glucoside content), sampling date was removed from the analysis to assess the effect of treatments for each sampling date. Finally, correlations between the percentage of mycorrhizal inoculation and flavonoid contents were calculated with the Pearson's test using the same software.

RESULTS

Weather Conditions, Mycorrhizal Colonization, and Grapevine Performance

The comparison between the growing season of the experiment and the reference data for the same period within the last 20 years showed that 2019–2020 was warmer and drier (**Figure 1**). Thus, average daily temperature was 0.5°C higher, especially in August, which reached 1.8°C more, and precipitation of 530 mm less compared to the average, hence, the 2020 growing season was an extreme year regarding temperature and rainfall.

Native mycorrhizal colonization was determined before treatment application and no differences between them were observed (**Table 1**). The mycorrhizal colonization intensity was analyzed (**Supplementary Figure 1**) after 3 months of treatment application to ensure the establishment of the mycorrhizal symbiosis, which frequently take place after 2–4 months of inoculation. Similar patterns in AMF colonization intensity were observed in both, 3 months after inoculation and at harvest, where roots from inoculated grapevines showed percentages of colonization values threefold higher than non-inoculated ones (**Table 1**). In addition, we observed increased AMF colonization rates along the growing season as shows the significant effect of the sampling date (T, $p \leq 0.0001$, **Supplementary Table 1**) and its interaction with the AMF inoculation (M × T, $p \leq 0.0001$, **Supplementary Table 1**).

Relative mycorrhizal dependency (RMD) index allows assessing the dependency of a crop on the mycorrhizal symbiosis to achieve its maximum growth at a given environmental condition. Under FI conditions, RMD values were lower than 100% indicating that the mycorrhizal association impairs the vegetative growth of grapevines; however, RMD values for HI conditions highlighted the role of the mycorrhizal symbiosis for improving grapevine growth under water deficit conditions (**Table 1**).

Grapevine vegetative growth was also monitored during the 2020 growing season by measuring the green pruning weight, trunk diameter, and leaf area (**Table 2**). Measurements before treatment showed no differences between the different plants concerning trunk diameter (data not shown), corroborating the effect of treatments modulating vegetative growth of vines. Irrigation amount was the main factor affecting both vegetative growth and yield, with grapevines subjected to HI decreasing them (**Table 2**). However, as RMD reported AMF inoculation impair the grapevine growth estimated as trunk diameter and as green pruning weight when vines were FI, whereas under

TABLE 2 | Vegetative growth, yield, and leaf area to fruit ratio of Merlot/3309C grapevines subjected to different irrigation amounts (FI, Full Irrigated; HI, Half Irrigated), AMF inoculation (I, inoculated; NI, non-inoculated), and their combinations during the 2020 growing season (first productive year) in Oakville (California).

	Green pruning (kg/plant)	Trunk diameter (cm)	Leaf area (cm^2)	Yield (kg/plant)	Leaf area to fruit ratio (m^2/kg)
Treatments					
FINI	0.239 ± 0.023 a	1.44 ± 0.04 a	10498.0 ± 2980.3	0.317 ± 0.03	3.32 ± 0.91
FII	0.212 ± 0.016 ab	1.34 ± 0.04 ab	7402.8 ± 835.8	0.257 ± 0.03	3.00 ± 0.50
HINI	0.156 ± 0.015 b	1.19 ± 0.05 b	4694.4 ± 921.0	0.246 ± 0.01	1.89 ± 0.30
HII	0.185 ± 0.013 ab	1.30 ± 0.03 ab	5274.8 ± 857.9	0.209 ± 0.02	2.62 ± 0.56
LMEM					
Irrigation amount (I)	***	**	*	*	ns
AMF inoculation (M)	*	ns	ns	ns	ns
I × M	*	*	ns	ns	ns

*Values represent means ± SE (n = 4) separated by Kenward–Roger method and Tukey's p-value adjustment (P ≤ 0.05). Different letters within column, indicate significant differences as affected by 'Irrigation amount, I,' 'AMF inoculation, M' and their interaction (I × M). ns, *, **, and *** indicate non-significance or significance at 5, 1, and 0.1% probability levels, respectively. LMEM, linear mixed-effect model.*

HI conditions, inoculated vines improved their growth (I × M, $p \leq 0.05$). Finally, the leaf area to fruit ratio was not affected by treatments applied. The contents of minerals measured in leaf blades were not affected by AMF inoculation or applied water amount in our experiment (**Table 3**).

Plant Water Status and Gas Exchange Parameters During the Growing Season

Plant water status was determined by monitoring the SWP each 2 weeks at noon during the growing season. The SWP values ranged between −0.8 and −1.3 MPa at harvest (**Figure 2A**) suggesting that the amount of applied water was successful in reaching the SWP target during the growing season. Irrigation amount was the main factor affecting the water status of vines especially at the end of the growing season. However, before *veraison* AMF inoculation could increase the grapevine water status under HI conditions (I × M, $p \leq 0.05$, **Figure 2A**). The calculation of the seasonal integral of SWP showed the same pattern; hence, $_{si}$SWP was mainly affected by irrigation system with HI plants being the most stressed vines (**Figure 2B**).

Gas exchange parameters monitored during the season are shown in **Figure 3**. Carbon assimilation (A_N) rates increased through the growing season, and were affected by the interaction between AMF inoculation and irrigation amounts (**Figure 3A**). Thus, FII plants showed the highest values of A_N at fruit set and harvest, while FINI grapevines increased A_N after *veraison*. Leaf evapotranspiration (E) was slightly modified by treatments at the beginning on the season (I × M, $p \leq 0.0001$, **Figure 3B**) but no effect was observed later in the season. On the other hand, although no differences in instantaneous water use efficiency (WUE) were recorded at harvest, AMF inoculated plants showed a better WUE during berry development and ripening (**Figure 3C**). Finally, stomatal conductance (g_s) was highly affected by the interaction between AMF inoculation and irrigation system during the whole season (**Figure 3D**). Thereby, AMF inoculation of HI plants mitigated the reduction of g_s.

Effect of AMF Inoculation and Irrigation Amounts on Merlot Berry Primary and Secondary Metabolites

Primary metabolites and berry fresh weight (BFW) are presented in **Table 4**. Must pH, TA, and TSS were not affected by treatments. However, BFW was modified by treatments; hence, AMF inoculation increased BFW of FI plants and decreased in HI (I × M, $p = 0.05$).

Flavonols and anthocyanins were monitored through berry ripening. The effect of AMF inoculation and irrigation systems on berry skin flavonol content and composition was modulated during the growing season as indicated by the significant interaction of treatments with the sampling dates (**Supplementary Table 2**). At mid ripening, the berry skin flavonol content increased in HINI grapevines (I × M, $p \leq 0.05$, **Table 5**). Similarly, quercetin-3-*O*-glucoside and laricitrin-3-*O*-glucoside decreased with AMF inoculation under HI

TABLE 3 | Leaf mineral content of Merlot/3309C grapevines subjected to different irrigation amounts (FI, Full Irrigated; HI, Half Irrigated), AMF inoculation (I, inoculated; NI, non-inoculated) and their combinations during the 2020 growing season (first productive year) in Oakville (California).

	N	P	K	Zn	Mn	Na	Ca	B	Mg	Fe	Cu
	%	%	%	mg/kg	mg/kg	%	mg/kg	%	%	mg/kg	mg/kg
Treatments											
FINI	3.26 ± 0.09	0.26 ± 0.01	1.23 ± 0.12	472.0 ± 73.0	156.0 ± 24.0	0.018 ± 0.003	1.73 ± 0.18	77.50 ± 9.60	0.39 ± 0.02	220.25 ± 12.80	100.3 ± 34.7
FII	3.13 ± 0.06	0.26 ± 0.02	1.34 ± 0.03	389.8 ± 49.5	119.5 ± 12.4	0.010 ± 0.004	1.39 ± 0.08	62.25 ± 2.56	0.35 ± 0.02	201.75 ± 13.97	96.5 ± 41.8
HINI	3.26 ± 0.06	0.27 ± 0.01	1.28 ± 0.05	406.0 ± 52.5	134.5 ± 18.6	0.013 ± 0.003	1.51 ± 0.09	63.25 ± 9.32	0.38 ± 0.01	225.75 ± 10.25	122.0 ± 22.3
HII	3.25 ± 0.05	0.26 ± 0.02	1.29 ± 0.11	351.8 ± 32.9	123.8 ± 21.0	0.010 ± 0.004	1.48 ± 0.23	58.50 ± 6.46	0.37 ± 0.05	227.50 ± 31.67	122.0 ± 55.5
LMEM											
Irrigation amount (I)	ns	ns	ns	ns	ns	ns	ns	ns	ns	ns	ns
AMF inoculation (M)	ns	ns	ns	ns	ns	ns	ns	ns	ns	ns	ns
I × M	ns	ns	ns	ns	ns	ns	ns	ns	ns	ns	ns

Values represent means ± SE (n = 4) separated by Kenward–Roger method and Tukey's p-value adjustment ($P \leq 0.05$). ns indicate non-significance. All values are expressed as % or mg of the mineral per kg of leaf dry weight. LMEM, linear mixed-effect model.

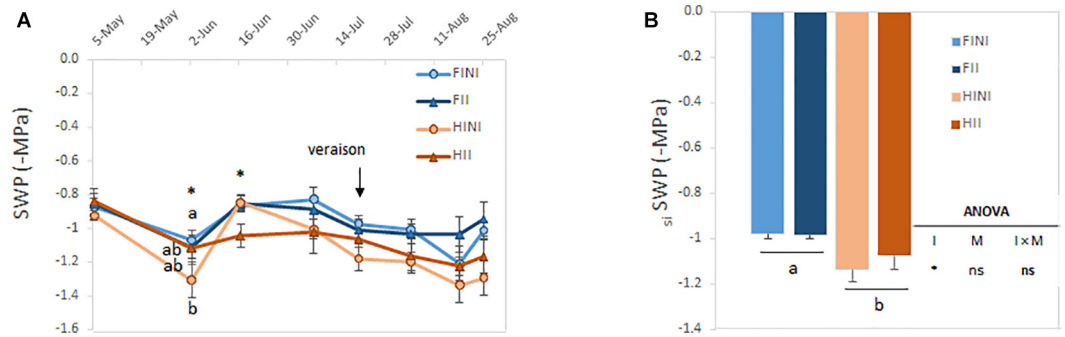

FIGURE 2 | Mid-day stem water potentials (SWP) monitored every 2 weeks during the growing season **(A)** and seasonal integrals of the SWP ($_{si}$SWP, **B**) of field grown Merlot/C3309 grapevines subjected to different irrigation amounts (FI, Full Irrigated; HI, Half Irrigated), AMF inoculation (I, inoculated; NI, non-inoculated), and their combinations. Values represent means ± SE (n = 4) separated by Kenward–Roger method and Tukey's p-value adjustment ($P \leq 0.05$). At each time point, different letters indicate significant differences as affected by Irrigation amount, I, AMF inoculation, M and their interaction (I × M) according to the linear mixed-effect model. ns and * indicate non-significance and significance at 5% probability levels, respectively.

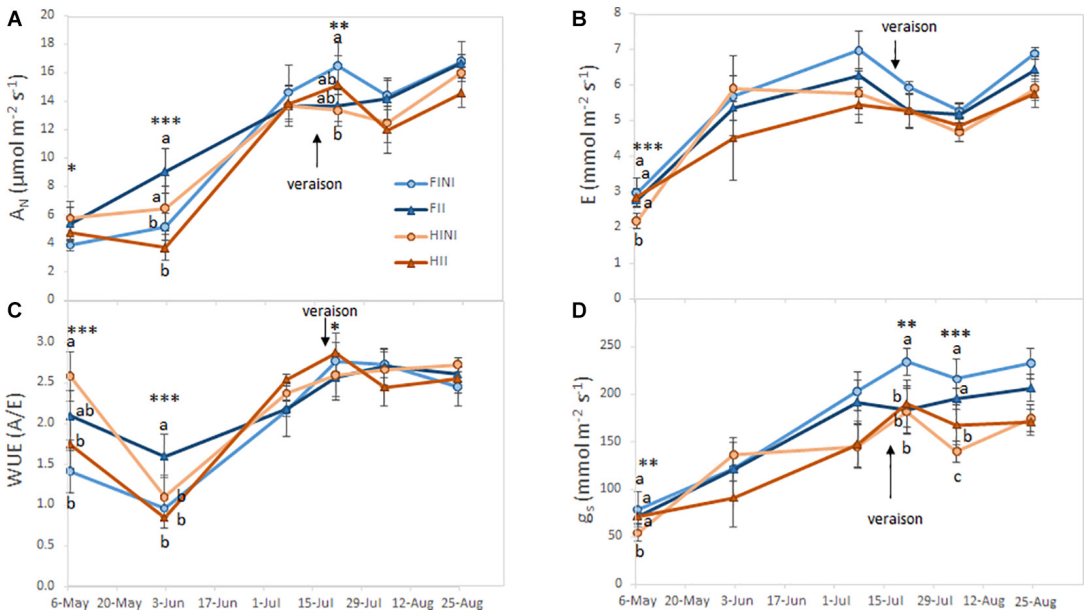

FIGURE 3 | Net carbon assimilation (A_N; **A**), leaf evapotranspiration (E; **B**), instantaneous water use efficiency (WUE; **C**), and stomatal conductance (g_s; **D**) measured during the growing season of field grown Merlot/C3309 grapevine subjected to different irrigation amounts (FI, Full Irrigated; HI, Half Irrigated), AMF inoculation (I, inoculated; NI, non-inoculated) and their combinations. Values represent means ± SE (n = 4) separated by Kenward–Roger method and Tukey's p-value adjustment ($P \leq 0.05$). At each time point, different letters indicate significant differences as affected by Irrigation amount, I, AMF inoculation, M, and their interaction (I × M) according to the linear mixed-effect model. *, **, and *** indicate significance at 5, 1, and 0.1% probability levels, respectively.

conditions (**Table 5**, I × M, $p \leq 0.05$). At harvest, myricetin and quercetin derivatives were the most abundant flavonols found in Merlot berry skins, accounting for more than 40% of the total flavonols. Irrigation treatment was the main factor affecting flavonol content and composition as indicated by the decrease in quercetin, laricitrin, kaempferol, isorhanmetin, and syringetin derivative contents in HI grapevines (**Table 5**). It is noteworthy to highlight the increased content of quercetin-3-*O*-galactoside in HII grapevines (I × M, $p \leq 0.001$).

At mid ripening the main anthocyanin was cyanidin-3-*O*-glucoside, which accounted for *ca.* 20% (**Table 6** and **Supplementary Figure 4**). The total anthocyanin content of Merlot berry skins was not affected by treatments but HI treatment decreased the contents of some anthocyanin derivatives (**Table 6**). At harvest, the total anthocyanin content in Merlot berry skins was not affected by different treatments (**Table 6**). Malvidin was the most abundant anthocyanin detected in Merlot berry skins (**Supplementary Figure 5**), with contents ranged between 23.1% for HINI plants and 28.7% from FII but none of the malvidin derivatives were affected by treatments (**Table 6**). The main changes in anthocyanin composition were due to irrigation treatments, thus, HI led

TABLE 4 | Primary metabolites of Merlot/3309C grapevines subjected to different irrigation amounts (FI, Full Irrigated; HI, Half Irrigated), AMF inoculation (I, inoculated; NI, non-inoculated), and their combinations during the 2020 growing season (first productive year) in Oakville (California).

	Juice pH	TA (g/L)	TSS (°Brix)	BFW (g/berry)
Treatments				
FINI	3.31 ± 0.02	0.76 ± 0.03	25.43 ± 0.98	0.89 ± 0.06 ab
FII	3.32 ± 0.02	0.73 ± 0.01	25.08 ± 0.26	0.99 ± 0.05 a
HINI	3.31 ± 0.03	0.73 ± 0.02	26.48 ± 0.58	0.89 ± 0.02 ab
HII	3.32 ± 0.02	0.71 ± 0.05	24.60 ± 1.87	0.84 ± 0.03 b
LMEM				
Irrigation amount (I)	ns	ns	ns	.
AMF inoculation (M)	ns	ns	ns	.
I × M	ns	ns	ns	.

Values represent means ± SE (n = 4) separated by Kenward–Roger method and Tukey's p-value adjustment (P ≤ 0.05). Different letters within column, indicate significant differences as affected by Irrigation amount, I, AMF inoculation, M, and their interaction (I × M). ns indicate non-significance or significance at 10% probability level, respectively. BFW, berry fresh weight; LMEM, linear mixed-effect model.

to decreased contents of cyanidin and peonidin derivatives (I, $p \leq 0.05$).

Finally, an analysis of the relationship between the percentage of AMF colonization and the main flavonoid contents was conducted (**Figure 4**). The intensity of the AMF colonization had a significant positive relationship with total cyanidins (**Figure 4B**; $R = 0.57$; $p \leq 0.05$), total peonidins (**Figure 4D**; $R = 0.52$; $p \leq 0.05$), and total quercetins (**Figure 4G**; $R = 0.56$; $p \leq 0.05$).

Analysis of the Economic and Environmental Profitability of Cultural Practices

Analysis of the cost of implementing the different treatments in vineyards indicated that HI irrigation led to decreased yields per hectare (**Table 7**, I, $p \leq 0.05$). However, the reduction in yield did not lead to a significant diminution of the gross income per hectare (I, $p = 0.873$). Regarding their impact of water resources, HI irrigation system increased the green component of the WF and decreased the blue WF (I, $p \leq 0.05$, and $p \leq 0.001$, respectively). Therefore, HI contributed to a decrease of total WF in the first productive year of Merlot vineyard.

DISCUSSION

In the last decades, warming trends in viticulture areas have been described worldwide (Petrie and Sadras, 2008; Fraga et al., 2013; Hannah et al., 2013; Neethling et al., 2017). Likewise, weather data recorded during 2020 growing season in Oakville, CA, United States (**Figure 1**), suggested more stressful conditions for grapevines comparing to the average of last 20 years, challenging their production and quality. Indeed, a recent study based on climate indices suggested a reduction of 8,700 km^2 for the California land suitable for grapevine cultivation by mid-21st century (Monteverde and De Sales, 2020). Within this scenario, smart-farming techniques are mandatory for adaptation and mitigation to guarantee the future of the winemaking industry and for reducing potential water conservation issues.

AMF Inoculation and Irrigation Amounts Modulated Water Status, Photosynthetic Performance, Growth, and Mineral Content of Young Merlot Vines

Colonization analysis of Merlot grapevine roots indicated that AMF inoculated integrated with the native communities colonizing grapevine roots (**Table 1**). Thus, we found that the percentage of mycorrhizal colonization was two to three fold higher in mycorrhizal inoculated treatments compared to non-inoculated ones. However, no differences in mycorrhizal colonization due to water amount received by plants were evident in accordance with a previous study conducted on fruit-bearing cuttings (Torres et al., 2018d). In contrast, a study conducted on own-rooted Cabernet Sauvignon field grapevines reported increased frequency of arbuscules and reduced fine root production when an additional water deficit was applied to the regulated deficit irrigation (RDI) plot, suggesting that plants could compensate the lower density of fine roots in vines facing water deficit by increasing AMF colonization (Schreiner et al., 2007). These discrepancies between studies may be explained by the fact that grapevines responded to the degree of water deficit from the previous growing season. Thus, Schreiner et al. (2007) observed increased arbuscular colonization at bloom, before the onset of differences between the treatments they applied whereas under our experimental conditions, water amounts received by Merlot grapevines the previous season did not differ. AMF colonization data also confirmed the seasonality effect on mycorrhizal colonization (Nogales et al., 2009) and the reinforcement that AMF inoculation exerts on native mycorrhizal colonization (Nicolás et al., 2015). Without imposed water stress, AMF inoculation impaired vegetative growth as indicated in the RMD index. However, when grapevines were subjected to HI treatment, AMF-inoculated vines grew better as indicated by the RMD, green pruning, and trunk diameter. Nevertheless, leaf area was not enhanced after AMF inoculation according to previous studies (Nogales et al., 2019), which would explain that AMF inoculation was not sufficient to avoid the yield loss due to HI treatment.

It is well established that AMF inoculation enhances mineral nutrition of grapevines presumably by a greater exploration of soil by the external hyphal network of the AMF resulting in more efficient roots for obtaining nutrients from soils (Smith and Read, 2008). Moreover, it was recently reported that the inoculation of grapevines with AMF under controlled conditions led to the upregulation of nutrient transport genes (Balestrini et al., 2017). In spite of the consensus about AMF enhancing grapevine nutrient uptake, contradictory results are reported about increased mineral nutrient content due to the symbiosis (Nicolás et al., 2015; Torres et al., 2018a). Leaf or petiole mineral nutrient content

TABLE 5 | Berry skin flavonol content and composition of Merlot/3309C grapevines subjected to different irrigation amounts (FI, Full Irrigated; HI, Half Irrigated), AMF inoculation (I, inoculated; NI, non-inoculated), and their combinations during the 2020 growing season (first productive year) in Oakville (California).

100% veraison	**Myricetin-3-*O*-galactoside**	**Myricetin-3-*O*-glucoside**	**Quercetin-3-*O*-galactoside**	**Quercetin-3-*O*-glucoside**	**Laricitrin-3-*O*-glucoside mg/g**	**Kaempferol-3-*O*-glucoside**	**Isorhamnetin-3-*O*-glucoside**	**Syringetin-3-*O*-glucoside**	**Total flavonols**
Treatments									
FINI	0.027 ± 0.004	0.022 ± 0.001	0.235 ± 0.029	2.296 ± 0.145 ab	1.430 ± 0.235	0.152 ± 0.038	0.189 ± 0.064	0.020 ± 0.003	4.37 ± 0.28 ab
FII	0.025 ± 0.005	0.025 ± 0.008	0.245 ± 0.027	2.306 ± 0.125 ab	1.558 ± 0.026	0.174 ± 0.013	0.186 ± 0.037	0.024 ± 0.004	4.54 ± 0.21 ab
HINI	0.025 ± 0.005	0.016 ± 0.007	0.285 ± 0.024	2.564 ± 0.142 a	1.842 ± 0.046	0.214 ± 0.013	0.205 ± 0.047	0.028 ± 0.006	5.18 ± 0.34 a
HII	0.028 ± 0.008	0.025 ± 0.009	0.340 ± 0.115	2.052 ± 0.102 b	1.432 ± 0.050	0.178 ± 0.007	0.181 ± 0.033	0.023 ± 0.001	4.26 ± 0.29 b
LMEM									
Irrigation amount (I)	ns	ns	ns	ns	ns	·	ns	ns	ns
AMF inoculation (M)	ns	ns	ns	*	*	ns	ns	ns	*
I × M	ns	ns	ns	*	*	ns	ns	ns	*
Harvest									
Treatments									
FINI	0.18 ± 0.01	0.45 ± 0.04	0.20 ± 0.02 b	0.60 ± 0.07	1.49 ± 0.14	0.24 ± 0.02	0.16 ± 0.02	0.06 ± 0.01 ab	3.24 ± 0.46
FII	0.19 ± 0.02	0.53 ± 0.06	0.17 ± 0.02 b	0.63 ± 0.05	1.77 ± 0.18	0.27 ± 0.03	0.22 ± 0.03	0.07 ± 0.01 a	3.11 ± 0.27
HINI	0.17 ± 0.02	0.44 ± 0.05	0.12 ± 0.01 c	0.50 ± 0.06	1.28 ± 0.14	0.21 ± 0.02	0.14 ± 0.01	0.04 ± 0.00 bc	3.51 ± 0.47
HII	0.17 ± 0.02	0.41 ± 0.04	0.25 ± 0.03 a	0.55 ± 0.02	1.27 ± 0.15	0.19 ± 0.02	0.15 ± 0.02	0.03 ± 0.00 c	3.30 ± 0.08
LMEM									
Irrigation amount (I)	ns	ns	ns	*	*	*	*	***	*
AMF inoculation (M)	ns	ns	***	ns	ns	ns	ns	*	ns
I × M	ns	ns	***	ns	ns	ns	ns	*	ns

Berries were sampled at 100% veraison (July 28) and at harvest (August 26, 2020).
*Values represent means ± SE (n = 4) separated by Kenward–Roger method and Tukey's p-value adjustment (P ≤ 0.05). Different letters within column, indicate significant differences as affected by Irrigation amount, I, AMF inoculation, M, and their interaction (I × M). ns, indicate non-significance. * and *** indicate non-significance or significance at 10, 5, and 0.1% probability levels, respectively. All values are expressed as mg of the compound per gram of skin dry weight. LMEM, linear mixed-effect model.*

TABLE 6 | Berry skin anthocyanin content and composition of Merlot/3309C grapevines subjected to different irrigation amounts (FI, full irrigated; HI, half irrigated), AMF inoculation (I, inoculated; NI, non-inoculated), and their combinations during the 2020 growing season (first productive year) in Oakville (California).

	3-Monoglucoside anthocyanins					3-Acetyl-glucoside					3-*p*-Coumaroyl-glucoside					Total
100% veraison	**Delphinidin**	**Cyanidin**	**Petunidin**	**Peonidin**	**Malvidin**	**Delphinidin**	**Cyanidin**	**Petunidin**	**Peonidin**	**Malvidin**	**Delphinidin**	**Cyanidin**	**Petunidin**	**Peonidin**	**Malvidin**	
									mg/g							
Treatments																
FINI	1.06 a ± 0.12	1.22 ± 0.19	0.50 a ± 0.05	1.07 ± 0.12	0.71 ± 0.03	0.18 a ± 0.02	0.17 ± 0.03	0.11 ± 0.01	0.12 ± 0.01	0.14 ± 0.01	0.10 ± 0.01	0.16 a ± 0.01	0.04 ± 0.00	0.14 ± 0.01	0.06 ± 0.01	4.56 ± 0.19
FII	1.00 a ± 0.10	1.19 ± 0.14	0.48 a ± 0.04	1.02 ± 0.10	0.67 ± 0.05	0.17 a ± 0.01	0.14 ± 0.02	0.11 ± 0.01	0.12 ± 0.01	0.15 ± 0.00	0.10 ± 0.01	0.14 b ± 0.01	0.05 ± 0.00	0.13 ± 0.01	0.07 ± 0.00	5.36 ± 0.48
HINI	0.70 b ± 0.17	0.86 ± 0.27	0.34 b ± 0.07	0.78 ± 0.21	0.55 ± 0.09	0.12 ± 0.03	0.13 ± 0.04	0.08 ± 0.02	0.09 ± 0.02	0.11 ± 0.02	0.08 ± 0.02	0.13 b ± 0.03	0.04 ± 0.01	0.11 ± 0.03	0.05 ± 0.01	5.19 ± 1.18
HII	0.91 ab ± 0.08	1.06 ± 0.11	0.45 a ± 0.05	0.98 ± 0.07	0.69 ± 0.09	0.15 b ± 0.01	0.15 ± 0.01	0.10 ± 0.01	0.12 ± 0.01	0.14 ± 0.01	0.10 ± 0.02	0.16 a ± 0.01	0.05 ± 0.01	0.15 ± 0.01	0.06 ± 0.02	5.01 ± 0.62
LMEM																
Irrigation amount (I)	*	ns	.	ns	ns	*	ns	ns	ns	ns	ns	.	ns	ns	ns	ns
AMF inoculation (M)	ns	ns	ns	ns	ns	ns	ns	ns	ns	ns	ns	ns	ns	.	ns	ns
I × M	ns	ns	ns	ns	ns	ns	ns	ns	ns	ns	ns	.	ns	ns	ns	ns
Harvest																
Treatments																
FINI	7.54 ± 0.75	3.05 b ± 0.38	4.51 ± 0.36	3.85 ± 0.50	9.21 ± 0.52	1.20 ± 0.15	0.47 b ± 0.06	0.94 ± 0.10	0.43 b ± 0.04	2.32 ± 0.15	0.74 ± 0.08	0.36 b ± 0.04	0.53 ± 0.03	0.59 ± 0.06	1.63 ± 0.08	37.4 ± 4.5
FII	7.46 ± 0.76	3.79 a ± 0.48	4.40 ± 0.42	4.49 ± 0.52	9.06 ± 0.82	1.15 ± 0.10	0.52 a ± 0.05	0.89 ± 0.08	0.48 a ± 0.03	2.25 ± 0.22	0.74 ± 0.09	0.41 a ± 0.04	0.53 ± 0.05	0.66 ± 0.06	1.63 ± 0.19	38.5 ± 2.6
HINI	6.68 ± 0.79	2.43 c ± 0.13	4.08 ± 0.47	3.28 ± 0.43	8.87 ± 1.16	1.03 ± 0.13	0.34 c ± 0.03	0.83 ± 0.10	0.37 c ± 0.04	2.25 ± 0.31	0.67 ± 0.08	0.29 c ± 0.03	0.51 ± 0.06	0.52 ± 0.06	1.83 ± 0.07	33.8 ± 3.4
HII	6.87 ± 0.78	2.76 c ± 0.24	4.14 ± 0.47	3.67 ± 0.38	8.55 ± 1.12	1.10 ± 0.10	0.43 b ± 0.03	0.86 ± 0.09	0.38 c ± 0.03	2.12 ± 0.24	0.73 ± 0.09	0.33 b ± 0.03	0.49 ± 0.06	0.63 ± 0.05	1.40 ± 0.18	34.5 ± 2.8
LMEM																
Irrigation amount (I)	ns	*	ns	ns	ns	ns	*	ns	*	ns	ns	*	ns	ns	ns	ns
AMF inoculation (M)	ns	ns	ns	ns	ns	ns	ns	ns	ns	ns	ns	ns	ns	ns	ns	ns
I × M	ns	ns	ns	ns	ns	ns	ns	ns	ns	ns	ns	ns	ns	ns	ns	ns

Berries were sampled at 100% veraison (July 28) and at harvest (August 26, 2020).

*Values represent means ± SE (n = 4) separated by Kenward–Roger method and Tukey's p-value adjustment ($P \leq 0.05$). Different letters within column, indicate significant differences as affected by Irrigation amount, I, AMF inoculation, M, and their interaction (I × M). ns, indicate non-significance and * indicate non-significance or significance at 10%, and 5% probability levels, respectively. All values are expressed as mg of the compound per gram of skin dry weight. LMEM, linear mixed-effect model.*

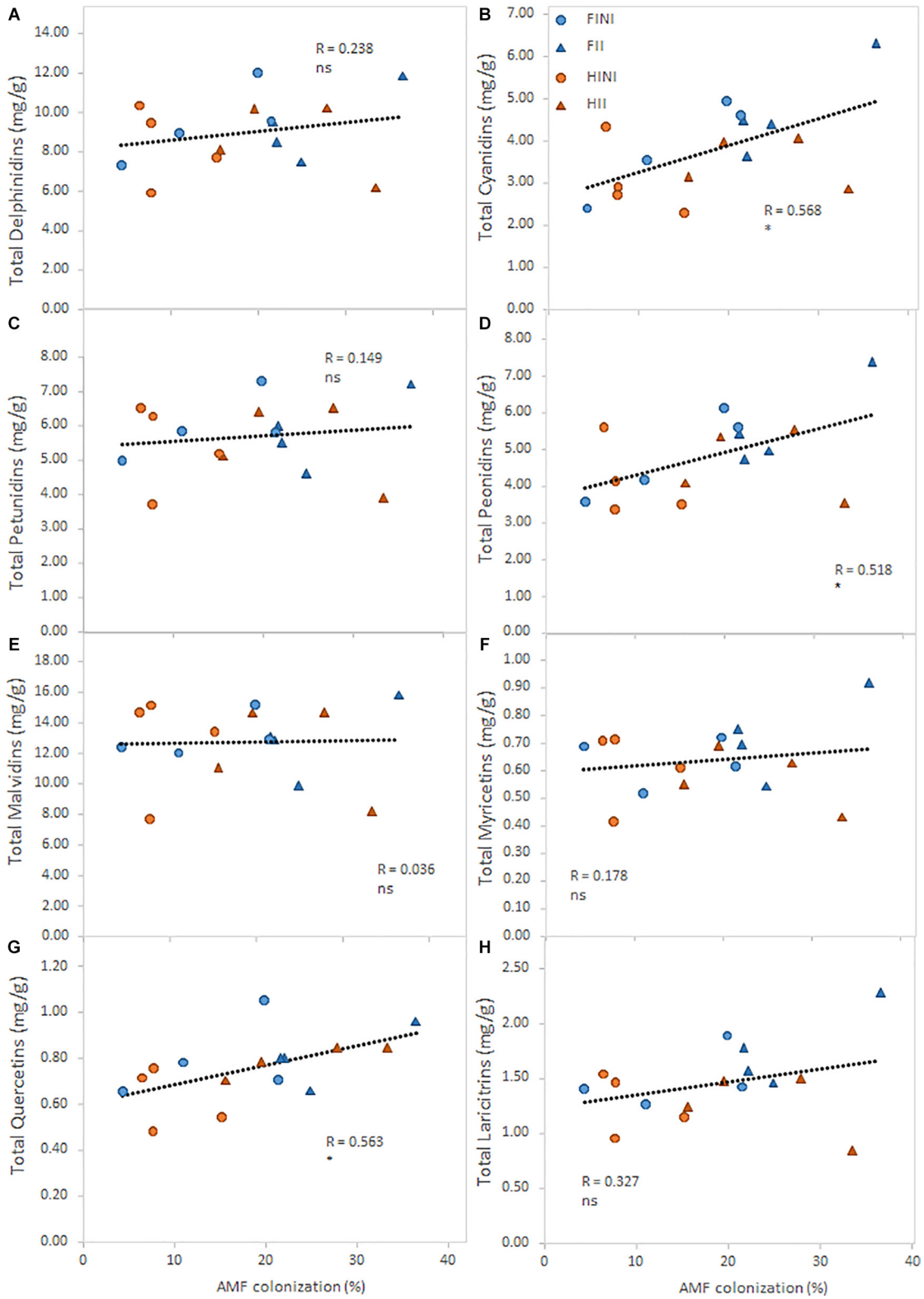

FIGURE 4 | Relationships between total content of the main flavonoid group measured in berry skins and the incidence of the mycorrhizal colonization (%) of field grown Merlot/C3309 grapevines subjected to different irrigation amounts (FI, Full Irrigated; HI, Half Irrigated), AMF inoculation (I, inoculated; NI, non-inoculated), and their combinations. For each flavonoid compound, straight lines correspond to the linear regression lines fitted for the pooled data of all treatments, ns and * indicate non-significance and significance at 5% probability levels, respectively.

TABLE 7 | Cost estimates on labor operations (Kurtural et al., 2020) and water footprint (Zotou and Tsihrintzis, 2017) of Merlot/3309C grapevines subjected to different irrigation amount (FI, full irrigated; HI, half irrigated), AMF inoculation (I, inoculated; NI, non-inoculated), and their combinations during the 2020 growing season (first productive year) in Oakville (California).

Labor operation cost	AMF inoculation ($/Ha)	Irrigation ($/Ha)	Total ($/Ha)	Yield (kg/Ha)	Gross income ($/Ha)
Treatment					
FINI	0	529.25	529.25	528.3 a ± 50.0	264.5 ± 30.6
FII	15	529.25	544.25	428.3 b ± 50.0	104.5 ± 47.1
HINI	0	363.86	363.86	410.0 b ± 16.7	251.1 ± 30.2
HII	15	363.86	378.86	348.3 c ± 33.3	127.4 ± 22.5
LMEM					
Irrigation amount (I)	–	–	–	*	ns
AMF inoculation (M)	–	–	–	ns	ns
I × M	–	–	–	ns	ns
Water footprint (WF)	Green WF (m^3/ton)	Blue WF (m^3/ton)	Gray WF (m^3/ton)	Total WF (m^3/ton)	
Treatment					
FINI	1061.7 c ± 121.5	1706.1 b ± 195.3	ND	2767.8 b ± 316.8	
FII	1331.3 ab ± 180.9	2139.2 a ± 290.7	ND	3470.5 a ± 471.5	
HINI	1326.7 b ± 65.2	1065.8 d ± 52.4	ND	2392.5 c ± 117.6	
HII	1597.7 a ± 152.5	1283.6 c ± 122.5	ND	2881.3 b ± 275.0	
LMEM					
Irrigation amount (I)	*	***	–	.	
AMF inoculation (M)	ns	ns	–	ns	
I × M	ns	ns	–	ns	

*Values represent means ± SE (n = 4) separated by Kenward–Roger method and Tukey's p-value adjustment ($P \leq 0.05$). Different letters within column, indicate significant differences as affected by Irrigation amount, I, AMF inoculation, M, and their interaction (I × M). ns, indicate non-significance, * and *** indicate non-significance or significance at 10, 5, and 0.1% probability levels, respectively. All values are expressed as mg of the compound per gram of skin dry weight. LMEM, linear mixed-effect model; ND, not determined.*

might be useful for the diagnostic of soil mineral deficiencies allowing growers to manage them. However, concentration of mineral nutrients does not provide accurate information on nutrient uptake or allocation of nutrient in various organs (Schreiner, 2016). Therefore, although no differences on the mineral nutrient content in leaf blades were observed, mineral uptake was presumably enhanced by AMF inoculation given the growth promotion recorded in mycorrhizal plants under HI conditions. Furthermore, Balestrini et al. (2017) recently reported that although mineral nutrient uptake genes were upregulated after inoculation with different inoculants (*F. mosseae* vs. a fungal and bacterial consortium), the degree of upregulation differed between them, suggesting a specific response to a specific inoculum. Similarly, Nogales et al. (2019) did not find accumulation of minerals in grapevine leaves after AMF inoculation with the exception of P, which was enhanced and decreased after *F. mosseae* and *R. irregulare* inoculations, respectively.

Grapevine water status monitored during the growing season showed that irrigation amounts were the main factor affecting the plant water status. Thus, according to previous work FI plants were maintained under well-watered conditions with values of midday SWP higher than −0.9 MPa (van Leeuwen et al., 2009) and/or g_s higher than 200 mmol m^{-2} s^{-1} (Medrano et al., 2002). On the other hand, grapevines subjected to HI were not exposed to a severe water stress as they never reached values of SWP and g_s lower than −1.5 MPa and 50 mmol m^{-2} s^{-1}, respectively, considered detrimental for grapevine development (Medrano et al., 2002; Villalobos-González et al., 2019).

We did not measure any SWP differences due to the AMF inoculation when plants were subjected to FI. However, within HI plants, AMF inoculation tended to result in higher SWP values (**Figure 2**) in accordance with previous studies (Nicolás et al., 2015). Therefore, a higher AMF occurrence in the root zone has been related to improve water status of vines by increasing water uptake presumably by increasing the mycorrhizal structures, mainly arbuscules (Schreiner et al., 2007). Accordingly, we observed that photosynthetic performance of AMF inoculated Merlot grapevines was improved (namely, A_N or WUE) (**Figure 3**). Likewise, Nicolás et al. (2015) found a better photosynthetic performance after inoculating Crimson grapevines grown in a commercial vineyard. Indeed, a recent meta-analysis demonstrated that AMF exert a positive influence on photosynthetic rates, stomatal conductance, and water use efficiency on both C3 and C4 plants subjected to salt stress (Chandrasekaran et al., 2019).

Flavonoid Composition of Berry Skins From Young Merlot Grapevines Is Modulated by AMF and Irrigation Amounts

Merlot grapevines did not show changes on their berry primary metabolites as affected by the treatments applied (**Table 4**).

Similarly, a recent study evaluating the effect of different sustained deficit irrigation (SDI) and RDI showed no differences in must pH and TSS in Merlot berries in a 4-year field experiment conducted in a hot climate (Munitz et al., 2017). This lack of effect of the irrigation systems on berry primary metabolism might be due to grapevines were not subjected to a severe water stress (discussed above). On the other hand, previous studies showed that inoculation with AMF of grapevines vineyards did not affect TSS or TA under field conditions (Nicolás et al., 2015) or under controlled conditions (Torres et al., 2018d, 2019) and our results corroborated these findings.

Regarding secondary metabolism, neither irrigation systems nor AMF inoculation modified flavonol and anthocyanin total content at harvest (**Tables 5**, **6**). Similarly, a 2-year field study conducted in Central valley in California with Merlot did not report differences on flavonol or anthocyanin skin content due to different irrigation amounts (Yu et al., 2016). A previous study conducted on Cabernet Sauvignon subjected to water deficit reported that although flavonol synthesis related genes were up-regulated after the onset of fruit ripening, this did not affect berry flavonol concentration at harvest (Castellarin et al., 2007). Similarly, previous studies with Tempranillo grown under controlled conditions did not observed differences due to AMF inoculation on the total content of flavonol and anthocyanins in berry skins (Torres et al., 2019).

Flavonol composition was affected by treatments. Thus, HII grapevines increased quercetin and decreased syringetin contents in berry skins at harvest in accordance to a previous study (Torres et al., 2019). Indeed, it is known that AMF inoculation up-regulated phenyl-propanoid biosynthesis key genes in grapevines in response to pathogens (Bruisson et al., 2016). On the other hand, HI led to decreased contents of quercetins, laricitrins, kaempferols, syringetins, and isorhamnetins. Likewise, Martínez-Lüscher et al. (2014) found that in spite of the increase in *O*-methyl-transferase (OMT) transcript level, methylated flavonols (i.e., isorhamnetins, laricitrins, and syringetins) did not increase under water deficit. These authors suggested that given the higher affinity of OMT for quercetins, the lower concentration of quercetins under water deficit could act as a limiting factor for the synthesis of methylated forms, and our findings corroborated this hypothesis.

Regarding anthocyanin composition, berry skins from HI grapevines showed lower contents of di-substituted anthocyanins (cyanidin and peonidin derivatives) than the ones of FI grapevines. It is well known that water deficit regulates the expression of key genes of the flavonoid pathway such as the flavonoid 3′-hydroxylase, flavonoid 3′,5′-hydroxylase, and *O*-methyltransferase in red cultivars (Castellarin et al., 2007; Deluc et al., 2009). Therefore, these decreased contents of di-substituted anthocyanins were likely explained by a different regulation of these genes when grapevines are subjected to water deficit.

The role of AMF for enhancing phenolic compounds was reported in several studies with potted grapevines. Thus, AMF grapevines showed increased content of resveratrol, viniferins, and pterostilbene (Bruisson et al., 2016), total phenols and quercetin content (Eftekhari et al., 2012), and total flavonoids (Torres et al., 2018a) in leaves of different grapevine varieties facing different biotic and/or abiotic stresses. Moreover, increased anthocyanin contents were reported in berries from grapevines grown under water deficit and warming conditions (Torres et al., 2018d). Similarly, we found a strong relationship between the percentage of mycorrhizal colonization and some flavonoids (**Figure 4**).

The economic analysis data indicated that AMF inoculation and water management did not affect the cost of labor operations, in spite of irrigating with half amount may lead to decreases in yield. However, this came with reductions of the water footprint that have to be taken into account. It is noteworthy that extreme weather recorded in 2020 could modulate the effects described in this work. Moreover, the mycorrhizal extraradical mycelium coexists with soil microbial communities and the synergistic activity between the AMF, the bacterial communities, and the grapevine modulates the benefits of symbiosis on nitrogen fixation, P solubilization, and production of phytohormones, siderophores, and antibiotics (Giovannini et al., 2020). On the other hand, previous studies demonstrated that the microbiome of vineyards is shaped by cropping management (Coller et al., 2019), and little is known about whether these communities stimulate or suppress the extraradicular mycelium activity (Svenningsen et al., 2018). Therefore, given the effect of AMF inoculation and different irrigation amounts had on grapevine physiology and berry composition, further studies should consider the potential effects of these management practices on vineyard soil living microbiota.

CONCLUSION

Current research aimed to study how Merlot grapevines responded to AMF inoculation and different water amounts in their first productive year *in situ*. Our results highlighted the role of AMF inoculation for improving vegetative growth, photosynthetic activity, and water status of grapevines, especially when facing mild water deficits in field grown grapevines. Additionally, a strong relationship between the mycorrhizal colonization of roots and some flavonoids was found, corroboration the effect of AMF for regulating anthocyanin and flavonol metabolisms. Finally, although some berry quality traits and grapevine performance (i.e., water status or gas exchange parameters) were improved by AMF inoculation under water deficit, AMF inoculation was not sufficient to avoid the yield losses due to water deficit in the first productive year of Merlot when facing a hyper-arid growing season. It is noteworthy that these results may be affected by edaphoclimatic characteristics and living microbiota in vineyard soils, which should be taken into account before making the decision of inoculating the vineyard. Therefore, this study offer a starting point to assess the effect of AMF inoculation on young vines under real field conditions.

DATA AVAILABILITY STATEMENT

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

AUTHOR CONTRIBUTIONS

NT and SK conceived the project. SK acquired the funding. NT and SK designed the project. NT curated the data and wrote the first version of the manuscript. RY curated and proofed the data. All authors approved the final version of the manuscript.

FUNDING

A post-doctoral student fellowship was provided to NT from Department of Viticulture and Enology at University of California, Davis during the execution of this study.

SUPPLEMENTARY MATERIAL

The Supplementary Material for this article can be found online at: https://www.frontiersin.org/articles/10.3389/fpls.2020.622209/full#supplementary-material

REFERENCES

Agnolucci, M., Avio, L., Pepe, A., Turrini, A., Cristani, C., Bonini, P., et al. (2019). Bacteria associated with a commercial mycorrhizal inoculum: community composition and multifunctional activity as assessed by Illumina Sequencing and culture-dependent tools. *Front. Plant Sci.* 9:1956. doi: 10.3389/fpls.2018.01956

Agnolucci, M., Battini, F., Cristani, C., and Giovannetti, M. (2015). Diverse bacterial communities are recruited on spores of different arbuscular mycorrhizal fungal isolates. *Biol. Fertil. Soils* 51, 379–389. doi: 10.1007/s00374-014-0989-5

Bagyaraj, D. J. (1994). "Vesicular-arbuscular mycorrhiza: application in agriculture," in *'Techniques for the Study of Mycorrhiza*', eds J. R. Norris, D. J. Read, and A. K. Varma (London: Academic Press), 819–833.

Balestrini, R., Salvioli, A., Dal Molin, A., Novero, M., Gabelli, G., Paparelli, E., et al. (2017). Impact of an arbuscular mycorrhizal fungus versus a mixed microbial inoculum on the transcriptome reprogramming of grapevine roots. *Mycorrhiza* 27, 417–430. doi: 10.1007/s00572-016-0754-8

Bates, D., Maechler, M., Bolker, B., and Walker, S. (2015). Fitting linear mixed-effects models usinglme4. *J. Stat. Softw.* 67, 1–48. doi: 10.18637/jss.v067.i01

Bates, D., Maechler, M., Bolker, B., Walker, S., Christensen, R. H. B., Singmann, H., et al. (2020). *Package 'lme4'. Linear Mixed-Effects Models using 'Eigen' and S4. 1.1-25*. Madison, WI: R Project.

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

Bruisson, S., Maillot, P., Schellenbaum, P., Walter, B., Gindro, K., and Deglène-Benbrahim, L. (2016). Arbuscular mycorrhizal symbiosis stimulates key genes of the phenylpropanoid biosynthesis and stilbenoid production in grapevine leaves in response to downy mildew and grey mould infection. *Phytochemistry* 131, 92–99. doi: 10.1016/j.phytochem.2016.09.002

California Department of Water Resources (2020). *CIMIS Weather Observation Data: University of California at Davis, 2020*. Available online at: www.cimis.water.ca.gov (accessed August 20, 2020).

Castellarin, S. D., Pfeiffer, A., Sivilotti, P., Degan, M., Peterlunger, E., and Di Gaspero, G. (2007). Transcriptional regulation of anthocyanin biosynthesis in ripening fruits of grapevine under seasonal water deficit. *Plant Cell Environ*. 30, 1381–1399. doi: 10.1111/j.1365-3040.2007.01716.x

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

Coller, E., Cestaro, A., Zanzotti, R., Bertoldi, D., Pindo, M., Larger, S., et al. (2019). Microbiome of vineyard soils is shaped by geography and management. *Microbiome* 7:140. doi: 10.1186/s40168-019-0758-7

Costa, J. M., Vaz, M., Escalona, J., Egipto, R., Lopes, C., Medrano, H., et al. (2016). Modern viticulture in southern Europe: vulnerabilities and strategies for adaptation to water scarcity. *Agric. Water Manage.* 164, 5–18. doi: 10.1016/j.agwat.2015.08.021

Deluc, L. G., Quilici, D. R., Decendit, A., Grimplet, J., Wheatley, D., Schlauch, A., et al. (2009). Water deficit alters differentially metabolic pathways affecting important flavour and quality traits in grape berries of Cabernet Sauvignonand Chardonnay. *BMC Genomics* 10:212. doi: 10.1186/1471-2164-10-212

Eftekhari, M., Alizadeh, M., and Ebrahimi, P. (2012). Evaluation of the total phenolics and quercetin content of foliage in mycorrhizal grape (*Vitis vinifera* L.) varieties and effect of postharvest drying on quercetin yield. *Ind. Crops Prod.* 38, 160–165. doi: 10.1016/j.indcrop.2012.01.022

Fraga, H., Malheiro, A. C., Moutinho-Pereira, J., and Santos, J. A. (2013). Future scenarios for viticultural zoning in Europe: ensemble projections and uncertainties. *Int. J. Biometeorol.* 57, 909–925. doi: 10.1007/s00484-012-0617-8

Gavlak, R. G., Horneck, D. A., and Miller, R. O. (1994). *"Plant, Soil and Water Reference Methods for the Western Region," Western Regional Extension Publication (WREP)*. Fairbanks, AK: University of Alaska, 125.

Gianinazzi, S., and Vosátka, M. (2004). Inoculum of arbuscular mycorrhizal fungi for production systems: science meets business. *Can. J. Bot.* 82, 1264–1271. doi: 10.1139/b04-072

Giovannini, L., Palla, M., Agnolucci, M., Avio, L., Sbrana, C., Turrini, A., et al. (2020). Arbuscular mycorrhizal fungi and associated microbiota as plant biostimulants: research strategies for the selection of the best performing inocula. *Agronomy* 10:106. doi: 10.3390/agronomy10010106

Hamilton, C. E., Bever, J. D., Labbé, J., Yang, X., and Yin, H. (2016). Mitigating climate change through managing constructed-microbial communities in agriculture. *Agric. Ecosyst. Environ*. 216, 304–308. doi: 10.1016/j.agee.2015.10.006

Hannah, L., Roehrdanz, P. R., Ikegami, M., Shepard, A. V., Shaw, M. R., Tabor, G., et al. (2013). Climate change, wine, and conservation. *Proc. Natl. Acad. Sci. U.S.A.* 110, 6907–6912. doi: 10.1073/pnas.1210127110

Hao, Z., van Tuinen, D., Fayolle, L., Chatagnier, O., Lid, X., Chen, B., et al. (2018). Arbuscular mycorrhiza affects grapevine *fanleaf* virus transmission by the nematode vector *Xiphinema index*. *App. Soil Ecol*. 129, 107–111. doi: 10.1016/j.apsoil.2018.05.007

Holland, T., Bowen, P., Kokkoris, V., Urbez-Torres, J. R., and Hart, M. (2019). Does inoculation with arbuscular mycorrhizal fungi reduce trunk disease in grapevine rootstocks? *Horticulturae* 5:61. doi: 10.3390/horticulturae5030061

Holland, T. C., Hart, M. M., Bogdanoff, C., and Bowen, P. (2018). Response of grapevine rootstocks to soil inocula from different sources. *Am. J. Enol. Vitic.* 69, 94–100. doi: 10.5344/ajev.2017.17090

Karagiannidis, N., Nikolaou, N., Ipsilantis, I., and Zioziou, E. (2007). Effects of different N fertilizers on the activity of *Glomus* mosseae and on grapevine nutrition and berry composition. *Mycorrhiza* 18, 43–50. doi: 10.1007/s00572007-0153-2

Koske, R. E., and Gemma, J. N. (1989). A modified procedure for staining roots to detect VA mycorrhizas. *Mycol. Res*. 92, 486–488. doi: 10.1016/s0953-7562(89)80195-9

Kurtural, S. K., Steward, D., and Sumner, D. (2020). Sample costs to establish a vineyard and produce wine grapes Napa County Crush District 4. *Univ. Calif. Coop. Ext. Serv. Bul.* , 1–27. (accessed October 8, 2020).

Kuznetsova, A., Brockhoff, P. B., Christensen, R. H. B., and Jensen, S. P. (2020). *Package 'lmerTest'. Tests in Linear Mixed Effects Models. Version 3.1-3*. Denmark: R Project.

Lenth, R. (2018). *Package 'lsmeans'. Least-Squares Means. Version 2.30-0*. Ames, IA: R Project.

Linderman, R. G., and Davis, E. A. (2001). Comparative response of selected grapevine rootstocks and cultivars to inoculation with different mycorrhizal fungi. *Am. J. Enol. Vitic.* 52, 8–11.

Martínez-Lüscher, J., Brillante, L., and Kurtural, S. K. (2019). Flavonol profile is a reliable indicator to assess canopy architecture and the exposure of red wine grapes to solar radiation. *Front. Plant Sci.* 10:10. doi: 10.3389/fpls.2019.00010

Martínez-Lüscher, J., Sánchez-Díaz, M., Delrot, S., Aguirreolea, J., Pascual, I., and Gomès, E. (2014). Ultraviolet-B radiation and water deficit interact to alter flavonol and anthocyanin profile in grapevine berries through transcriptomic regulation. *Plant Cell Physiol.* 55, 1925–1936. doi: 10.1093/pcp/pcu121

Medrano, H., Escalona, J. M., Bota, J., Gulías, J., and Flexas, J. (2002). Regulation of photosynthesis of C3 plants in response to progressive drought: stomatal conductance as a reference parameter. *Ann. Bot.* 89, 895–905. doi: 10.1093/aob/mcf079

Monteverde, C., and De Sales, F. (2020). Impacts of global warming on southern California's winegrape climate suitability. *Adv. Clim. Chang. Res.* 11, 279–293. doi: 10.1016/j.accre.2020.08.002

Munitz, S., Netzer, Y., and Schwartz, A. (2017). Sustained and regulated deficit irrigation of field-grown Merlot grapevines. *Aust. J. Grape Wine Res.* 23, 87–94. doi: 10.1111/ajgw.12241

Neethling, E., Petitjean, T., Quénol, H., and Barbeau, G. (2017). Assessing local climate vulnerability and winegrowers' adaptive processes in the context of climate change. *Mitig. Adapt. Strat. Global Change* 22, 777–803. doi: 10.1007/s11027-015-9698-0

Nicolás, E., Maestre-Valero, J. F., Alarcón, J. J., Pedrero, F., VicenteSánchez, J., Bernabé, A., et al. (2015). Effectiveness and persistence of arbuscular mycorrhizal fungi on the physiology, nutrient uptake and yield of Crimson seedless grapevine. *J. Agric. Sci.* 153, 1084–1096. doi: 10.1017/s002185961400080x

Nikolaou, N. A., Koukourikou, M., Angelopoulos, K., and Karagiannidis, N. (2003). Cytokinin content and water relations of 'Cabernet Sauvignon' grapevine exposed to drought stress. *J. Hortic. Sci. Biotechnol.* 78, 113–118. doi: 10.1080/14620316.2003.11511576

Nogales, A., Luque, J., Estaún, V., Camprubí, A., Garcia-Figueres, F., and Calvet, C. (2009). Differential growth of mycorrhizal field-inoculated grapevine rootstocks in two replant soils. *Am. J. Enol. Vitic.* 60, 484–489.

Nogales, A., Santos, E. S., Abreu, M. M., Arán, D., Victorino, G., Pereira, H. S., et al. (2019). Mycorrhizal inoculation differentially affects grapevine's performance in copper contaminated and non-contaminated soils. *Front. Plant Sci.* 9:1906. doi: 10.3389/fpls.2018.01906

Petrie, P. R., and Sadras, V. O. (2008). Advancement of grapevine maturity in Australia between 1993 and 2006: putative causes, magnitude of trends and viticultural consequences. *Aust. J. Grape Wine Res.* 14, 33–45. doi: 10.1111/j.1755-0238.2008.00005.x

Resco, P., Iglesias, A., Bardají, I., and Sotés, V. (2016). Exploring adaptation choices for grapevine regions in Spain. *Reg. Environ. Change* 16, 979–993. doi: 10.1007/s10113-015-0811-4

Schreiner, R. P. (2016). Nutrient uptake and distribution in young Pinot Noir grapevines over two seasons. *Am. J. Enol. Vitic.* 67, 436–448. doi: 10.5344/ajev.2016.16019

Schreiner, R. P., Tarara, J. M., and Smithyman, R. P. (2007). Deficit irrigation promotes arbuscular colonization of fine roots by mycorrhizal fungi in grapevines (*Vitis vinifera* L.) in an arid climate. *Mycorrhiza* 17, 551–562. doi: 10.1007/s00572-007-0128-3

Smith, S. E., and Read, D. (2008). "The symbionts forming arbuscular mycorrhizas," in *Mycorrhizal Symbiosis*, 3rd Edn, eds S. E. Smith and D. Read (London: Academic Press), 13–41. doi: 10.1016/B978-012370526-6

Svenningsen, N. B., Watts-Williams, S. J., Joner, E. J., Battini, F., Efthymiou, A., Cruz-Paredes, C., et al. (2018). Suppression of the activity of arbuscular mycorrhizal fungi by the soil microbiota. *ISME J.* 12, 1296–1307. doi: 10.1038/s41396-018-0059-3

Torres, N., Antolín, M. C., Garmendia, I., and Goicoechea, N. (2018a). Nutritional properties of Tempranillo grapevine leaves are affected by clonal diversity, mycorrhizal symbiosis and air temperature regime. *Plant. Physiol. Biochem.* 130, 542–554. doi: 10.1016/j.plaphy.2018.08.004

Torres, N., Goicoechea, N., and Antolín, M. C. (2018b). Arbuscular Mycorrhial Symbiosis as a promising resource for improving berry quality in grapevines under changing environments. *Front. Plant Sci.* 9:897. doi: 10.3389/fpls.2018.00897

Torres, N., Goicoechea, N., and Antolín, M. C. (2018c). Influence of irrigation strategy and mycorrhizal inoculation on fruit quality in different clones of Tempranillo grown under elevated temperatures. *Agric. Water Manag.* 202, 285–298. doi: 10.1016/j.agwat.2017.12.004

Torres, N., Goicoechea, N., Morales, F., and Antolín, M. C. (2016). Berry quality and antioxidant properties in Vitis vinifera cv. Tempranillo as affected by clonal variability, mycorrhizal inoculation and temperature. *Crop Past. Sci.* 67, 961–977. doi: 10.1071/CP16038

Torres, N., Hilbert, G., Antolín, M. C., and Goicoechea, N. (2019). Aminoacids and flavonoid profiling in Tempranillo berries can be modulated by the Arbuscular mycorrhizal fungi. *Plants*. 8:400. doi: 10.3390/plants8100400

Torres, N., Zamarreño, A., Goicoechea, N., and Antolín, M. C. (2018d). Changes in ABA conjugation/catabolism could account for the effects of AMF inoculation on Tempranillo (Vitis vinifera L.) fruit quality under climate change scenarios. *Plant Sci.* 274, 383–393. doi: 10.1016/j.plantsci.2018.06.009

Trouvelot, S., Bonneau, L., Redecker, D., van Tuinen, D., Adrian, M., and Wipf, D. (2015). Arbuscular mycorrhiza symbiosis in viticulture: a review. *Agron. Sustain. Dev.* 35, 1449–1467. doi: 10.1007/s13593-015-0329-7

Van Geel, M., Verbruggen, E., De Beenhouwer, M., Rennes, G., Lievens, B., and Honnay, O. (2017). High soil phosphorus levels overrule the potential benefits of organic farming on arbuscular mycorrhizal diversity in northern vineyards. *Agr. Ecosyst. Environ.* 248, 144–152. doi: 10.1016/j.agee.2017.07.017

van Leeuwen, C., Tregoat, O., Choné, X., Bois, B., Pernet, D., and Gaudillère, J.-P. (2009). Vine water status is a key factor in grape ripening and vintage quality for red Bordeaux wine. How can it be assessed for vineyard management purposes? *J. Int. Des. Sci. la Vigne du Vin* 43, 121–134. doi: 10.20870/oeno-one.2009.43.3.798

Villalobos-González, L., Muñoz-Araya, M., Franck, M., and Pastenes, C. (2019). Controversies in midday water potential regulation and stomatal behavior might result from the environment, genotype, and/or rootstock: evidence from Carménère and Syrah grapevine varieties. *Front. Plant Sci.* 10:1522. doi: 10.3389/fpls.2019.01522

Wilson, T. G., Kustas, W. P., Alfieri, J. G., Anderson, M. C., Gaoa, F., Prueger, J. H., et al. (2020). Relationships between soil water content, evapotranspiration, and irrigation measurements in a California drip-irrigated Pinot noir vineyard. *Agric. Water Manag.* 237:106186. doi: 10.1016/j.agwat.2020.106186

Wine Institute (2020). Available online at: https://wineinstitute.org/our-industry/statistics/ (accessed October 08, 2020).

Yu, R., Cook, M. G., Yacco, R. S., Watrelot, A. A., Gambetta, G., Kennedy, J. A., et al. (2016). Effects of leaf removal and applied water on flavonoid accumulation in grapevine (*Vitis vinifera* L. cv. Merlot) berry in a hot climate. *J. Agric. Food Chem.* 64, 8118–8127. doi: 10.1021/acs.jafc.6b03748

Zotou, I., and Tsihrintzis, V. A. (2017). The Water Footprint of Crops in the Area of Mesogeia, Attiki, Greece. *Environ. Process.* 4(Suppl. 1), S63–S79. doi: 10.1007/s40710-017-0260-9

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

frontiers in Plant Science

ORIGINAL RESEARCH
published: 15 January 2021
doi: 10.3389/fpls.2020.612299

Arbuscular Mycorrhizal Fungi Improve Tolerance of the Medicinal Plant *Eclipta prostrata* (L.) and Induce Major Changes in Polyphenol Profiles Under Salt Stresses

*Nguyen Hong Duc[1], Au Trung Vo[1], Imane Haddidi[1], Hussein Daood[2] and Katalin Posta[1]**

[1] Department of Genetics, Microbiology and Biotechnology, Szent István University, Gödöllő, Hungary, [2] Regional Knowledge Center, Szent István University, Gödöllő, Hungary

Eclipta prostrata (L.) is an important and well-known medicinal plant due to its valuable bioactive compounds. Microorganisms, including arbuscular mycorrhizal fungi (AMF), and salinity could directly impact plant metabolome, thus influencing their secondary metabolites and the efficacy of herbal medicine. In this study, the role of different single AMF species (*Funneliformis mosseae*, *Septoglomus deserticola*, *Acaulospora lacunosa*) and a mixture of six AMF species in plant growth and physio-biochemical characteristics of *E. prostrata* under non-saline conditions was investigated. Next, the most suitable AM treatment was chosen to examine the impact of AMF on physio-biochemical features and polyphenol profiles of *E. prostrata* under saline conditions (100 and 200 mM NaCl). The findings indicated that AMF mixture application resulted in more effective promotion on the aboveground part of non-saline plants than single AMF species. AM mixture application improved growth and salt tolerance of *E. prostrata* through increasing the activity of catalase, peroxidase (at 4 weeks), proline, and total phenolic content (at 8 weeks). Such benefits were not observed under high salinity, except for a higher total phenolic concentration in mycorrhizal plants at 8 weeks. Through high-performance liquid chromatography, 14 individual phenolic compounds were analyzed, with wedelolactone and/or 4,5-dicaffeoylquinic acid abundant in all treatments. Salinity and mycorrhizal inoculation sharply altered the polyphenol profiles of *E. prostrata*. Moderate salinity boosted phenolic compound production in non-AM plants at 4 weeks, while at 8 weeks, the decline in the content of phenolic compounds occurred in uncolonized plants subjected to both saline conditions. Mycorrhization augmented polyphenol concentration and yield under non-saline and saline conditions, depending on the growth stages and salt stress severity. Plant age influenced polyphenol profiles with usually a higher content of phenolic compounds in older plants and changed the production of individual polyphenols of both non-AM and AM plants under non-stress and salt stress conditions. A better understanding of factors (involving mycorrhiza and salinity) affecting the phenolic compounds of *E. prostrata* facilitates the optimization of individual polyphenol production in this medicinal plant.

Keywords: arbuscular mycorrhizal fungi, *Eclipta prostrata*, medicinal plant, phenolic compounds, bioactive compounds, secondary metabolites, salinity, salt tolerance

OPEN ACCESS

Edited by:
Sergio Saia,
University of Pisa, Italy

Reviewed by:
Miroslav Vosatka,
Institute of Botany (ASCR), Czechia
Philipp Franken,
Friedrich Schiller University Jena, Germany

****Correspondence:***
Katalin Posta
Posta.Katalin@mkk.szie.hu

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

Received: *30 September 2020*
Accepted: *16 December 2020*
Published: *15 January 2021*

Citation:
Duc NH, Vo AT, Haddidi I, Daood H and Posta K (2021) Arbuscular Mycorrhizal Fungi Improve Tolerance of the Medicinal Plant Eclipta prostrata (L.) and Induce Major Changes in Polyphenol Profiles Under Salt Stresses. Front. Plant Sci. 11:612299. doi: 10.3389/fpls.2020.612299

INTRODUCTION

Eclipta prostrata (L.), belonging to a family of medicinal plants (Asteraceae), is a native plant of Asia but is widely distributed in subtropical, tropical, and warm temperate regions on the globe (Liu et al., 2012). It is an important medicinal plant, which has been used in conventional systems of medicine and also by traditional healers, particularly in China, Japan, India, Vietnam, and other regions in the cure for various diseases (Yu et al., 2020). In fact, this medicinal plant has been utilized for the medication of respiratory diseases such as asthma, diphtheria, pertussis, and tuberculosis; as anti-HIV 1; and for diabetes type II; loose teeth; graying of hair; dizziness; hemoptysis; antihyperlipidemic, antihyperglycemic, and antioxidant activities (Chung et al., 2017; Feng et al., 2019; Yu et al., 2020); and mitigating the cognitive impairment induced by scopolamine (Jung et al., 2016). The plant possesses an outstanding therapeutic and medicinal value due to its valuable chemical composition such as alkaloids; alkenynes; cardiac glycosides; coumarins; flavonoids; flavones, e.g., luteolin; lipids; polyacetylene compounds; essentials oil; steroids; saponins; phytosterol; β-amyrin; triterpenes, e.g., ecalbatin and echinocystic acid; and coumestans, e.g., wedelolactone (Gani and Devi, 2015). Wedelolactone has been shown to have potent hepatoprotective or antihepatotoxicity, anti-inflammatory, and antitumor effects and neutralization of myotoxic and lethal activities of snake venom (Chung et al., 2017). Phenolic compounds, for instance, flavonoids, phenolic acids, and tannins extracted from this plant, have various biological activities, including anti-inflammatory, anti-atherosclerotic, and anticarcinogenic (Chung et al., 1998; Soobrattee et al., 2005). Noticeably, the environment and other factors such as harvest time, storage time, and geographical sources may influence the chemical components in *E. prostrata* (Chung et al., 2017).

Salinization is a growing problem in agricultural ecosystems, which jeopardizes plant growth and productivity. Salinity causes ion toxicity (Na^+ and Cl^-), nutritional imbalance, pigment degradation and inhibition of photosynthesis, oxidative and osmotic stress, limited leaf diffusion (flux) of CO_2, modification of metabolic pathways, cell deformation, premature senescence, and ultimately cell death in the plant (Zelm et al., 2020). It is predicted that around one billion hectares traversing more than 100 countries face salt problems, and salinization is rapidly increasing with an estimated annual addition of 0.3–1.5 million hectares of farmland (Food and Agriculture Organization [FAO], 2015). Therefore, efficient strategies to cope with soil salinity under agricultural management systems could include salt-tolerant varieties and biotechnological approaches such as applying beneficial microbes capable of improving plant tolerance.

Arbuscular mycorrhizal fungi (AMF), one of the prevalent soil microbes, can colonize most terrestrial plant species' roots. These symbiotic fungi have been reported to considerably offer various benefits to their host plants, such as enhanced uptake of mineral nutrients and water and increased tolerance to stressful environments (Baum et al., 2015). Remarkably, arbuscular mycorrhizal (AM) fungi could improve host plant tolerance to salinity stress by an array of physiological and biochemical mechanisms, including higher water-use efficiency, photosynthetic capacity, ionic homeostasis maintenance, osmoprotection, cell ultrastructure preservation, and strengthened antioxidant metabolism (Evelin et al., 2019). In lettuce, AM inoculation elevated proline accumulation and the activities of antioxidants such as catalase (CAT), superoxide dismutase (SOD), and ascorbate peroxidase, but lowered phenolic compound synthesis and oxidative stress with high growth in plants exposed to salt stress (Santander et al., 2019). Recently, AMF have been demonstrated to increase growth, nutrient acquisition (P, K^+, Mg^{2+}), and the ratios of total chlorophyll:carotenoids, Ca^{2+}:Na^+, Mg^{2+}:Na^+, and K^+:Na^+ in the shoots of the medicinal plant *Valeriana officinalis* under salinity stress (Amanifar and Toghranegar, 2020). The authors also showed a higher stimulated root proline, total soluble sugars, and total phenolics in the shoots of mycorrhizal plants subjected to saline conditions.

Bioactive compounds accumulated in medicinal plants are susceptible to changes in the growing seasons, growth years, and environmental factors (Li et al., 2020). Indeed, abiotic stresses are robust elicitors of secondary metabolite production in plants since they use their energy in defense mechanisms by activating specific biosynthesis pathways (Caretto et al., 2015; Toscano et al., 2019). Despite the deleterious effects of salinity, this abiotic stress is one of the major factors influencing the physiology, biochemistry, and synthesis of bioactive compounds in many herbs (Bistgani et al., 2019; Behdad et al., 2020; Boughalleb et al., 2020). Bistgani et al. (2019) illustrated that total phenolic content in *Thymus vulgaris* (L.) and *T. daenensis* Celak was inclined by approximately 20% after treatment with 60 mM NaCl, while leaf flavonoid content was enhanced by 38.6 and 36.6% in plants in response to 60 and 90 mM NaCl, respectively. Cinnamic acid, the main constituent in both plant species, was elevated by 31.4% in *T. vulgaris* in the presence of 60 mM NaCl (Bistgani et al., 2019). In a recent work, total flavonoid compounds, phenolic acids, and phenolic compounds in *Polygonum equisetiforme* plants were elevated with salinity levels remarkably at 300 mM NaCl (Boughalleb et al., 2020). An augmentation in total phenolic acids resulted mainly from an incline in gallic, protocatechuic, and quinic acids, followed by quercetin-3-*O*-galactoside, catechin, and epicatechin (Boughalleb et al., 2020).

On the other hand, the potential of AM inoculation to boost the biosynthesis of bioactive compounds such as phenols, alkaloids, and terpenes has been described in numerous medicinal plants; thus, fungal symbiosis could inflict significant changes in the pharmacological properties of medicinal plants (Kapoor et al., 2017). Previously, we reported that mycorrhizal inoculation modified the content of some polyphenols such as protocatechuic, 4-*O*-caffeoylquinic acid, 4,5-dicaffeoylquinic acid, luteolin, and quercetin-3-arabinoside of *E. prostrata* cultivated under different substrates with various nutrient supplies (Vo et al., 2019). In rosemary, mycorrhization altered polyphenolic profile distribution in the leaves. Four substances possessing strong antioxidant properties, namely, asiatic acid, ferulic acid, vanillin, and carnosol, were linked to rosemary plants treated with *Rhizoglomus irregulare* (Seró et al., 2019). Amanifar and Toghranegar (2020) pointed out that moderate

salt stress stimulated a higher valerenic acid production in the medicinal plant *V. officinalis* L., which was more profound in the plants colonized by *Funneliformis mosseae*. Intriguingly, the potential of AMF, in combination with salinity, has not been fully noticed yet (Kapoor et al., 2017). AM application for the cultivation of medicinal plants in saline soils could be used to address the growing demand of medicinal plants for the pharmaceutical industry (Amanifar and Toghranegar, 2020). Moreover, it may offer the possibility of re-utilizing salt-affected soils under agricultural systems.

It is noteworthy that the synergistic effects of salt stress with other environmental factors on bioactive compounds in medicinal plants have been poorly understood (Isah, 2019). To the best of our knowledge, no studies on the interactive effects of salinity stress and AM inoculation on physio-biochemical parameters and polyphenol profiles of *E. prostrata* have been reported yet. Different AMF species or isolates could differently influence host plant growth and responses to stresses (Lee et al., 2013; Duc et al., 2018). Thus, this study aimed to explore the role of single AMF species and a mixture, including six AMF species on plant growth and physio-biochemical characteristics of *E. prostrata* under non-saline conditions. Next, the most suitable AM treatment was chosen to examine the impact of AMF on physio-biochemical features and polyphenol profiles of *E. prostrata* during plant growth and under salinity stresses. In practice, *E. prostrata* plants are usually cultivated and harvested before flowering; however, there is no information available to select the right harvest time to optimize individual bioactive compounds for pharmaceutical and cosmetic industries. Our hypotheses were as follows: (1) that mycorrhizal colonization could improve plant growth and tolerance to salt stresses, particularly moderate salinity, and (2) that AMF and salinity had interactive effects on the polyphenol profiles of this medicinal plant, which depended on plant age.

MATERIALS AND METHODS

Plant Material, Arbuscular Mycorrhizal Preparation, and Inoculation

Seeds of *E. prostrata* (L.) from Hong Dai Viet Ltd (Vietnam) were used in our experiments. The mycorrhizal commercial inoculant Symbivit containing six AMF species, *Claroideoglomus etunicatum*, *Rhizoglomus microaggregatum*, *Rhizophagus intraradices*, *Claroideoglomus claroideum*, *Funneliformis mosseae*, and *Funneliformis geosporum*, was provided by Symbiom Ltd., Czechia. *Septoglomus deserticola* BEG 73 and *Acaulospora lacunose* BEG 78 were obtained from the International Bank for the Glomeromycota. *F. mosseae* SZIE originated from the collection of Szent István University. Three single AMF species were separately propagated by using *Medicago truncatula* and *Zea mays* as host plants cultured in autoclaved sand for 6 months. A mixture of spores, mycelia, infected root fragments, and sand from cultures was harvested for mycorrhizal inoculation. The inoculation dosage was 15 g of inocula per pot with about 2,400 infective propagules evaluated by the most probable number test (Porter, 1979). Mycorrhizal inocula were applied before transferring germinated seeds to pots of the experiments. In the preliminary experiment, control treatment representing pots without AMF inoculation was prepared in the following way. 15 g of autoclaved combined inoculum including *F. mossea*, *A. lacunose*, *Symbivit, S. deserticola* (each inoculum accounted for one-fourth of the amount) and 3 ml of a filtrate (<20 μm) of this combined inoculum to provide a microbial population with non-AM propagules. In the salt stress experiment, 15 g of sterilized Symbivit and 3 ml of a filtrated solution of Symbivit were used for pots without mycorrhizal inoculation.

Plant Growth and Experiment Design

Preliminary Experiment: Impact of Different Single AMF Species and a Mixture of AM Inoculation on *E. prostrata* Plant Performance Under Non-stress Conditions

The seeds were sterilized with NaOCl 1%, then washed with distilled water several times and put on a filter paper in Petri dishes at 26°C for germination for 3 days. Germinated seeds were placed in 0.5-L plastic pots filled with an autoclaved mixture of sand and peat (60:40%) (v/v). The chemical properties of the sand:peat substrate have pH 6.9, N (%) 0.6%, P 681.29 mg kg^{-1}, K 2,819 mg kg^{-1}, carbonate (%) 17.18%, and dry matter content (m/m%) 54% (Vo et al., 2019). The AM inocula were placed adjacent to seedling roots. An experiment setup according to a randomized complete block design included five different treatments: (1) plants inoculated with *S. deserticola*, (2) plants inoculated with *A. lacunose*, (3) plants inoculated with *F. mosseae*, (4) plants inoculated with a mixture of six AMF species (Symbivit), and (5) plants without AM inoculation (control). Each treatment had 12 biological replicates, equivalent to 12 pots (one plant per pot). Therefore, five treatments (four different kinds of AM treatment and control) with 12 replicates resulted in a total of 60 pots. Pots were put in a climatic chamber EKOCHL 1500 (24/28°C, 60% relative humidity, 16/8 h photoperiod, light intensity 600 μmol m^{-2} s^{-1}) and watered once a week. At 4 and 8 weeks of growth, plants were harvested for measurements. Root colonization, plant height, fresh root and shoot weight, leaf number, and leaf area were examined. Fully expanded leaves (excluding petioles) were immediately frozen in liquid nitrogen and stored at −80°C until total phenolics and proline content determination.

Salt Stress Experiment: Impact of AM Inoculation and Salinity Stress on Plant Performance and Polyphenol Profiles of *E. prostrata*

Based on the preliminary experiment results, the mixture of six AMF species (Symbivit) was chosen for AM treatment in this experiment. A factorial experiment was performed using a randomized complete block design with two factors: (1) salinity levels (0, 100, and 200 mM NaCl) (Chauhan and Johnson, 2008) and (2) mycorrhizal inoculation (inoculated with either the mixture of six AMF species or the sterilized AM inoculant as control). After surface-disinfected seeds were germinated, they were sown in each plastic pot (10 × 6 × 14 cm in size) containing 3 kg of sterilized sand and peat (60:40%) (v/v) substrate. Each treatment had 10 biological replicates; therefore, six treatments (3 salinity levels × 2 mycorrhizal inoculations) with 10 replicates

resulted in a total of 60 pots (one plant per pot). Pots were put in a climatic chamber EKOCHL 1500 (24/28°C, 60% relative humidity, 16/8 h photoperiod, light intensity 600 $\mu mol\ m^{-2}\ s^{-1}$). During plant growth (8 weeks), non-stress plants were watered with 100 ml of tapping water per pot once a week, while salt stress treatments were applied by watering with 100 ml of 100 or 200 mM NaCl for each pot once a week. Fresh shoot and root weight, plant height, leaf number, leaf area, stem diameter, chlorophyll fluorescence, and mycorrhizal colonization rate were examined at 4 and 8 weeks of growth. The fully expanded leaves (excluding petioles) were immediately frozen in liquid nitrogen and stored at -80°C until analysis of proline, superoxide dismutase, peroxidase, catalase, and polyphenol components.

Assessment of Arbuscular Mycorrhizal Colonization

Five plants per treatment were randomly selected, then their roots were washed to remove the substrate and cleared with 10% KOH for 10 min, acidified using 2% HCl and 0.05% Trypan blue in 1:1:1 water:glycerin:lactic acid overnight. Thirty root fragments (1 cm long) were mounted on a glass slide, and four glass slides per plant were examined according to Trouvelot et al. (1986) using the MYCOCALC software in the preliminary experiment. In the salt stress experiment, 60 root fragments per technical replicate and four technical replicates per plant were used to evaluate mycorrhizal colonization according to the gridline intersect method (Giovanetti and Mosse, 1980).

Leaf Area and Chlorophyll Fluorescence Measurement

Leaf area was determined according to the method of Glozer (2008). The maximum quantum efficiency of photosystem II photochemistry (F_v/F_m), a chlorophyll fluorescence parameter, was measured after 30 min of dark adaptation using a Walz-PAM 2500 (Germany) fluorometer according to the method of Oxborough and Baker (1997). The measurements were implemented on the fourth leaf from a single plant's shoot apex in each treatment with five biological replicates.

Proline Content Determination

Proline content was quantified by the acid ninhydrin procedure of Bates et al. (1973). A half gram of leaf samples from each treatment was homogenized in 10 ml of 3% aqueous sulfosalicylic acid. Afterward, it was centrifuged at 10,000 rpm for 15 min. Two milliliters of the supernatant, 2 ml of glacial acetic acid, and ml ninhydrin acid were blended, then incubated at 100°C for 1 h. The reaction was terminated in an ice bath; subsequently, the chromophore was extracted with 4 ml toluene. Its absorbance at 520 nm was measured by U-2900 UV-VIS spectrophotometer (Hitachi). Proline concentration (μmol proline per g of fresh weight) was estimated from the standard curve.

Measurement of Total Phenolic Content

Total phenolic concentration was measured by the Folin–Ciocalteu assay (Lister and Wilson, 2001). Briefly, 2 g of leaves were blended well with 20 ml of 60% ethanol and subsequently filtered. One milliliter of filtrate and 0.5 ml of Folin–Denis reagent were transferred to a tube, then mixed completely. Next, 1 ml of saturated Na_2CO_3 was added after 3 min at room temperature. The mixture was completed to 10 ml with distilled water and incubated for 30 min at room temperature. The absorbance at 760 nm was recorded, and total phenolics content was presented as mg gallic acid per g fresh weight.

Measurement of Antioxidant Enzymatic Activities

Frozen leaves (0.5 g) were homogenized in 3 ml of 50 mM Tris–HCl buffer (pH 7.8) containing 1 mM Na_2EDTA and 7.5% (w/v) polyvinylpyrrolidone K25 and centrifuged at 10,000 × *g* at 4°C for 20 min. The supernatants were used for peroxidase, superoxide dismutase, and catalase assays. The protein content of all leaf extracts was estimated by the method of Bradford (1976).

Peroxidase (POD, EC 1.11.1.7) activity was measured by the method of Rathmell and Sequeira (1974). Shortly, the reaction mixture (2.2 ml) containing 0.1 M sodium phosphate buffer (pH 6.0), 100 μl of 12 mM H_2O_2, and 100 μl of 50 mM guaiacol with 10 μl of plant extract was used to measure the POD activity at 436 nm in 5 min. The enzyme activity was expressed as the changes in absorbance mg^{-1} protein min^{-1}.

Superoxide dismutase (SOD, EC 1.15.1.1) activity was determined spectrophotometrically at 560 nm following by the method of Beyer and Fridovich (1987). Briefly, the reaction mixture (2 ml) consisted 50 mM phosphate buffer (pH 7.8), 2 mM EDTA, 0.025% Triton X-100, 55 μM Nitroblue tetrazolium (NBT), 9.9 mM L-methionine, 20 μl of crude extract, and 20 μl of 1 mM riboflavin. The absorbance was read at 560 nm. One unit of SOD activity (U) was defined as the required enzyme volume to lead to 50% inhibition of the NBT decline under the assay conditions.

Catalase (CAT, EC 1.11.1.6) activity was measured by the method of Aebi (1984). The reaction mixture consisted of 1 ml of 10 mM of hydrogen peroxide and 2 ml of 50 mM potassium phosphate buffer (pH 7.0) and 20 μl leaf extract. The absorbance decrease at 240 nm of the reaction was recorded as the deposition level of H_2O_2. The enzyme activity was presented as the changes in absorbance mg^{-1} protein min^{-1}.

HPLC Determination of Polyphenols

From each well-homogenized aerial part of fresh material of *E. prostrata*, a 0.5-g sample was taken and crushed in a crucible mortar with quartz sand. Twenty milliliters of a mixture of 44% EtOH, 4% MeOH, 10% water, and 2% acetic acid was gradually added with crushing and then transferred to a 100-ml Erlenmeyer flask. The macerate was subjected to an ultrasonication force using an ultrasonic water bath device (Model USD-150, Raypa) for 4 min, followed by mechanical shaking (GLF3005) for 15 min. The mixture was kept overnight at 4°C and filtered through Albet-DF400125 type filter paper. Before injection onto the HPLC column, it was further cleaned up by passing through a 0.22-mm PTFE HPLC syringe filter. Nucleosil C18-100, 3 μm, 240 × 4.6 mm Protect-1 HPLC column (Macherey-Nagel, Duren, Germany) was used to separate phenolic compounds using a

gradient elution of 1% formic acid in water (A) and acetonitrile (B) with a flow rate of 0.6 ml min^{-1}. Gradient elution began with 2% B, changed to 13, 25, and 40% B in 10, 5, and 15 min, respectively, and finally turned to 2% B in 5 min. The HPLC determination was performed using a Hitachi Chromaster HPLC with a Model 5160 pump, a Model 5260 autosampler, a Model 5310 column oven, and a Model 5430 diode-array detector. The separation and data processing were operated by OpenLab CDS software. The peaks were identified by comparing their retention times and spectral characteristics with available standards such as quercetin-3-arabinoside, luteolin-glucoside, luteolin-7-*O*-glucoside, luteolin, wedelolactone, demethyl wedelolactone, caffeic acid, 3,4-*O*-dicaffeoylquinic acid, 3,5-dicaffeoylquinic acid, 4-*O*-caffeoylquinic acid, 4,5-dicaffeoylquinic acid, 5-*O*-caffeoylquinic acid, ferulic acid, and feruloylquinic acid (Sigma-Aldrich Ltd., Hungary). For the quantification of phenolic compounds, each peak area was integrated at the maximum absorption wavelength, and the concentrations were calculated by relating the areas of the peaks to those of the available external standards (Merken and Beecher, 2000). The standard materials were singly injected as external standards and chromatographed with the samples as well.

Statistical Analysis

Statistical analysis was implemented using the SAS 9.1 (SAS Institute, Cary, NC, United States) package for Windows. In the preliminary experiment, differences in plant growth traits, mycorrhizal colonization, proline, and total phenolics among AMF treatments were analyzed by one-way analysis of variance and Tukey *post hoc* tests. In the salinity stress experiment, two-way analysis of variance (GLM procedure in SAS) was applied with explanatory variables (factors) of AM inoculation and salt stress levels as well as their interaction. Moreover, Tukey *post hoc* tests were applied. In addition, two-sample, two-tailed *t*-test was applied using MS Excel to compare each particular treatment result at 4 and 8 weeks. PCA was carried out by the XLSTAT program to identify patterns, i.e., interactions among the studied variables and treatments, in polyphenolic data of *E. prostrata* with and without AMF under different salinity stress levels including no salinity stress.

RESULTS

Inoculation of the AM Mixture Improved Aboveground Biomass and Total Phenolic Content of *E. prostrata* Under Non-stress Conditions (the Preliminary Experiment)

Mycorrhizal Colonization and Plant Growth Parameters

Microscopic observation of the roots showed that no AM colonization in non-AM plants (control plants) was detected. In contrast, plants in mycorrhizal treatments were successfully colonized by three single AMF species and the mixed AM inoculant. The colonization rate of plants infected by *A. lacunose* was lowest (22.9%) and significantly lower than plants treated with other single AMF species and the mixture of AMF (from 47.8 to 54.1%) 4 weeks after inoculation. Interestingly, no significant differences in this rate among 8-week plants treated with different mycorrhizal inoculants (ranged from 49.5 to 59.5%) were found (**Table 1**).

Regarding plant growth parameters, there were no significant differences in fresh root weight and leaf number among all treatments at 4 and 8 weeks (**Table 1**). Treatment with the mixture of six AMF dramatically increased fresh shoot weight by 76% in relation to non-AM plants after 8 weeks of growth. By contrast, plants inoculated with *S. deserticola* displayed a substantial decline in fresh shoot weight by 52.9 and 47.5% after 4 and 8 weeks of plant growth, respectively, in comparison with that of the corresponding non-mycorrhizal plants. Notably, inoculation of *F. mosseae*, *S. deserticola*, and *A. lacunose* considerably reduced plant height compared with uncolonized plants at 4 weeks, while the decline did not occur in the mixed AM treatment at both stages of plant growth. In terms of leaf area, no beneficial effects of different AM inoculations were observed in host plants.

Proline and Total Phenolic Concentration

Under non-stress conditions, inoculation with different species of AMF did not induce substantially higher proline concentrations in plants compared with non-AM plants during plant growth (**Figure 1A**). Remarkable decreases in the proline content were recorded in plants inoculated with *S. deserticola* (by 64.6%) and *A. lacunose* (49.3%) at the later stage compared with plants at the early stage of plant growth ($P < 0.05$). Similarly, there were no significant differences in total phenolic content between control plants and mycorrhizal plants after 4 weeks of growth (**Figure 1B**). Nonetheless, the treatment of Symbivit remarkably reduced the total phenolic concentration in plants by 56.8 and 51% relative to plants inoculated with *F. mosseae* and *S. deserticola*, respectively. At 8 weeks, the content of total phenolics in plants treated by the mixed inoculant was dramatically enhanced by 178.5% versus that at 4 weeks ($P < 0.001$). No significant differences in total phenolic content among control plants and plants treated with *A. lacunose* or Symbivit were detected 8 weeks after inoculation. Conversely, the total phenolic level in *S. deserticola* colonized plants at 8 weeks considerably declined by 31.2% ($P < 0.05$) in relation to that at 4 weeks. Moreover, *S. deserticola* caused a sharp reduction in total phenolic concentration in plants, as compared with non-AM plants (by 37.8%) and plants colonized by other fungal symbionts (by 46% versus *F. mosseae*, 50.2% versus *A. lacunose*, and 49.5% versus Symbivit) after 8 weeks of growth.

Arbuscular Mycorrhizal Fungi Enhanced Plant Tolerance of *E. prostrata* to Moderate Salt Stress (the Salt Stress Experiment)

Root Colonization and Growth Parameters

Non-AM plants had no mycorrhizal colonization during plant growth. After 4 weeks of growth, the mycorrhizal colonization

TABLE 1 | Growth parameters of *Eclipta prostrata* inoculated with different arbuscular mycorrhizal fungi or not inoculated under non-saline conditions 4 and 8 weeks after inoculation (the preliminary experiment).

Treatment	Fresh root weight (g plant^{-1})		Fresh shoot weight (g plant^{-1})		Leaf number (leaf plant^{-1})		Plant height (cm plant^{-1})		Leaf area (cm^2 plant^{-1})		Mycorrhizal colonization (%)	
	4 weeks	8 weeks	4 weeks	8 weeks	4 weeks	8 weeks	4 weeks	8 weeks	4 weeks	8 weeks	4 weeks	8 weeks
No AMF	1.49 ± 1.05^{a}	1.81 ± 0.13^{a}	4.44 ± 1.12^{ab}	6.01 ± 0.91^{b}	14.89 ± 4.8^{a}	20 ± 5.53^{a}	15.38 ± 3.07^{a}	52.71 ± 7.41^{a}	15.18 ± 2.73^{a}	17.48 ± 0.91^{ab}	0	0
F. mosseae	1.67 ± 0.19^{a}	2.38 ± 0.62^{a}	3.26 ± 0.30^{bc}	6.05 ± 1.16^{b}	12.89 ± 5.1^{a}	20.86 ± 5.8^{a}	9.44 ± 2.28^{b}	40.88 ± 10.05^{ab}	14.87 ± 2.00^{ab}	16.38 ± 5.43^{b}	54.1 ± 4.2^{a}	59.5 ± 4.7^{a}
S. deserticola	1.69 ± 0.40^{a}	2.02 ± 0.18^{a}	2.09 ± 0.17^{c}	3.15 ± 0.55^{c}	13.78 ± 4.29^{a}	18.86 ± 4.14^{a}	8.51 ± 1.78^{b}	34 ± 7.57^{b}	11.22 ± 0.94^{b}	12.80 ± 2.62^{b}	47.8 ± 6.6^{a}	53.1 ± 5.8^{a}
A. lacunose	1.83 ± 0.76^{a}	2.33 ± 0.16^{a}	3.07 ± 0.90^{bc}	4.98 ± 1.20^{bc}	17 ± 6.68^{a}	22.85 ± 11.36^{a}	10.72 ± 4.36^{b}	38.71 ± 3.60^{b}	14.79 ± 1.67^{ab}	16.83 ± 1.22^{b}	22.9 ± 3.6^{b}	49.5 ± 9.2^{a}
Symbivit	1.53 ± 0.03^{a}	2.37 ± 0.30^{a}	4.95 ± 0.30^{a}	10.58 ± 1.86^{a}	12.89 ± 2.47^{a}	21.43 ± 4.86^{a}	11.64 ± 1.57^{ab}	46 ± 5.62^{ab}	16.58 ± 1.37^{a}	24.36 ± 1.79^{a}	50.5 ± 5.4^{a}	56.3 ± 6.8^{a}

The means ± standard deviation are shown (n = 5 for mycorrhizal colonization, n = 4 for other parameters). Different letters in each column indicate significant difference according to the Tukey test ($P < 0.05$) among treatments 4 and 8 weeks after inoculation. AMF, arbuscular mycorrhizal fungi.

rate of AM plants obtained 54% under non-stress conditions, while the rate was 58.4% in those treated with 100 mM NaCl (**Figure 2**). No significant differences could be found between mycorrhizal plants under non-stress conditions and salt stress at 100 mM NaCl. Nonetheless, high salinity (200 mM NaCl) considerably decreased the colonization percentage to 29.6% at this plant growth stage. Interestingly, we did not find any substantial differences in mycorrhizal colonization rates among colonized plants under non-stress and saline conditions at 8 weeks. Their rates were 51.9, 47.4, and 43% in mycorrhizal plants under non-stress and moderate and high salt stress. The percentage of AM colonization in AM plants under high saline conditions at the later stage was significantly elevated ($P < 0.05$) relative to those at the early stage.

Exposure of *E. prostrata* plants to salt stresses, particularly at 200 mM NaCl, led to a considerable decrement in most growth parameters tested at both plant growth stages (**Table 2**). Under non-stress conditions, mycorrhizal inoculation substantially enhanced fresh root weight (only 75.6% at 8 weeks), fresh shoot weight (101 and 125%), leaf number (89 and 107%), stem diameter (70 and 47.1%), and leaf area (81.5 and 99.6%) at 4 and 8 weeks as compared with those of non-AM plants, while plant height remained unchanged in AM plants. In the presence of 100 mM NaCl, increases in fresh shoot weight (93% at 8 weeks), leaf number (68.1 and 96.3% at 4 and 8 weeks, respectively), and leaf area (59.7 and 88.5%) at both times measured in colonized plants were observed, compared with those of non-AM plants. AM colonization also markedly elevated the leaf area at 4 weeks (by 101%) in plants treated with 200 mM NaCl in comparison with the corresponding uncolonized plants.

Chlorophyll Fluorescence

Although salt stresses slightly increased the maximal photochemical efficiency of photosystem II (F_v/F_m) in plants, no significant differences between mycorrhizal and non-mycorrhizal plants were found under the same conditions at 4 and 8 weeks of growth (**Figure 3**). Mycorrhizal treatment was the main factor substantially influencing F_v/F_m at 4 and 8 weeks ($P < 0.01$), whereas the effect of salt stress was statistically significant on this parameter at 4 weeks ($P < 0.05$).

Proline Concentration

Salinity heightened proline concentrations in mycorrhizal and non-mycorrhizal plants at 4 weeks (**Figure 4**). In detail, 4.7- and 8.2-folds of proline content in non-AM plants exposed to 100 and 200 mM NaCl over the control (non-AM plants) were detected, while 5.3- and 6.8-folds of proline level in AM plants under moderate and high saline conditions over non-stress mycorrhizal plants, respectively, were recorded. There are no significant differences between AM and non-AM plants under the same conditions (no stress and moderate and high salinity). A nearly similar trend was observed at 8 weeks of growth. Plants exposed to salt stresses substantially accumulated a higher proline content in comparison with non-exposed ones. Notably, under moderate salinity, the proline level in AM plants was 116% higher than non-AM plants. The effects of mycorrhizal inoculation (M) and salt stress (S) were statistically significant on proline concentration measured at 4 and 8 weeks (at least $P < 0.05$) with the existence of interaction between the two factors at 8 weeks ($P < 0.05$).

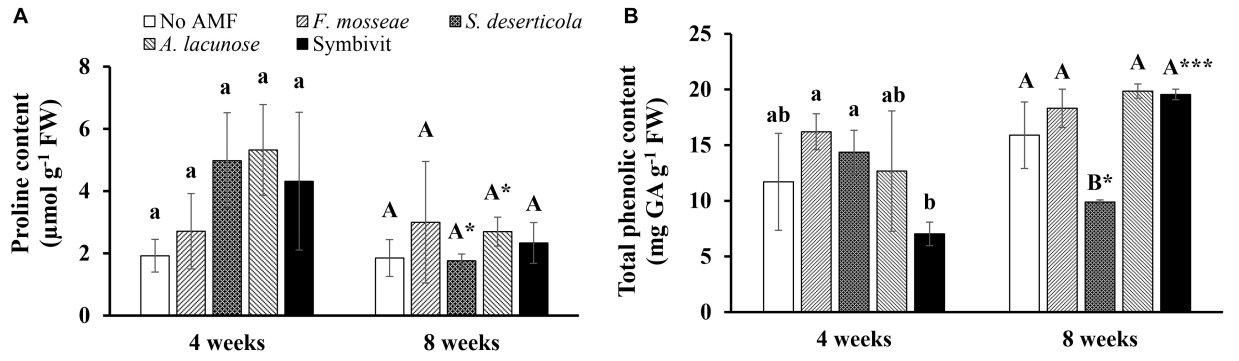

FIGURE 1 | Proline **(A)** and total phenolic content **(B)** of *Eclipta prostrata* inoculated with *Funneliformis mosseae*, *Septoglomus deserticola*, *Acaulospora lacunose*, and Symbivit or not inoculated 4 and 8 weeks after inoculation (the preliminary experiment). AMF, arbuscular mycorrhizal fungi. Each bar shows the mean ± standard deviation ($n = 3$). Different regular and capital letters indicate significant differences among treatments according to the Tukey test ($P < 0.05$) 4 and 8 weeks, respectively, after inoculation. *, *** Indicate significant difference at $P < 0.05$ and $P < 0.001$, respectively, according to the two-tailed *t*-test for the same treatments between 4 and 8 weeks after inoculation.

Antioxidant Enzymatic Activities

At the early stage of plant growth, mycorrhizal plants gained the highest POD activity under moderate salt stress, while the activity of this enzyme was lowest in non-AM plants subjected to 100 mM NaCl (**Figure 5A**). No significant differences could be seen in other treatments. At the later stage, POD activity was considerably lowered (by 80.8%, $P < 0.05$) in non-AM plants under non-stress conditions and (by 37.4%, $P < 0.05$) in moderate-salted mycorrhizal plants, but it substantially leaped (by 140%, $P < 0.05$) in uncolonized plants exposed to 100 mM NaCl. Salt treatments remarkably induced almost three- and seven-folds higher POD activity in non-stress uncolonized plants subjected to 100 and 200 mM NaCl, respectively, at 8 weeks. In contrast, both saline levels did not elevate POD activity in colonized plants. However, AM inoculation triggered an increase in POD activity by nearly sixfold in non-stress plants. Under moderate and high salt stress, no significant differences in POD activity were found between non-AM and AM plants.

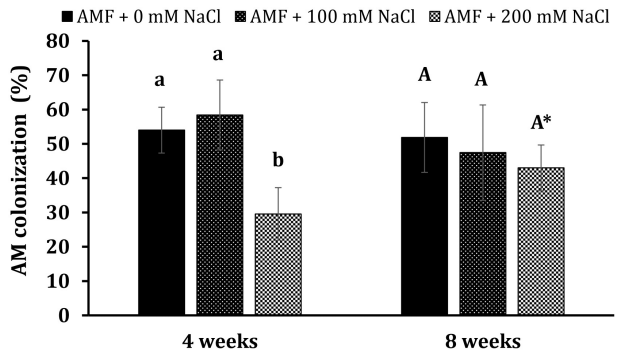

FIGURE 2 | Arbuscular mycorrhizal colonization in the roots of *Eclipta prostrata* plants under non-stress and moderate (100 mM NaCl) and high saline (200 mM NaCl) conditions 4 and 8 weeks after inoculation. * Indicates a significant difference at $P < 0.05$ according to the two-tailed *t*-test in the same treatments between 4 and 8 weeks after inoculation.

Mycorrhizal treatment markedly impacted POD at 4 weeks ($P < 0.01$), while salinity remarkably affected POD at 8 weeks ($P < 0.05$).

In terms of SOD activity, the observed differences between mycorrhizal and non-mycorrhizal plants under non-stress and salt levels were not statistically significant at 4 and 8 weeks (**Figure 5B**). There were substantial increments in this enzyme activity in non-AM plants under high salt stress (by 257%, $P < 0.05$), non-stress AM plants (by 131%, $P < 0.05$), and mycorrhizal plants exposed to moderate saline conditions (by 112%, $P < 0.05$) at the later stage versus the early stage of plant growth. Salinity considerably affected SOD at 4 weeks ($P < 0.05$).

Under non-stress conditions, mycorrhizal application significantly dropped CAT activity in plants at 4 weeks (**Figure 5C**). Moderate salt stress triggered a substantially higher level (by 205%) of this enzyme activity in colonized plants but remarkably lessened it (by 65.2%) in non-AM plants as compared with the corresponding ones. When plants were exposed to high salt concentration, no changes in CAT activity were recorded in mycorrhizal plants. Conversely, CAT activity was markedly reduced (by 90.9%) in uncolonized plants in comparison with those under non-stress conditions. No significant differences in CAT activity in both mycorrhizal and non-mycorrhizal plants under all conditions were found at 8 weeks of growth. Nevertheless, profound declines in CAT activity in non-AM plants under non-stress (by 292%, $P < 0.001$) and AM plants exposed to moderate salt stress (by 70.8%, $P < 0.05$) 8 versus 4 weeks after inoculation were observed. Salinity remarkably affected CAT at 4 ($P < 0.001$) and 8 weeks ($P < 0.05$).

Arbuscular Mycorrhizal Fungi Altered Individual Phenolic Compounds of *E. prostrata* Under Non-saline and Saline Conditions (the Salt Stress Experiment)

The quantitative and qualitative measurements of polyphenols in the leaves of *E. prostrata* were implemented by HPLC-DAD analysis. The gradient elution applied was able to

TABLE 2 | Growth parameters of *Eclipta prostrata* inoculated with arbuscular mycorrhizal fungi or not inoculated under non-stress and moderate and high saline conditions 4 and 8 weeks after inoculation (the salt stress experiment).

Treatment		Fresh root weight (g plant^{-1})		Fresh shoot weight (g plant^{-1})		Leaf number (leaf plant^{-1})		Plant height (cm plant^{-1})		Stem diameter (mm plant^{-1})		Leaf area (cm^2 plant^{-1})	
Stress conditions	**Mycorrhizal inoculation**	**4 weeks**	**8 weeks**	**4 weeks**	**8 weeks**	**4 weeks**	**8 weeks**	**4 weeks**	**8 weeks**	**4 weeks**	**8 weeks**	**4 weeks**	**8 weeks**
Non-stress	AMF-	1.17 ± 0.05^{ab}	1.68 ± 0.56^{b}	2.43 ± 0.42^{b}	4.4 ± 0.7^{b}	10.0 ± 1.5^{c}	15.2 ± 1.5^{bc}	11.3 ± 6.0^{a}	24.6 ± 6.6^{ab}	2.0 ± 0.3^{b}	2.93 ± 0.2^{b}	8.73 ± 0.7^{b}	11.93 ± 2.8^{bc}
	AMF +	1.68 ± 0.4^{a}	2.95 ± 0.72^{a}	4.02 ± 0.4^{a}	9.9 ± 2.6^{a}	18.9 ± 2.9^{a}	31.5 ± 10.1^{a}	13.7 ± 6.0^{a}	25.5 ± 6.5^{a}	3.4 ± 0.7^{a}	4.31 ± 0.8^{a}	15.85 ± 2.6^{a}	23.81 ± 3.7^{a}
100 mM NaCl	AMF-	0.53 ± 0.3^{bcd}	0.15 ± 0.05^{c}	0.99 ± 0.33^{cd}	1.4 ± 0.1^{c}	9.4 ± 0.4^{c}	11.1 ± 1.5^{c}	6.9 ± 2.2^{a}	10.9 ± 3.0^{c}	1.9 ± 0.1^{b}	2.13 ± 0.1^{b}	5.24 ± 0.8^{cd}	7.63 ± 1.0^{c}
	AMF +	0.78 ± 0.3^{bc}	0.41 ± 0.06^{c}	1.82 ± 0.6^{bc}	2.7 ± 0.3^{b}	15.8 ± 3.2^{ab}	21.8 ± 3.7^{b}	7.9 ± 2.1^{a}	13.3 ± 2.2^{bc}	2.4 ± 0.1^{ab}	2.45 ± 0.1^{b}	8.37 ± 0.6^{b}	14.38 ± 3.7^{b}
200 mM NaCl	AMF-	0.03 ± 0.00^{d}	0.13 ± 0.02^{c}	0.44 ± 0.1^{d}	0.9 ± 0.4^{c}	9.4 ± 0.5^{c}	9.0 ± 1.7^{c}	3.8 ± 0.5^{a}	5.0 ± 0.6^{c}	1.4 ± 0.2^{b}	2.06 ± 0.4^{b}	3.28 ± 0.6^{d}	7.28 ± 1.5^{c}
	AMF +	0.11 ± 0.02^{cd}	0.19 ± 0.04^{c}	0.66 ± 0.5^{cd}	1.6 ± 0.6^{bc}	12.8 ± 1.1^{bc}	11.7 ± 0.3^{c}	5.6 ± 0.7^{a}	10.1 ± 2.1^{c}	1.8 ± 0.3^{b}	2.36 ± 0.4^{b}	6.60 ± 0.5^{bc}	9.15 ± 1.8^{bc}
Source of variation (ns, not significant, *$P \leq 0.05$, **$P \leq 0.01$, ***$P \leq 0.001$)													
Mycorrhizal inoculation (M)		*	*	***	***	***	***	ns	ns	**	**	***	***
Salt stress (S)		***	***	***	***	**	**	*	***	***	***	***	***
M × S		ns	*	ns	**	*	ns	ns	ns	ns	Ns	*	*

The means ± standard deviation are presented (n = 5 for mycorrhizal colonization, n = 3 for other parameters). Different letters in each column indicates significant difference according to the Tukey test ($P < 0.05$) among treatments 4 and 8 weeks after inoculation. AMF, arbuscular mycorrhizal fungi; ns, non-significant difference.

FIGURE 3 | Maximal photochemical efficiency of photosystem II (F_v/F_m) in the leaves of *Eclipta prostrata* inoculated with arbuscular mycorrhizal fungi or not inoculated under non-stress and moderate (100 mM NaCl) and high saline (200 mM NaCl) conditions 4 and 8 weeks after inoculation. AMF, arbuscular mycorrhizal fungi. Each bar shows the mean ± standard deviation ($n = 5$). Different regular and capital letters indicate significant differences among treatments according to the Tukey test ($P < 0.05$) 4 and 8 weeks, respectively, after inoculation. **, significant differences at $P < 0.01$. ns, not significant. AMF, arbuscular mycorrhizal fungi. M, mycorrhizal inoculation effect. S, salt stress effect. M × S, the interaction between mycorrhizal inoculation and salt stress.

FIGURE 4 | Proline concentration in the leaves of *Eclipta prostrata* inoculated with arbuscular mycorrhizal fungi or not inoculated under non-stress and moderate (100 mM NaCl) and high saline (200 mM NaCl) conditions 4 and 8 weeks after inoculation. Each bar shows the mean ± standard deviation ($n = 3$). Different regular and capital letters indicate significant differences among treatments according to the Tukey test ($P < 0.05$) 4 and 8 weeks, respectively, after inoculation. *, ***, significant differences at $P < 0.05$, 0.001. ns, not significant. AMF, arbuscular mycorrhizal fungi. M, mycorrhizal inoculation effect. S, salt stress effect. M × S, the interaction between mycorrhizal inoculation and salt stress.

efficiently separate 14 phenolic constituents in plants 4 weeks after growth, namely eight hydroxycinnamates (caffeic acid, ferulic acid, 3,4-*O*-dicaffeoylquinic acid, 3,5-dicaffeoylquinic acid, 4-*O*-caffeoylquinic acid, 4,5-dicaffeoylquinic acid, 5-*O*-caffeoylquinic acid, feruloylquinic acid), four flavonoids (luteolin-glucoside, luteolin, luteolin-7-*O*-glucoside, quercetin-3-arabinoside), and two coumarins (wedelolactone and demethyl wedelolactone) (**Figure 6A**), whereas only 13 components of polyphenols (feruloylquinic acid was under detection limit) were determined in 8-week plants (**Figure 6B**). Among polyphenols, wedelolactone and/or 4,5-dicaffeoylquinic was abundant in all plants under different conditions. At the early stage of growth,

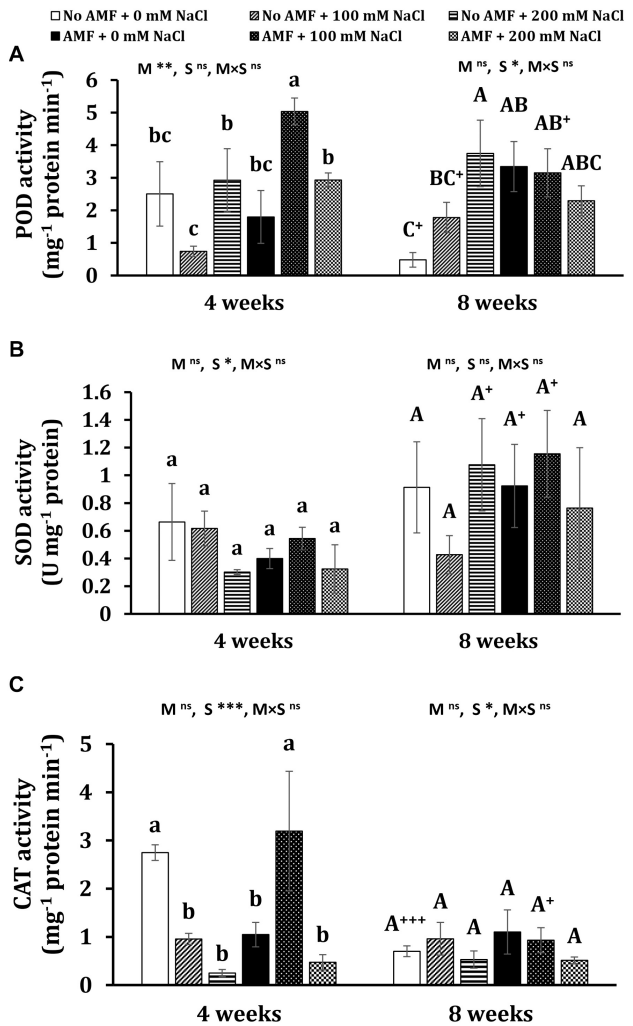

FIGURE 5 | Peroxidase (POD) **(A)**, superoxidase dismutase (SOD) **(B)**, and catalase (CAT) **(C)** activity in the leaves of *Eclipta prostrata* inoculated with arbuscular mycorrhiza or not inoculated under non-stress and moderate (100 mM NaCl) and high saline (200 mM NaCl) conditions 4 and 8 weeks after inoculation. AMF, arbuscular mycorrhizal fungi. Each bar shows the mean ± standard deviation (n = 3). Different regular and capital letters indicate significant differences among treatments according to the Tukey test ($P < 0.05$) 4 and 8 weeks, respectively, after inoculation. +, ++, +++ indicate a significant difference between the same treatments 4 and 8 weeks after inoculation at $P < 0.05$, $P < 0.01$, and $P < 0.001$, respectively, according to the two-tailed test. ns, non-significant. *, **, ***, significant differences at $P < 0.05$, 0.01, 0.001. AMF, arbuscular mycorrhizal fungi. M, mycorrhizal inoculation effect. S, salt stress effect. M × S, the interaction between mycorrhizal inoculation and salt stress.

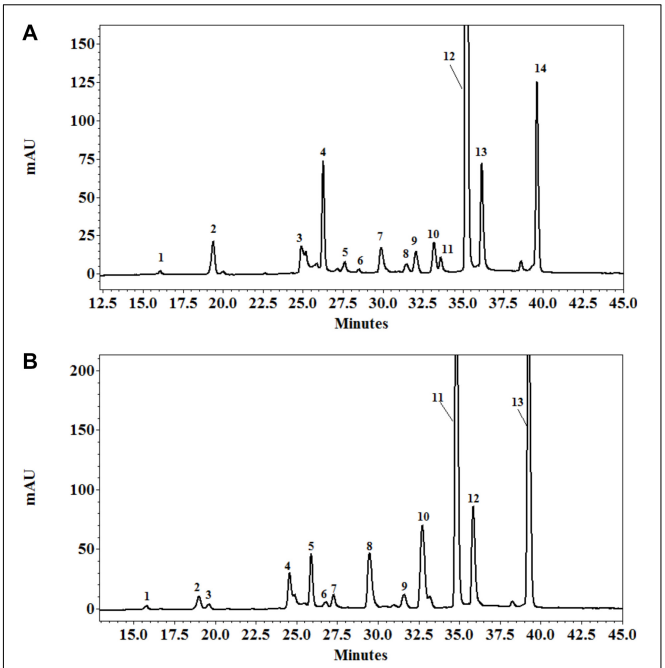

FIGURE 6 | HPLC profile of polyphenols from the leaves of *Eclipta prostrata* separated on C18 Protect-1, 250 × 4.6 mm eluated with a gradient of acetonitrile in 1% formic acid solution 4 **(A)** and 8 weeks **(B)** after inoculation. Peak identifications of **(A)**: 1 = 5-*O*-caffeoylquinic acid; 2 = 4-*O*-caffeoylquinic acid; 3 = caffeic acid; 4 = 3,4-*O*-dicaffeoylquinic acid; 5 = 3,5-dicaffeoylquinic acid; 6 = luteolin-glucoside; 7 = luteolin-7-*O*-glucoside; 8 = ferulic acid; 9 = quercetin-3-arabinoside; 10 = demethyl wedelolactone; 11 = feruloylquinic acid; 12 = 4,5-*O*-dicaffeoylquinic acid; 13 = luteolin; 14 = wedelolactone. Peak identifications of **(B)**: 1 = 5-*O*-caffeoylquinic acid; 2 = 4-*O*-caffeoylquinic acid; 3 = caffeic acid; 4 = 3,4-*O*-dicaffeoylquinic acid; 5 = 3,5-dicaffeoylquinic acid; 6 = luteolin-glucoside; 7 = luteolin-7-*O*-glucoside; 8 = ferulic acid; 9 = quercetin-3-arabinoside; 10 = demethyl wedelolactone; 11 = 4,5-dicaffeoylquinic acid; 12 = luteolin; 13 = wedelolactone.

the content of the total and individual polyphenols was mainly affected by salinity, whereas both mycorrhizal inoculation and salt stress influenced phenolic production at the later growth stage (**Figures 7**, **8**). In detail, after 4 weeks of growth, there was a considerable effect of mycorrhizal inoculation (M) on the contents of four flavonoids (at least $P < 0.05$), five hydroxycinnamic acids (at least $P < 0.01$), and demethyl wedelolactone ($P < 0.001$). Salinity had a substantial impact on the level of all polyphenol compounds tested (at least $P < 0.05$), except demethyl wedelolactone and 5-*O*-caffeoylquinic acid. Interactions between two main effects on 3,5-dicaffeoylquinic acid ($P < 0.001$), ferulic acid ($P < 0.01$), feruloylquinic acid ($P < 0.001$), 4,5-dicaffeoylquinic acid ($P < 0.05$), and luteolin ($P < 0.001$) were found. When plants reached 8 weeks of age, mycorrhizal colonization significantly influenced all polyphenol compounds (at least $P < 0.05$), except demethyl wedelolactone. Likewise, salinity elicited sharp changes in all polyphenols (with at least $P < 0.01$). Interactions between two main effects on most polyphenols were recorded (at least $P < 0.05$, except luteolin-glucoside and demethyl wedelolactone).

After 4 weeks of growth, mycorrhizal colonization resulted in a significant increase in the total polyphenols (by 139%) in non-stress plants. Such a tendency was observed in the content of wedelolactone (105%), 3,5-dicaffeoylquinic acid (404%), 4,5-dicaffeoylquinic acid (1,281%), and feruloylquinic acid (2,901%). Moderate salinity significantly induced higher total phenolics (166%) and seven individual polyphenols such as wedelolactone (134%), ferulic acid (239%), 3,5-dicaffeoylquinic acid (842%), 4,5-dicaffeoylquinic acid (1,436%), 4-*O*-caffeoylquinic acid (336%),

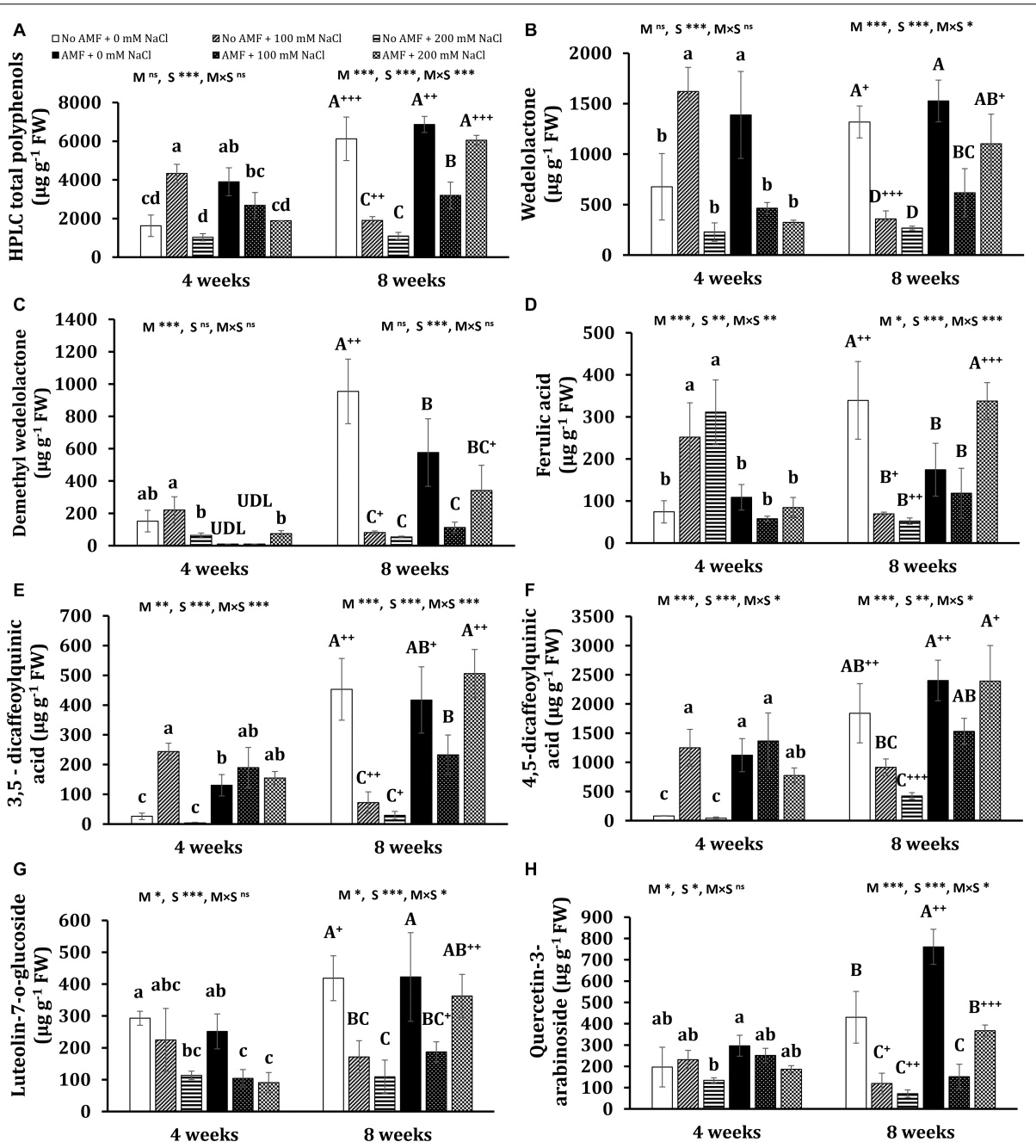

FIGURE 7 | Contents of HPLC total polyphenols **(A)** and major polyphenols: wedelolactone **(B)**, demethyl wedelolactone **(C)**, ferulic acid **(D)**, 3,5-dicaffeoylquinic acid **(E)**, 4,5-dicaffeoylquinic acid **(F)**, luteolin-7-*O*-glucoside **(G)**, and quercetin-3-arabinoside **(H)** in the leaves of *Eclipta prostrata* inoculated with arbuscular mycorrhiza or not inoculated under non-stress and moderate (100 mM NaCl) and high saline (200 mM NaCl) conditions 4 and 8 weeks after inoculation. Each bar shows the mean ± standard deviation (n = 3). Different regular and capital letters indicate significant differences among treatments 4 and 8 weeks after inoculation, respectively, according to the Tukey test ($P < 0.05$). +, ++, +++ Indicate significant differences between the same treatments 4 and 8 weeks after inoculation at $P < 0.05$, $P < 0.01$, and $P < 0.001$, respectively, according to the two-tailed test. ns, non-significant. *, **, ***, significant differences at $P < 0.05$, 0.01, 0.001. AMF, arbuscular mycorrhizal fungi. M, mycorrhizal inoculation effect. S, salt stress effect. M × S, the interaction between mycorrhizal inoculation and salt stress. UDL, under the detection limit.

caffeic acid (171%), and luteolin (287%) in uncolonized plants at 4 weeks, while these increments were not found under high salt stress, except ferulic acid, 5-*O*-caffeoylquinic acid, and luteolin. By contrast, under both salt stresses, the decrement trend was seen in the content of total polyphenols and wedelolactone, luteolin-7-glucoside, and feruloylquinic acid in mycorrhizal plants, being more severe under high salt stress, whereas there were no significant changes in the concentrations of ferulic acid,

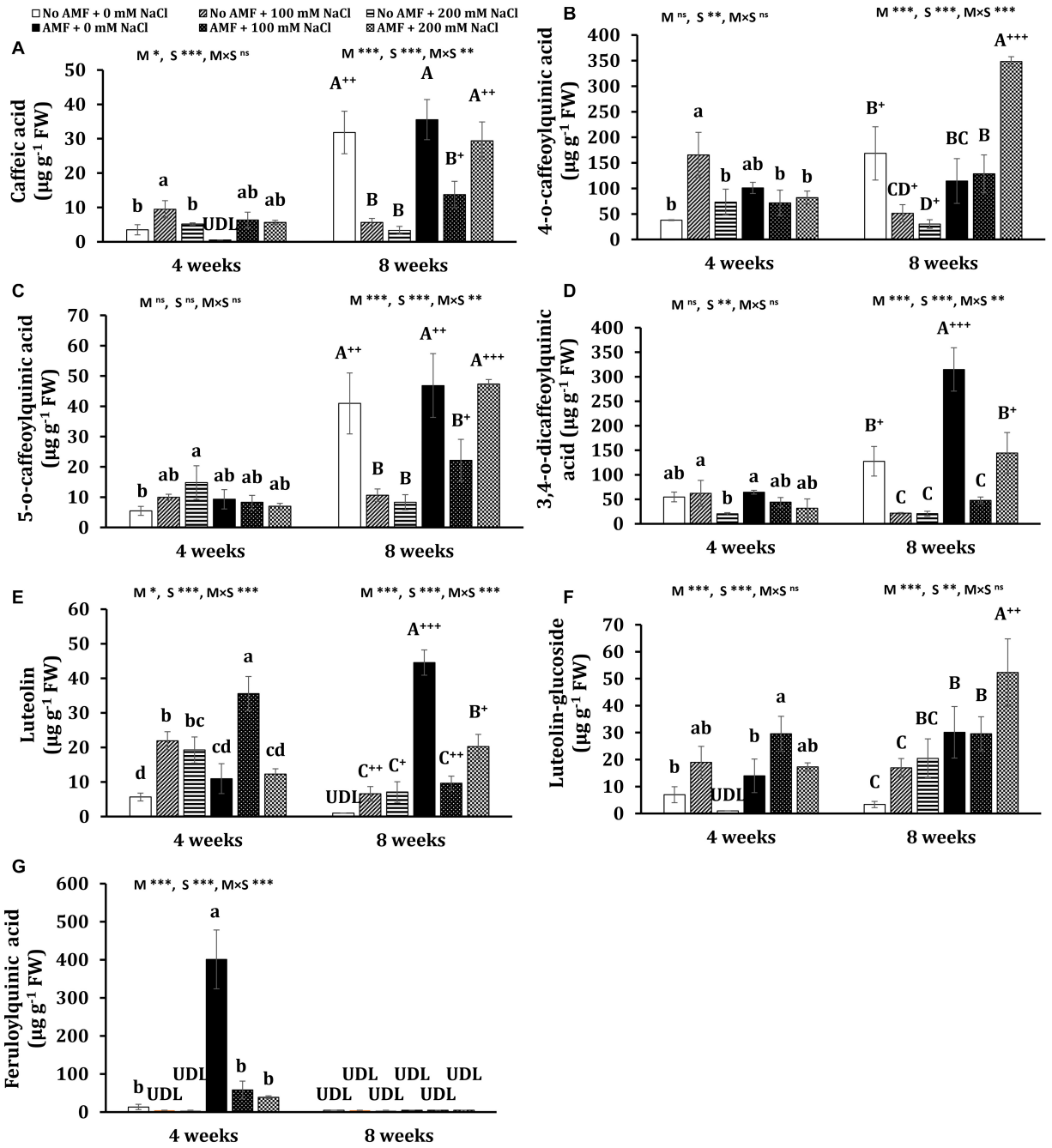

FIGURE 8 | Contents of polyphenols: caffeic acid **(A)**, 4-*O*-caffeoylquinic acid **(B)**, 5-*O*-caffeoylquinic acid **(C)**, 3,4-*O*-dicaffeoylquinic acid **(D)**, luteolin **(E)**, luteolin-glucoside **(F)**, and feruloylquinic acid **(G)** in the leaves of *Eclipta prostrata* inoculated with arbuscular mycorrhiza or not inoculated under non-stress and moderate (100 mM NaCl) and high saline (200 mM NaCl) conditions 4 and 8 weeks after inoculation. Each bar shows the mean ± standard deviation (n = 3). Different regular and capital letters indicate significant differences among treatments 4 and 8 weeks after inoculation, respectively, according to the Tukey test ($P < 0.05$). +, ++, +++ Indicate significant differences between the same treatments 4 and 8 weeks after inoculation at $P < 0.05$, $P < 0.01$, and $P < 0.001$, respectively, according to the two-tailed test. ns, non-significant. *, **, ***, significant differences at $P < 0.05$, 0.01, 0.001. AMF, arbuscular mycorrhizal fungi. M, mycorrhizal inoculation effect. S, salt stress effect. M × S, the interaction between mycorrhizal inoculation and salt stress. UDL, under the detection limit.

4,5-dicaffeoylquinic acid, 3,5-dicaffeoylquinic acid, quercentin-3-arabinoside, 4-*O*-caffeoylquinic acid, 5-*O*-caffeoylquinic acid, and 3,4-*O*-dicaffeoylquinic acid in colonized plants as compared with the counterparts of non-stress mycorrhizal ones. Noticeably, under moderate salinity, the concentrations of wedelolactone, ferulic acid, and 4-*O*-caffeoylquinic acid were substantially higher in non-AM plants than in AM plants. Nevertheless, the fungal symbiont markedly enhanced the content of luteolin

(62.3%) in the host plants in relation to those of non-AM plants. Besides, demethyl wedelolactone was under the detection limit in colonized plants under such stress, but their feruloylquinic acid was detectable. When exposed to high salinity (200 mM NaCl), fungal colonization positively influenced the level of 3,5-dicaffeoylquinic acid (37-folds more than that of the corresponding uncolonized plants), 4,5-dicaffeoylquinic acid (17-folds), feruloylquinic acid (detectable versus undetectable), and luteolin-glucoside (detectable), but negatively affected the content of ferulic acid (decreased by 268% over the corresponding uncolonized plants) in colonized plants at 4 weeks.

After 8 weeks of growth, salinity led to a significant reduction in the content of total polyphenols and 11 phenolic compounds in non-AM plants, being more severe under high saline conditions. In 8-week mycorrhizal plants, moderate salinity also depressed the content of total polyphenols and eight phenolic substances, but the descending trend was alleviated in most bioactive compounds under high saline conditions. The level of few metabolites such as ferulic acid, 4-*O*-caffeoylquinic acid, and luteolin-glucoside was even profoundly enhanced by 93.7, 204, and 74%, respectively, in AM plants exposed to high salinity relative to non-stress AM plants. Noticeably, after 8 weeks of growth in the presence of 200 mM NaCl, the concentrations of all phenolic compounds were sharply inclined in mycorrhizal plants in relation to the counterparts in non-AM plants, except demethyl wedelolactone. The highest and lowest increases induced by AMF were 4-*O*-caffeoylquinic acid (more than 10-folds) and luteolin-glucoside (160%), respectively.

Interestingly, significant changes in the content of phenolic compounds in non-AM and AM plants were observed over time. Under non-stress conditions, there were substantial increases in the content of most polyphenols in AM (eight phenolics) and non-AM plants (10 phenolics) at 8 weeks versus their levels in the corresponding plants at 4 weeks. Considerable decreases in the content of seven individual phenolics were found in uncolonized plants treated with 100 mM NaCl 8 weeks after growth versus those 4 weeks after growth. By contrast, substantial inclines in the concentration of three polyphenols and a dramatic decrement in luteolin level (73%) were detected in colonized plants exposed to moderate salinity at 8 weeks relative to their counterparts at 4 weeks. A significant augmentation in the level of two polyphenols and remarkable declines in four phenolic concentrations were found in uncolonized plants subjected to 200 mM NaCl at 8 versus 4 weeks. Contrariwise, pronounced increases in the concentration of all phenolic compounds were recorded in colonized plants exposed to high salinity at the early stage of plant growth relative to those at the later stage.

Principal Component Analysis of Individual Polyphenols

Principal component analysis of individual polyphenols were performed, independently for each harvest time, to correlate variables determined under different conditions at 4 and 8 weeks. Four (at 4 weeks) and three (at 8 weeks) components showed eigenvalues higher than 1 (**Supplementary Tables S1, S2**). The results demonstrated that 55.6 and 80.8% of the total variation were explained by the first two principal components (PC1 and PC2) at 4 and 8 weeks, respectively (**Figure 9**). After 4 weeks of growth, 33.6% of the total variation was covered by the PC1, which had strong positive associations mainly with wedelolactone, 4,5-dicaffeoylquinic acid, quercetin-3-arabinoside, 4-*O*-caffeoylquinic acid, 3,4-*O*-dicaffeoylquinic acid, 3,5-*O*-dicaffeoylquinic acid, and luteolin-glucoside. PC2, covering 21.9%, was contributed primarily by caffeic acid (positive association) and feruloylquinic acid (negative association). In the next stage of plant growth (8 weeks), as much as 67.5% of the total variation was covered by the PC1, which was positively influenced by all phenolic compounds (13 individual polyphenols with luteolin and luteolin-glucoside having fewer impacts). PC2 explaining 13.3% of the total variance is positively influenced mainly by luteolin-glucoside and luteolin but negatively impacted principally by demethyl wedelolactone. At 4 weeks, high positive correlations between ferulic acid and 5-*O*-caffeoylquinic acid, demethyl wedelolactone and caffeic acid, 4-*O*-caffeoylquinic acid and 3,5-*O*-dicaffeoylquinic acid, 3,5-*O*-dicaffeoylquinic acid and luteolin-glucoside, luteolin-glucoside and 4,5-*O*-dicaffeoylquinic acid, quercetin-3-arabinoside and 3,4-*O*-dicaffeoylquinic acid, and luteolin-7-*O*-glucoside and feruloylquinic acid could be seen, whereas there were negative associations between feruloylquinic acid/luteolin-7-*O*-glucoside and ferulic acid/5-*O*-caffeoylquinic acid (**Figure 9A**). At 8 weeks, there were robust positive correlations between 4-*O*-caffeoylquinic acid and 4,5-*O*-dicaffeoylquinic acid, 5-*O*-caffeoylquinic acid and 3,5-*O*-dicaffeoylquinic acid, 3,5-*O*-dicaffeoylquinic acid and quercetin-3-arabinoside, and wedelolactone and luteolin-7-*O*-glucoside (**Figure 9B**). The biplot also demonstrated a relatively clear discrimination among the groups of the control treatment (C_0) and mycorrhizal treatment (A_0) under non-saline conditions, the control treatment under 200 mM NaCl (C_{200}), and the other group ($A_{100} + A_{200} + C_{100}$) at 4 weeks. Differences among C_{200}, C_0, and A_0 groups were distinguished by PC1, while PC2 discriminated between the salinity stresses and non-saline groups at 4 weeks. Four different clusters—C_0, A_{100} (AM inoculation under 100 mM NaCl), $A_0 + A_{200}$ (AM treatment under non-stress and high salt stress conditions), and $C_{100} + C_{200}$ (control treatment in the presence of 100 and 200 mM NaCl)—were recognized at 8 weeks. Obviously, AM inoculation under non-saline and high saline conditions influencing individual polyphenols was different from the other groups at the later stage of plant growth.

DISCUSSION

In our preliminary experiment, although *A. lacunose* treatment had considerably lower mycorrhizal colonization than others at 4 weeks, no significant differences in AM colonization among colonized plants in different AM treatments were recognized after 8 weeks of growth. In contrast to observations in artichoke, six AMF isolates exhibited different colonization dynamics at 7, 12, and 23 weeks after inoculation in two cultivars (Avio et al., 2020).

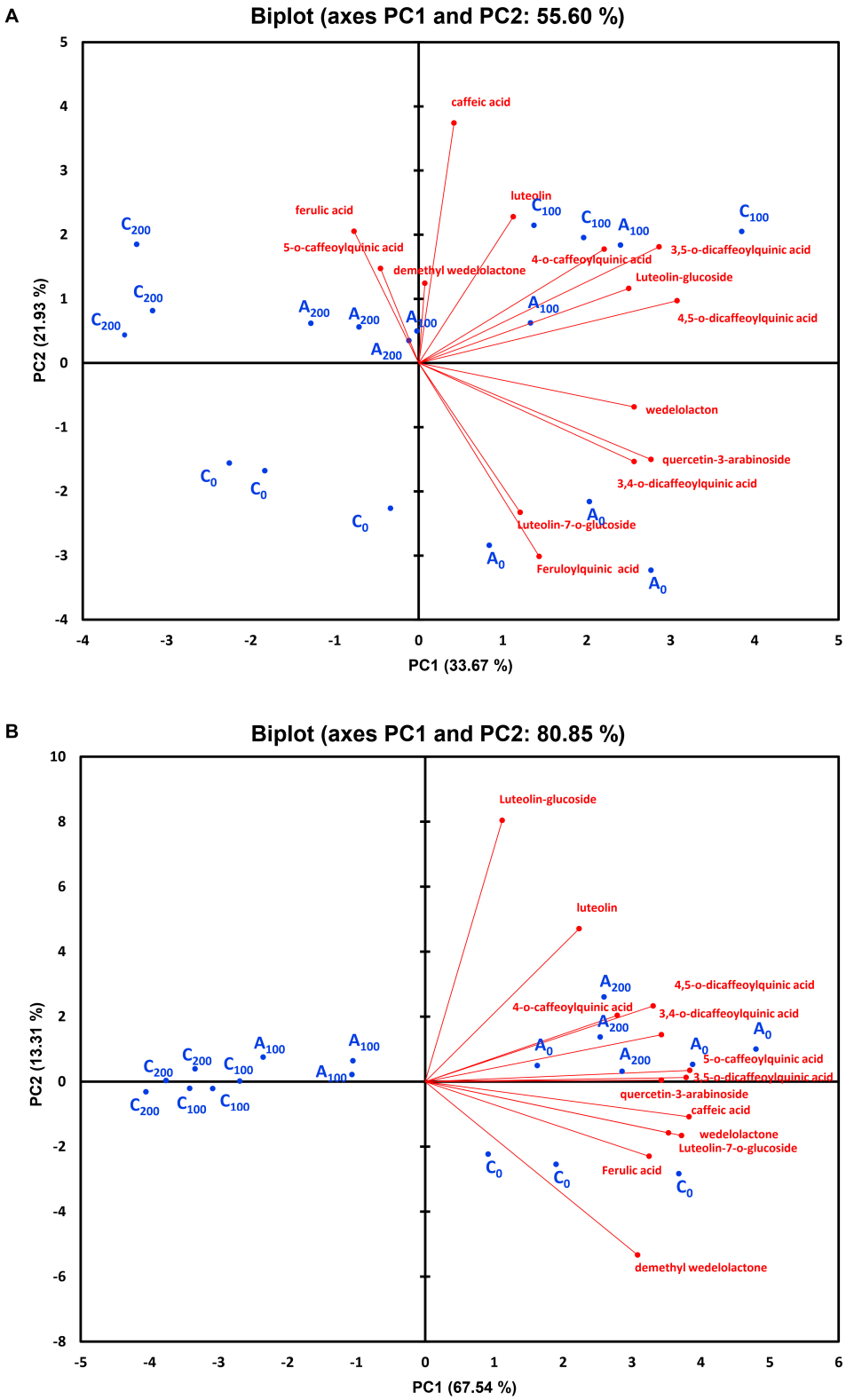

FIGURE 9 | Principal component analysis of individual polyphenols in the leaves of *Eclipta prostrata* inoculated with arbuscular mycorrhiza or not inoculated under non-stress, saline conditions 4 **(A)** and 8 weeks **(B)** after inoculation. C_0, control treatment (plants without mycorrhiza) under non-saline conditions; C_{100}, control treatment exposed to 100 mM NaCl; C_{200}, control treatment exposed to 200 mM NaCl; A_0, mycorrhizal treatment under non-saline conditions; A_{100}, mycorrhizal treatment exposed to 100 mM NaCl; A_{200}, mycorrhizal treatment exposed to 200 mM NaCl.

Indeed, different colonization of AMF species or isolates may reflect various AM colonization strategies, and/or AMF strains had a particular selectivity to their host plants (Duc and Posta, 2018). However, our different results might be the outcome of the specific combination of AM isolates and this medicinal plant. It is worth mentioning that root mycorrhizal colonization has been shown not necessarily to correlate with the host performances' promotion (Duc et al., 2018). Our findings showed that the effect of AMF species on growth characteristics of host plants was different (**Table 1**). *S. deserticola* remarkably lowered shoot weight and plant height in relation to non-AM plants, while plant height of *A. lacunose* treatment was considerably reduced as compared with that of uncolonized plants. These growth reductions of colonized plants may result from the unbalanced trade between the host plant and AMF, i.e., the AMF–plant interaction could be parasitism (Schmidt et al., 2011) due to the carbon drainage in the host inflicted by the fungi (Fitter, 2006). Interestingly, the application of the AMF mixture considerably increased shoot weight but not other growth parameters. This may highlight that the responsiveness of the host plant varies between different inocula. Besides growth characteristics, AM treatments did not elicit any substantial changes in proline and total phenolic content during plant growth, except lower total phenolic concentration in *S. deserticola* colonized plants 8 weeks after inoculation. Hence, the AM mixture (Symbivit) was chosen for mycorrhizal inoculation in our salt stress experiment.

Although AMF have been shown to improve plant performance under salinity in many plant species (Santander et al., 2019; Ait-El-Mokhtar et al., 2020; Amanifar and Toghranegar, 2020), responses of mycorrhizal *E. prostrata* plants to salinity, especially in terms of bioactive compounds, have not been addressed. In the present work, mycorrhizal colonization rate was markedly reduced after 4 weeks of plant growth due to the high saline level (200 mM NaCl), but not at the later stage of plant growth (**Figure 2**). The adverse impact of high salt stress on AM colonization capacity at the early stage of plant growth could be the consequence of the direct inhibitory effect of NaCl on extraradical hyphal growth, sporulation, and spore germination (Garg and Chandel, 2015), but subsequently, AMF may adapt to such salt level. Previous reports illustrated that salinity lessened mycorrhizal colonization though it was dependent on cultivars and AMF isolates (Santander et al., 2019; Wang et al., 2019). Plants exposed to salt stresses display inhibited growth mainly as a consequence of the deleterious effects of the high osmotic potential of salt-affected soils and ionic imbalances, which disrupt normal metabolism, water, and nutrient uptake (Santander et al., 2017). Plant biomass is the most prominent and direct attribute showing symbiosis-mediated plant performance under salt stress. AM inoculation has been reported to enhance the growth characteristics of several plants subjected to saline conditions, such as lettuce (Santander et al., 2019), date palm (*Phoenix dactylifera* L.) (Ait-El-Mokhtar et al., 2020), and the medicinal plant *V. officinalis* (L.) (Amanifar and Toghranegar, 2020). These findings support that the beneficial effects of AM application on the growth of host plants under salinity are consistent with our observation (increased fresh shoot weight, leaf number, and leaf area) under moderate salinity. Several mechanisms have been proposed to explain the higher host growth under salinity, such as improved nutrient uptake, net photosynthetic rate, stomatal conductance, relative water content, and osmoprotection; enhanced antioxidative enzymes; and maintenance of ionic homeostasis (Evelin et al., 2019). Furthermore, fungal symbiosis has the ability to retain Na^+ in the roots, probably in intraradical hyphae, which reduces its availability to the host (Giri et al., 2007; Rivero et al., 2018). A substantial decrement in Na^+ translocation from roots to shoots was found in colonized plants under salinity (Moreira et al., 2020), which may contribute to the higher plant growth. However, our results illustrated that the host growth promotion failed to be detected under high salt conditions (200 mM NaCl), suggesting that AM benefits depended on the severity of salt stress. Thus, our findings suggest that AM application could be applied to this plant production in agricultural areas with saline irrigation below 100 mM NaCl (EC = 10 dS m^{-1}) and/or slightly and moderately saline soils (EC of the saturation extract from 4 to 8 dS m^{-1}).

Our findings also revealed that proline production was stimulated in AM symbiosis under moderate salt conditions 8 weeks after inoculation, in accordance with the report of Santander et al. (2019). Contrariwise, Amanifar and Toghranegar (2020) illustrated that under salt stresses, a lower proline level was recorded in the leaves of the medicinal plant *V. officinalis* inoculated by either *R. irregularis* or *F. mosseae*, which may indicate mitigation of the stress (e.g., maintaining the ratio of K^+/Na^+) upstream of proline synthesis (Evelin et al., 2019). A high proline level in our results may be associated with less oxidative damage in moderate-salted mycorrhizal plants. In fact, proline serves as an osmoprotectant and effective ROS scavenger, stabilizing cellular structures and membranes (Meena et al., 2019), thus decreasing ROS damage as shown by the findings in this study. Besides, proline lowers K^+ efflux induced by Na^+, leading to the increment in K^+ concentration within plant cells (Hossain et al., 2014). As crucial parts of the photosynthetic apparatus, photosystems (PS) I and II are susceptible to saline conditions. Salinity can demolish the reaction center of PS II, disturb electron transport from PS II to PS I, and eventually result in a drop in photosynthesis (Wang et al., 2019). We found that the maximal photochemical efficiency of PS II (F_v/F_m) was not affected by salinity, indicating that salinity did not impair the photosynthetic system under our experimental conditions. Contrast observations were obtained in early investigations (Wang et al., 2019). The reasons may be attributed to differences in growth conditions, stress treatments, and stress duration, as well as specific interaction between fungal and plant partners, as reported in earlier studies (Duc et al., 2018; Amanifar and Toghranegar, 2020).

It is well known that plants can activate antioxidant systems where SOD, POD, and CAT are important enzymes to protect themselves against oxidative stress caused by ROS. SOD functions as the first defense line to deal with ROS, catalyzing the dismutation of superoxide radical (O_2^-) or singlet oxygen ($^1O_2$) to H_2O_2 and O_2 (Mittler, 2002). H_2O_2 is a potentially destructive subproduct of oxygen metabolism and is scavenged from cell compartments *via* CAT and peroxidases (Mittler, 2002). Under

moderate salt stress, the activity of POD and CAT at 4 weeks was highly induced in mycorrhizal plants, while mycorrhizal application did not change the activity of SOD at both times of measurement. This may suggest that at the first stage of plant growth, POD and CAT were two major antioxidative enzymes in mycorrhizal *E. prostrata* plants to alleviate oxidative stress caused by moderate saline conditions. The present results concur with those in cucumber plants (Hashem et al., 2018) and date palm plants (Ait-El-Mokhtar et al., 2020). By contrast, at the later stage, fungal symbiosis did not change the activity of these enzymes in response to salt stresses. This may imply that AM-induced defense enzymes under abiotic stresses varied with plant age (Mayer et al., 2019). It is most likely that mycorrhizal plants activated different antioxidative enzymes and/or non-enzymatic antioxidants to cope with stress 8 weeks after inoculation.

Phenolic substances, the most pronounced secondary metabolites present in plants, play a crucial role in the formation of various biomolecules protecting plants against stresses (Saxena et al., 2015). Increasing the phenolics content may contribute to osmoregulation, ROS protection, or the general defense systems of salt-stressed plants (Alqarawi et al., 2014). Boosted total phenolic level has been shown in salt-stressed *Ephedra aphylla* plants (Alqarawi et al., 2014) and *V. officinalis* plants (Amanifar and Toghranegar, 2020) due to mycorrhization. Our results indicated that total phenolic accumulation (measured by HPLC) was positively influenced by AM inoculation under non-stress (at 4 weeks) and saline conditions (at 8 weeks). Under moderate salt stress, total polyphenol content was heightened at 4 weeks but declined at 8 weeks in non-AM plants. Conversely, in moderate-salted mycorrhizal plants, total phenolics remained unchanged at the first stage of plant growth but was reduced at the later stage as compared with that of non-stress colonized plants. Still, the diminishment was alleviated in AM plants relative to non-AM plants in the presence of 100 mM NaCl at 8 weeks. This may reflect different strategies between non-AM and AM plants exposed to moderate salinity during the growth stages in the activation of phenolic production to diminish oxidative damage caused by ROS. Indeed, mycorrhizal plants effectively activated POD and CAT at 4 weeks and produced higher total phenolic content at 8 weeks than uncolonized plants to cope with oxidative stress. In the presence of 200 mM NaCl, mycorrhizal inoculation promoted higher total phenolic content in plants, particularly 8 weeks after inoculation; however, this response was not effective in detoxifying ROS in colonized plants due to lower biomass production in AM plants subjected to high salinity.

No data on the production of phenolic compounds in *E. prostrata* plants with or without AM inoculation under salinity stress have been reported. Polyphenols possess antioxidant properties, therefore not only contributing to plant defenses against oxidative stress but also promoting human health benefits for their antioxidant, anticarcinogenic, cardioprotective, antihypertensive, anti-inflammatory, anti-allergic, anti-arthritic, and antimicrobial activities (Lin et al., 2016). Natural antioxidants such as polyphenols have been intensively studied in the last few years because of restrictions on the use of synthetic antioxidants and enhanced public awareness of health-related issues (Bhuyan and Basu, 2017). In our previous study, nine major polyphenols were identified and measured in *E. prostrata* plants (Vo et al., 2019). Noticeably, in the current work, we extended identifications to 14 individual phenolics. The findings showed that wedelolactone, an important phenolic compound to prevent inflammatory diseases and cancer in humans (Sarveswaran et al., 2012), was one of the two main components of phenolic compounds in plants during growth stages under different conditions, which is in line with the earlier results (Vo et al., 2019). The difference in the second main constituent of polyphenols (4,5-dicaffeoylquinic acid in the present experiment versus demethyl wedelolactone in our previous one) may be attributable mainly to the different substrate volume we applied. The results demonstrated that under non-stress conditions, AM colonization considerably altered the content of six polyphenols in plants during growth stages, which is a confirmation with our previous findings showing that individual phenolics were influenced by AMF in this medicinal plant (Vo et al., 2019). The reasons may be due to the mechanisms underlying AMF–plant interaction during mycorrhization. AM colonization could induce a secondary metabolism response in the leaves and enhance abscisic acid biosynthesis and flavonoid and terpenoid biosynthesis regulated by jasmonate in the leaves (Adolfsson et al., 2017). Moreover, the changes in phenolic compound accumulation may be related to global metabolic alterations such as the majority of sugars, organic acids, amino acids, fatty acids, and phenolic acids in mycorrhizal shoots (Saleh et al., 2020). In our findings, higher aboveground biomass in colonized plants may result from an improved nutrient, water uptake, and photosynthesis of AM plants, leading to higher production of primary metabolites, which are the main precursors for the biosynthesis of phenolic compounds through the shikimic acid pathway (Lin et al., 2016). Therefore, changes in carbohydrate metabolism in colonized plants could alter the biosynthesis of phenolic substances (Pedone-Bonfim et al., 2018). Interestingly, the AMF-induced changes in polyphenol profiles at both stages of plant growth in this work and our previous one were not the same, probably owing to differences in plant age (4, 8, and 7 weeks) and substrate volume. We also observed that plant age markedly influenced the content of total and individual phenolic substances in AM and non-AM plants (8 versus 4 weeks). In fact, many biological factors, including developmental ones, contribute to the accumulation of secondary metabolites in plants (Broun et al., 2006). Developmental factors have an influence on the initiation and consequent differentiation of cellular structures related to secondary metabolites' biosynthesis and storage (Broun et al., 2006). Notably, developmental stages of the plant impact the expression pattern of biosynthetic genes of secondary metabolites (Sanchita and Sharma, 2018), which could explain the changes in the content of phenolic constituents during plant growth in this study.

Salt stress stimulates phenolic compound accumulation in plants as a defense mechanism to stress (Parvaiz and Satyawati, 2008). Therefore, this abiotic stress is one of the robust elicitors of secondary metabolite production of many herbs (Bistgani et al., 2019; Behdad et al., 2020; Boughalleb et al., 2020). In this study, salinity had the trend toward increasing and remaining phenolic

compounds unchanged in non-AM plants in the presence of 100 and 200 mM NaCl, respectively, at the first stage of growth but dropped them at the later stage. On the contrary, the decline in polyphenols caused by salt treatments was observed in mycorrhizal plants during the growth stage, with the mitigation at the later growth stage. Different behaviors in individual phenolics accumulation between non-AM and AM plants under saline conditions may result from the difference in the biochemical and physiological status in the host due to mycorrhization and AM benefits. On top of that, mycorrhizal inoculation caused changes in the content of many tested secondary metabolites of *E. prostrata* plants under both salinity levels at the early stage of plant growth. Noticeably, at the later growth stage, AMF enhanced all phenolic components in the host plants under high salt stress (200 mM NaCl). According to Rivero et al. (2018), in response to salt stress, various compounds with antistress properties differentially accumulated in mycorrhizal roots. The fungal symbiont also influenced the age-related changes in the leaf metabolome and partially halted senescence in the leaves, possibly resulting in better metabolite accumulation (Shtark et al., 2019). Taken altogether, these metabolic alterations induced by AMF may be the reason for the profound impact on polyphenol profiles of this medicinal plant under saline conditions during growth stages. Discrepant observations on phenolic compound accumulation in mycorrhizal plants subjected to salt stress were reported. Santander et al. (2019) pointed out considerably lower phenolics in the leaves of two lettuce cultivars colonized by AMF under salt stresses. Contrariwise, several studies have illustrated significant inclines in phenolic substances in AM plants (Hashem et al., 2018; Amanifar and Toghranegar, 2020). However, most previous studies only examined polyphenol profiles at one harvest time. Here, we observed both trends (increase and decrease) in phenolic compounds under salt stress conditions, depending on plant age and stress severity. It may be owing to the fact that the secondary metabolic pathways and their regulation are incredibly susceptible to environmental factors and growth stages since the expression of genes involved in their pathways or their encoded protein activities are changed at different plant ages and/or in the presence of various stresses (Sanchita and Sharma, 2018; Li et al., 2020).

CONCLUSION

In this work, mycorrhization and co-treatment of AMF and salinity elicited significant changes in the accumulation of phenolic compounds in the medicinal plant *E. prostrata*. The findings illustrated that the positive effect of AM inoculation on polyphenol profiles was dependent on stress severity and plant growth stage. Mycorrhizal inoculation under moderate salinity showed a higher plant tolerance during plant growth, but under high saline conditions, the higher accumulated content of phenolic compounds was achieved at the later plant growth stage. Hence, mycorrhizal application individually or in combination with salinity and harvest time would be a practical approach for optimizing individual polyphenol production in this medicinal plant.

This study shows how important the selection of the right date of harvest is for obtaining the optimal composition of phenolic compounds for particular purposes (such as pharmaceutical, cosmetic industries); moreover, AMF and moderate salt stress can be used to manipulate the pattern of individual polyphenol production. Further studies should investigate other bioactive compounds in this medicinal plant colonized by AMF and/or exposure to different abiotic stresses to optimize their production.

DATA AVAILABILITY STATEMENT

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

AUTHOR CONTRIBUTIONS

ND: propagation of single AMF species for inoculants, data analysis, data presentation, writing of the original draft, revision, and editing. AV: conceptualization, experimental design, mycorrhizal inoculation, plant care, stress treatments, collecting samples, measurement of AM colonization, growth parameters, chlorophyll fluorescence, proline and total phenolics, extraction of leaf samples for enzyme measurements, sample preparation for HPLC, data curation, and data analysis. IH: measurement of AM colonization, enzymes, proline, and phenolic compounds (HPLC), extraction of leaf samples for enzyme measurements, sample preparation for HPLC, and preparation of **Figure 6** (HPLC diagram). HD: measurement of phenolic compounds (HPLC), writing, revision, and editing. KP: experimental design, funding acquisition, project administration, resources, supervision, writing, revision, and editing. All authors contributed to the article and approved the submitted version.

FUNDING

This work was supported by the Higher Education Institutional Excellence Program awarded by the Ministry of Human Capacities (NKFIH-1159-6/2019) and by the 2017-1.3.1-VKE-2017-00001 project provided from the National Research, Development and Innovation Fund of Hungary.

ACKNOWLEDGMENTS

The authors would like to thank Dr. Nemenyi Andras, Institute of Horticulture, Szent István University, Godollo for technical assistance in chlorophyll fluorescence.

SUPPLEMENTARY MATERIAL

The Supplementary Material for this article can be found online at: https://www.frontiersin.org/articles/10.3389/fpls.2020.612299/full#supplementary-material

REFERENCES

Adolfsson, L., Nziengui, H., Abreu, I. N., Šimura, J., Beebo, A., Herdean, A., et al. (2017). Enhanced secondary- and hormone metabolism in leaves of arbuscular mycorrhizal *Medicago truncatula*. *Plant Physiol.* 175, 392–411. doi: 10.1104/pp.16.01509

Aebi, H. (1984). Catalase in vitro. *Meth. Enzymol*. 105, 121–126. doi: 10.1016/S0076-6879(84)05016-3

Ait-El-Mokhtar, M., Baslam, M., Ben-Laouane, R., Anli, M., Boutasknit, A., Mitsui, T., et al. (2020). Alleviation of detrimental effects of salt stress on date palm (*Phoenix dactylifera* L.) by the application of arbuscular mycorrhizal fungi and/or compost. *Front. Sustain. Food Syst*. 4:131. doi: 10.3389/fsufs.2020.00131

Alqarawi, A. A., Abd Allah, E., and Hashem, A. (2014). Alleviation of salt-induced adverse impact via mycorrhizal fungi in *Ephedra aphylla* Forssk. *J. Plant Interact*. 9, 802–810. doi: 10.1080/17429145.2014.949886

Amanifar, S., and Toghranegar, Z. (2020). The efficiency of arbuscular mycorrhiza for improving tolerance of *Valeriana officinalis* L. and enhancing valerenic acid accumulation under salinity stress. *Ind. Crops Prod*. 147:112234. doi: 10.1016/j.indcrop.2020.112234

Avio, L., Maggini, R., Ujvári, G., Incrocci, L., Giovannetti, M., and Turrini, A. (2020). Phenolics content and antioxidant activity in the leaves of two artichoke cultivars are differentially affected by six mycorrhizal symbionts. *Sci. Hortic*. 264:109153. doi: 10.1016/j.scienta.2019.109153

Bates, L. S., Waldren, R. P., and Teare, I. D. (1973). Rapid determination of free proline for water stress studies. *Plant Soil*. 39, 205–207. doi: 10.1007/BF00018060

Baum, C., El-Tohamy, W., and Gruda, N. (2015). Increasing the productivity and product quality of vegetable crops using arbuscular mycorrhizal fungi: a review. *Sci. Hortic*. 187, 131–141. doi: 10.1016/j.scienta.2015.03.002

Behdad, A., Mohsenzadeh, S., Azizib, M., and Moshtaghi, N. (2020). Salinity effects on physiological and phytochemical characteristics and gene expression of two *Glycyrrhiza glabra* L. populations. *Phytochemistry* 171:112236. doi: 10.1016/j.phytochem.2019.112236

Beyer, W. F., and Fridovich, I. (1987). Assaying for superoxide dismutase activity: some large consequences of minor changes in conditions. *Anal. Biochem*. 161, 559–566. doi: 10.1016/0003-2697(87)90489-1

Bhuyan, D. J., and Basu, A. (2017). "Phenolic compounds potential health benefits and toxicity," in *Utilisation of Bioactive Compounds from Agricultural and Food Production Waste*, ed. Q. V. Vuong (Boca Raton, FL: CRC Press), 27–59. doi: 10.1201/9781315151540

Bistgani, Z. E., Hashemib, M., DaCostab, M., Crakerb, L., Maggic, F., and Morshedloo, M. R. (2019). Effect of salinity stress on the physiological characteristics, phenolic compounds and antioxidant activity of *Thymus vulgaris* L. and *Thymus daenensis* Celak. *Ind. Crops Prod*. 135, 311–320. doi: 10.1016/j.indcrop.2019.04.055

Boughalleb, F., Abdellaoui, R., Mahmoudi, M., and Bakhshandeh, E. (2020). Changes in phenolic profile, soluble sugar, proline, and antioxidant enzyme activities of *Polygonum equisetiforme* in response to salinity. *Turk. J. Bot*. 44, 25–35. doi: 10.3906/bot-1908-2

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

Broun, P., Liu, Y., Queen, E., Schwarz, Y., Abenes, M. L., and Leibman, M. (2006). Importance of transcription factors in the regulation of plant secondary metabolism and their relevance to the control of terpenoid accumulation. *Phytochem. Rev*. 5, 27–38. doi: 10.1007/s11101-006-9000-x

Caretto, S., Linsalata, V., Colella, G., Mita, G., and Lattanzio, V. (2015). Carbon fluxes between primary metabolism and phenolic pathway in plant tissues under stress. *Int. J. Mol. Sci*. 16, 26378–26394. doi: 10.3390/ijms161125967

Chauhan, B. S., and Johnson, D. E. (2008). Influence of environmental factors on seed germination and seedling emergence of Eclipta (*Eclipta prostrata*) in a tropical environment. *Weed Sci.* 56, 383–388. doi: 10.1614/WS-07-154.1

Chung, I. M., Rajakumar, G., Lee, J. H., Kim, S. H., and Thiruvengadam, M. (2017). Ethnopharmacological uses, phytochemistry, biological activities, and biotechnological applications of *Eclipta prostrata*. *Appl. Microbiol. Biotechnol.* 101, 5247–5257. doi: 10.1007/s00253-017-8363-9

Chung, K. T., Wong, T. Y., Wei, C. I., Huang, Y. W., and Lin, Y. (1998). Tannins and human health: a review. *Crit. Rev. Food Sci. Nutr*. 38, 421–464. doi: 10.1080/10408699891274273

Duc, N. H., Csintalan, Z., and Posta, K. (2018). Arbuscular mycorrhizal fungi mitigate negative effects of combined drought and heat stress on tomato plants. *Plant Physiol. Biochem*. 132, 297–307. doi: 10.1016/j.plaphy.2018.09.011

Duc, N. H., and Posta, K. (2018). Mycorrhiza-induced alleviation of plant disease caused by *Clavibacter michiganensis* subsp. *michiganensis* in tomato and role of ethylene in mycorrhiza-induced resistance. *Acta Biol. Hung.* 69, 170–181. doi: 10.1556/018.69.2018.2.6

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, L., Zhai, Y. Y., Xu, J., Yao, W. F., Cao, Y. D., Cheng, F. F., et al. (2019). A review on traditional uses, phytochemistry and pharmacology of *Eclipta prostrata* (L.) L. *J Ethnopharmacol*. 245:112109. doi: 10.1016/j.jep.2019.112109

Fitter, A. H. (2006). What is the link between carbon and phosphorus fluxes in arbuscular mycorrhizas? A null hypothesis for symbiotic function. *New Phytol.* 172, 3–6. doi: 10.1111/j.1469-8137.2006.01861.x

Food and Agriculture Organization [FAO] (2015). *Status of the World's's Soil Resources (SWSR) – Main Report*. Rome: Food and Agriculture Organization.

Gani, A. M. S., and Devi, N. D. (2015). Antioxidant activity of methanolic extract of *Eclipta prostrata* L. *Int. J. Phytopharm.* 5, 21–24.

Garg, N., and Chandel, S. (2015). Role of arbuscular mycorrhiza in arresting reactive oxygen species (ROS) and strengthening antioxidant defense in *Cajanus cajan* (L.) Millsp. nodules under salinity (NaCl) and cadmium (Cd) stress. *Plant Growth Regu*. 75, 521–534. doi: 10.1007/s10725-014-0016-8

Giovanetti, M., and Mosse, B. (1980). An evaluation of techniques for measuring vesicular arbuscular mycorrhizal infection in roots. *New Phytol.* 84, 489–500. doi: 10.1111/j.1469-8137.1980.tb04556.x

Giri, B., Kapoor, R., and Mukerji, K. G. (2007). Improved tolerance of *Acacia nilotica* to salt stress by arbuscular mycorrhiza, *Glomus fasciculatum*, may be partly related to elevated K^+/Na^+ ratios in root and shoot tissues. *Microb. Ecol.* 54, 753–760. doi: 10.1007/s00248-007-9239-9

Glozer, K. (2008). *Protocol for Leaf Image Analysis-Surface Area*. Davis, CA: University of California.

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

Hossain, A. M., Hoque, M. A., Burritt, D. J., and Fujita, M. (2014). "Proline protects plants against abiotic oxidative stress: biochemical and molecular mechanisms," in *Oxidative Damage to Plants*, ed. P. Ahmad (San Diego, CA: Academic Press), 477–522. doi: 10.1016/B978-0-12-799963-0.00016-2

Isah, T. (2019). Stress and defense responses in plant secondary metabolites production. *Biol. Res*. 52:39. doi: 10.1186/s40659-019-0246-3

Jung, W. Y., Kim, H., Park, H. J., Jeon, S. J., Park, H. J., Choi, H. J., et al. (2016). The ethanolic extract of the *Eclipta prostrata* L. ameliorates the cognitive impairment in mice induced by scopolamine. *J Ethnopharmacol.* 190, 165–173. doi: 10.1016/j.jep.2016.06.010

Kapoor, R., Anand, G., Gupta, P., and Mandal, S. (2017). Insight into the mechanisms of enhanced production of valuable terpenoids by arbuscular mycorrhiza. *Phytochem. Rev*. 16, 677–692. doi: 10.1007/s11101-016-9486-9

Lee, E. H., Eo, J. K., Ka, K. H., and Eom, A. H. (2013). Diversity of arbuscular mycorrhizal fungi and their roles in ecosystems. *Mycobiology* 41, 121–125. doi: 10.5941/MYCO.2013.41.3.121

Li, Y., Kong, D., Fu, Y., Sussmand, M. R., and Wu, H. (2020). The effect of developmental and environmental factors on secondary metabolites in medicinal plants. *Plant Physiol. Biochem*. 148, 80–89. doi: 10.1016/j.plaphy.2020.01.006

Lin, D., Xiao, M., Zhao, J., Li, Z., Xing, B., Li, X., et al. (2016). An overview of plant phenolic compounds and their importance in human nutrition and management of type 2 diabetes. *Molecules* 21:1374. doi: 10.3390/molecules21101374

Lister, E., and Wilson, P. (2001). *Measurement of Total Phenolics and ABTS Assay for Antioxidant Activity. Personal Communication*. Lincoln: Crop Research Institute.

Liu, Q. M., Zhao, H. Y., Zhong, X. K., and Jiang, J. G. (2012). *Eclipta prostrata* L. phytochemicals: isolation, structure elucidation, and their antitumor activity. *Food Chem. Toxicol.* 50, 4016–4022. doi: 10.1016/j.fct.2012.08.007

Mayer, Z., Juhász, A., Vo, T. A., and Posta, K. (2019). Impact of arbuscular mycorrhizal fungi on some defense enzyme activities at early stage of maize (*Zea mays* L.) under different abiotic stresses. *Appl. Ecol. Environ. Res.* 17, 6241–6253. doi: 10.15666/aeer/1703_62416253

Meena, M., Divyanshu, K., Kumar, S., Swapnil, P., Zehra, A., Shukla, V., et al. (2019). Regulation of L-proline biosynthesis, signal transduction, transport, accumulation and its vital role in plants during variable environmental conditions. *Heliyon* 5:e02952. doi: 10.1016/j.heliyon.2019.e02952

Merken, H. M., and Beecher, G. R. (2000). Measurement of food flavonoids by high-performance liquid chromatography: a review. *J. Agr. Food Chem.* 48, 577–599. doi: 10.1021/jf990872o

Mittler, R. (2002). Oxidative stress, antioxidants and stress tolerance. *Trends Plant Sci.* 7, 405–410. doi: 10.1016/S1360-1385(02)02312-9

Moreira, H., Pereira, S. I. A., Vega, A., Castro, P. M. L., and Marques, A. P. G. C. (2020). Synergistic effects of arbuscular mycorrhizal fungi and plant growth-promoting bacteria benefit maize growth under increasing soil salinity. *J. Environ. Manage.* 257:109982. doi: 10.1016/j.jenvman.2019.109982

Oxborough, K., and Baker, N. R. (1997). Resolving chlorophyll a fluorescence images of photosynthetic efficiency into photochemical and non-photochemical components – calculation of qP and Fv'/Fm' without measuring Fo'. *Photosynth. Res.* 54, 135–142. doi: 10.1023/A:1005936823310

Parvaiz, A., and Satyawati, S. (2008). Salt stress and phyto-biochemical responses of plants—a review. *Plant Soil Environ.* 54:89. doi: 10.17221/2774-PSE

Pedone-Bonfim, M. V. L., da Silva, D. K. A., da Silva-Batista, A. R., de Oliveira, A. P., Almeida, J. R. G., Yano-Melo, A. M., et al. (2018). Mycorrhizal inoculation as an alternative for the sustainable production of *Mimosa tenuiflora* seedlings with improved growth and secondary compounds content. *Fungal Biol.* 122, 918–927. doi: 10.1016/j.funbio.2018.05.009

Porter, W. M. (1979). The 'Most Probable Number' method for enumerating infective propagules of vesicular arbuscular mycorrhizal fungi in soil. *Aust. J. Soil Res.* 17, 515–519. doi: 10.1071/SR9790515

Rathmell, W. G., and Sequeira, L. (1974). Soluble peroxidase in fluid from the intercellular spaces of tobacco leaves. *Plant Physiol.* 53, 317–318. doi: 10.1104/pp.53.2.317

Rivero, J., Alvarez, D., Flors, V., Azcón-Aguilar, C., and Pozo, M. J. (2018). Root metabolic plasticity underlies functional diversity in mycorrhiza-enhanced stress tolerance in tomato. *New Phytol.* 220, 1322–1336. doi: 10.1111/nph.15295

Saleh, A. M., Abdel-Mawgoud, M., Hassan, A. R., Habeeb, T. H., Yehia, R. S., and AbdElgawad, H. (2020). Global metabolic changes induced by arbuscular mycorrhizal fungi in oregano plants grown under ambient and elevated levels of atmospheric CO_2. *Plant Physiol. Biochem.* 151, 255–263. doi: 10.1016/j.plaphy.2020.03.026

Sanchita, and Sharma, A. (2018). "Gene expression analysis in medicinal plants under abiotic stress conditions," in *Plant Metabolites and Regulation Under Environmental Stress*, eds P. Ahmad, M. A. Ahanger, V. P. Singh, D. K. Tripathi, P. Alam, and M. N. Alyemeni (Cambridge, MA: Academic Press), 407–414. doi: 10.1016/B978-0-12-812689-9.00023-6

Santander, C., Aroca, R., Ruiz-Lozano, J. M., Olave, J., Cartes, P., Borie, F., et al. (2017). Arbuscular mycorrhiza effects on plant performance under osmotic stress. *Mycorrhiza* 27, 639–657. doi: 10.1007/s00572-017-0784-x

Santander, C., Ruiz, A., García, S., Aroca, R., Cumming, J., and Cornejoa, P. (2019). Efficiency of two arbuscular mycorrhizal fungal inocula to improve saline stress tolerance in lettuce plants by changes of antioxidant defense mechanisms. *J. Sci. Food Agric.* 100, 1577–1587. doi: 10.1002/jsfa.10166

Sarveswaran, S., Gautam, S. C., and Ghosh, J. (2012). Wedelolactone, a medicinal plant-derived coumestan, induces caspase-dependent apoptosis in prostate cancer cells via downregulation of PKCϵ without inhibiting Akt. *Int. J. Oncol.* 41, 2191–2199. doi: 10.3892/ijo.2012.1664

Saxena, A., Raghuwanshi, R., and Singh, H. B. (2015). *Trichoderma* species mediated differential tolerance against biotic stress of phytopathogens in *Cicer arietinum* L. *J. Basic Microbiol.* 55, 195–206. doi: 10.1002/jobm.201400317

Schmidt, B., Gaşpar, S., Camen, D., Ciobanu, I., and Sumălan, R. (2011). Arbuscular mycorrhizal fungi in terms of symbiosis-parasitism continuum. *Commun. Agric. Appl. Biol. Sci.* 76, 653–659.

Seró, R., Núñez, N., Núñez, O., Camprubí, A., Grases, J. M., Saurina, J., et al. (2019). Modified distribution in the polyphenolic profile of rosemary leaves induced by plant inoculation with an arbuscular mycorrhizal fungus. *J. Sci. Food Agric.* 99, 2966–2973. doi: 10.1002/jsfa.9510

Shtark, O. Y., Puzanskiy, R. K., Avdeeva, G. S., Yurkov, A. P., Smolikova, G. N., Yemelyanov, V. V., et al. (2019). Metabolic alterations in pea leaves during arbuscular mycorrhiza development. *PeerJ.* 7:e7495. doi: 10.7717/peerj.7495

Soobrattee, M. A., Neergheen, V. S., Luximon-Ramma, A., Aruoma, O. I., and Bahorun, T. (2005). Phenolics as potential antioxidant therapeutic agents: mechanism and actions. *Mutat. Res. Fund. Mol. M.* 579, 200–213. doi: 10.1016/j.mrfmmm.2005.03.023

Toscano, S., Trivellini, A., Cocetta, G., Bulgari, R., Francini, A., Romano, D., et al. (2019). Effect of preharvest abiotic stresses on the accumulation of bioactive compounds in horticultural produce. *Front. Plant Sci.* 10:1212. doi: 10.3389/fpls.2019.01212

Trouvelot, A., Kough, J., and Gianinazzi-Pearson, V. (1986). "Evaluation of VA infection levels in root systems," in *Physiological and Genetical Aspects of Mycorrhizae*, ed. V. Gianinazzi-Pearson (Paris, France: INRA Press), 217–221.

Vo, A. T., Haddidi, I., Daood, H., Mayer, Z., and Posta, K. (2019). Impact of arbuscular mycorrhizal inoculation and growth substrate on biomass and content of polyphenols in *Eclipta prostrata*. *Hort. Sci.* 54, 1976–1983. doi: 10.21273/HORTSCI14227-19

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

Yu, S. J., Yu, J. H., Yu, Z. P., Yan, X., Zhang, J. S., Sun, J. Y., et al. (2020). Bioactive terpenoid constituents from *Eclipta prostrata*. *Phytochemistry* 170:112192. doi: 10.1016/j.phytochem.2019.112192

Zelm, E. V., 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

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

frontiers in Plant Science

PERSPECTIVE
published: 26 January 2021
doi: 10.3389/fpls.2020.627345

Understanding Multilevel Selection May Facilitate Management of Arbuscular Mycorrhizae in Sustainable Agroecosystems

Nancy Collins Johnson[1,2]* *and Kara Skye Gibson*[2]

[1]School of Earth & Sustainability, Northern Arizona University, Flagstaff, AZ, United States, [2]Department of Biological Sciences, Northern Arizona University, Flagstaff, AZ, United States

Studies in natural ecosystems show that adaptation of arbuscular mycorrhizal (AM) fungi and other microbial plant symbionts to local environmental conditions can help ameliorate stress and optimize plant fitness. This local adaptation arises from the process of multilevel selection, which is the simultaneous selection of a hierarchy of groups. Studies of multilevel selection in natural ecosystems may inform the creation of sustainable agroecosystems through developing strategies to effectively manage crop microbiomes including AM symbioses. Field experiments show that the species composition of AM fungal communities varies across environmental gradients, and that the biomass of AM fungi and their benefits for plants generally diminish when fertilization and irrigation eliminate nutrient and water limitations. Furthermore, pathogen protection by mycorrhizas is only important in environments prone to plant damage due to pathogens. Consequently, certain agricultural practices may inadvertently select for less beneficial root symbioses because the conventional agricultural practices of fertilization, irrigation, and use of pesticides can make these symbioses superfluous for optimizing crop performance. The purpose of this paper is to examine how multilevel selection influences the flow of matter, energy, and genetic information through mycorrhizal microbiomes in natural and agricultural ecosystems, and propose testable hypotheses about how mycorrhizae may be actively managed to increase agricultural sustainability.

Keywords: complex adaptive systems, emergent properties, local adaptation, mycorrhizal phenotype, arbuscular mycorrhizae, high-input agriculture

OPEN ACCESS

Edited by:
Sergio Saia,
University of Pisa, Italy

Reviewed by:
Guido Lingua,
University of Eastern Piedmont, Italy
David Paul Janos,
University of Miami, United States
Thomas Kuyper,
Wageningen University and Research, Netherlands

***Correspondence:**
Nancy Collins Johnson
nancy.johnson@nau.edu

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

Received: *09 November 2020*
Accepted: *30 December 2020*
Published: *26 January 2021*

Citation:
Johnson NC and Gibson KS (2021) Understanding Multilevel Selection May Facilitate Management of Arbuscular Mycorrhizae in Sustainable Agroecosystems. Front. Plant Sci. 11:627345. doi: 10.3389/fpls.2020.627345

INTRODUCTION

Although the term "mycorrhiza" is often equated with a root inhabiting fungus, technically, a mycorrhiza is not a fungus, but rather the *symbiosis* between a fungus and a plant root (Frank, 1885; Trappe, 2005). Acknowledging this fact immediately expands our perspective of mycorrhizae to include not only fungi, but also their complex interactions with plant hosts. The purpose of this essay is to expand this perspective even further and envision mycorrhizae as complex adaptive systems in which matter, energy and information move through a hierarchy of interconnected components (**Figure 1**). Asymmetrical trading partnerships between plant

hosts and arbuscular mycorrhizal (AM) fungi drive mycorrhizal systems: most plants can survive and – depending on the environment – possibly thrive in the absence of the symbiosis, while AM fungi are obligate symbionts and require a living plant host for survival. Most wild plants rely on mycorrhizae for normal nutrition, drought tolerance, and pathogen protection (Smith and Read, 2008). Although nearly all crops form AM symbioses, their value in production agriculture is debated (Ryan and Graham, 2018; Rillig et al., 2019). Envisioning mycorrhizae as constantly evolving symbiotic systems helps explain the reasons for this debate. This essay explores the hypothesis that local adaptation of mycorrhizal systems arises through multilevel selection, and that current agricultural practices uncouple critical feedbacks so that the mutualistic properties of mycorrhizas may diminish over time. An evolutionary framework can guide the design of experiments that test strategies to recouple feedbacks among plants, AM fungi and their associated microbiome so that the benefits of mycorrhizae can be harnessed in the development of sustainable agroecosystems.

MYCORRHIZAL PHENOTYPE IS AN EMERGENT PROPERTY OF MYCORRHIZAL SYSTEMS

Phenotypes of mycorrhizal symbioses arise from the interaction between the genotypes of plant and fungal symbionts and the environment, and range along a continuum of mutualism to parasitism (**Figure 1**; Johnson et al., 1997). Evolution of organisms engaged in symbiotic associations is often driven by tension between cooperative traits that benefit both partners and selfish traits that benefit individuals (Bahar, 2018), and mycorrhizae are no exception. Key cooperative traits for plants involve their support of fungi in return for resources and services, while selfish plant traits involve the ability to control and manipulate AM fungi for their own benefit with minimal investment of photosynthate. In turn, cooperative traits of AM fungi involve their potential to deliver resources to host plants, and selfish traits involve their ability to overcome plant control in order to gain photosynthate without providing nutrients or beneficial services (Hammer et al., 2011; Kiers et al., 2011; Whiteside et al., 2019). In naturally evolving ecosystems these traits interact to generate a dynamic process of reciprocal adaptation between plants and fungi that is mediated by environmental heterogeneity and variation in plant and fungal traits. Natural communities are often composed of plant species that vary in the degree to which they benefit from AM symbioses (Wilson and Hartnett, 1998). There appear to be no AM fungi that are consistently beneficial to all plant hosts (Klironomos, 2003), and with the exception of mycoheterotrophic plants, there is little evidence for codependency or specificity between particular species of plants and AM fungi (Kokkoris et al., 2020a).

The scope of AM systems is much greater than individual plant-fungal partnerships, and even greater than communities of plants and AM fungi. The location of mycorrhizal phenotypes

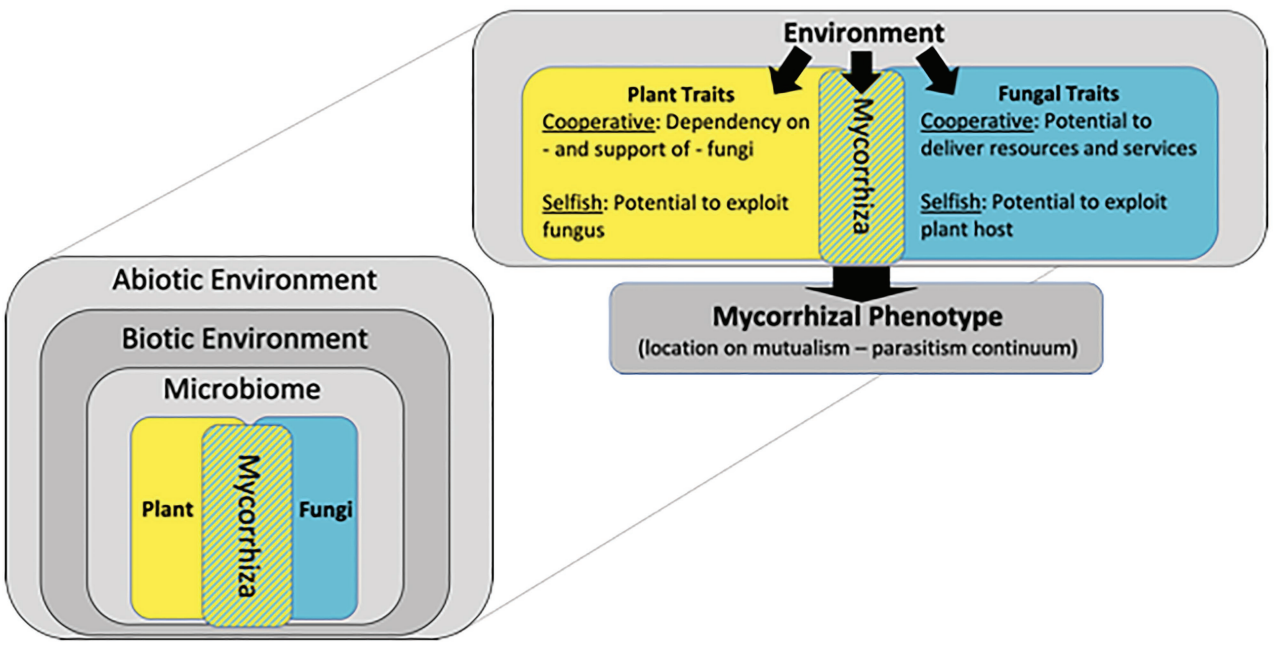

FIGURE 1 | Mycorrhizae are symbiotic associations between plant roots and fungi, and their phenotype is determined by interactions among plant and fungal genotypes and the environment. A hierarchy of environmental factors determines mycorrhizal phenotype including abiotic conditions such as climate and soil properties and biotic factors such as communities of plant competitors, animal herbivores, and microbial antagonists and mutualists. Genotypes of plant and fungal partners can be characterized by cooperative traits that strengthen the mutualism and selfish traits that weaken the mutualism. Figure modified from Johnson et al. (1997).

on the mutualism-parasitism continuum is an emergent property of interactions among a hierarchy of biotic and abiotic factors (**Figure 1**; Johnson et al., 1997). Like most organisms, plants and AM fungi host diverse microbial communities that can influence their fitness (Vandenkoornhuyse et al., 2015; Bakker et al., 2018). Currently, little is known about mycorrhizal microbiomes (Revillini et al., 2016), but there is solid evidence that undefined biotic interactions can influence plant-soil feedbacks and mediate mycorrhizal function (Hoeksema et al., 2010; De Long et al., 2019). The balance of trade between host plants and their symbiotic fungi is highly dependent on abiotic conditions and resource availability (Johnson, 2010). Temperature, precipitation, light availability, and soil chemistry can structure the environment to make AM symbioses more or less beneficial to plants. For example, symbioses between the same genotypes of plants and AM fungi have been shown to function as mutualism, commensalism, or parasitism depending on phosphorus and light availability (Johnson et al., 2015). Whether or not AM fungi form mutualistic symbioses with crop hosts depends on the biotic and abiotic conditions of the agricultural environment.

NATURAL SELECTION, COMMUNITY ASSEMBLY, AND AM FUNGAL INOCULUM

Multilevel selection is the simultaneous selection of a hierarchy of groups ranging from nucleotypes within an individual AM fungal clone to whole communities of plants and microbes within an ecosystem. Evolution through natural selection occurs when heritable variation among individuals makes some phenotypes reproduce more successfully than others (Darwin, 1859). This process can be scaled up to account for evolution that results in the differential extinction and proliferation of communities (Whitham et al., 2020). Theoretical and empirical studies over the past 4 decades expand Darwinian evolution to explain how, in addition to phenotypes of individual organisms, groups of organisms can be units of selection (Bahar, 2018; Wilson, 2019; Whitham et al., 2020). This expanded paradigm of evolution is necessary for understanding local adaptation of plants as holobionts that include all organisms within their microbiome (Vandenkoornhuyse et al., 2015). It also accommodates the unusual nuclear dynamics of AM fungi in which individual clones contain thousands of independently dividing nuclei that represent genetically independent units (Kokkoris et al., 2020b). Multilevel selection blurs the distinction between genetic selection and community assembly. Multilevel selection encompasses a continuum of interactions, ranging from processes that structure the genetic composition of communities of nuclei within individual AM fungal clones to processes that structure the community composition of interacting organisms. There is evidence that mycorrhizae play a role in the adaptation of plants to their local environment in natural ecosystems (Johnson et al., 2010; Rúa et al., 2016; Remke et al., 2020), and that certain aspects of agricultural management disrupt local adaptation and may inadvertently select AM fungal communities with less mutualistic properties (Johnson et al., 1992; Verbruggen and Kiers, 2010; Verbruggen et al., 2013). Recognition of the role of multilevel selection in generating mutualistic AM phenotypes will facilitate harnessing mycorrhizae in sustainable agriculture.

The resilience and resistance of AM fungi to disturbances can be remarkable (Johnson and Wedin, 1997; Lekberg et al., 2012), and it is important to recognize that agriculture does not eliminate AM symbioses, but rather, it changes the composition of AM fungal communities to be dominated by r-selected, disturbance resistant taxa (Verbruggen and Kiers, 2010; Moora et al., 2014; Banerjee et al., 2019). Commercially produced AM fungal inoculum is generally composed of a low diversity of easily propagated r-selected AM fungi that may or may not be beneficial for nutrient uptake, drought tolerance, and pathogen protection compared to diverse communities of indigenous fungi (Berruti et al., 2016). Also, the addition of exotic AM fungal inoculum to pre-established AM fungal communities may lead to increased competition among fungi and reduced host plant productivity (Janoušková et al., 2013). These concerns have led some to suggest that the expense and risks associated with widespread application of exotic AM fungal inoculum may outweigh its potential benefits (Hart et al., 2018). Furthermore, the existence of robust communities of indigenous AM fungi in most agricultural fields implies that adding commercially produced inoculum may be like sprinkling expensive salt in the ocean.

FLOW OF MATTER, ENERGY, AND INFORMATION THROUGH MYCORRHIZAS

Comparing mycorrhizae in natural and agricultural ecosystems provides useful insights. Exchange of energy and matter in the form of plant photosynthate in return for fungal access to limiting soil resources is at the core of all AM symbioses. The balance of trade between plant and fungal symbionts is very different in natural and agricultural ecosystems. In drought prone, nutrient limited soils, plant hosts and AM fungi exchange valuable commodities with their symbiotic partners, but crops in highly fertilized and irrigated agricultural systems have little to gain from mycorrhizae, and if AM fungi do not provide other services such as pathogen resistance, then mycorrhizae may depress crop yield (Modjo et al., 1987; Raya-Hernández et al., 2020).

Compared to crop monocultures, natural ecosystems have diverse plant and AM fungal communities and soil food-webs that create more complex trophic interactions, which maintain and cycle matter and energy within the system (**Figure 2**; Pérez-Jaramillo et al., 2016; Pärtel et al., 2017; Mariotte et al., 2018). Networks of AM hyphae have been shown to help conserve water and soil nutrients (Jia et al., 2020), and in undisturbed systems these networks remain intact year after year, throughout the seasons, in contrast to agroecosystems where regular tillage severs hyphal networks and long periods of fallow without cover-crops reduce

AM fungal populations (Bowles et al., 2017). Furthermore, irrigation and fertilization may accelerate decomposition, mineralization, leaching, and volatilization processes, which when coupled with biomass removal through harvest, tend to produce open and leaky nutrient cycles in agricultural systems compared to closed and conservative nutrient cycles in natural ecosystems (Mariotte et al., 2018).

Natural and artificial selection influence the flow of genetic information through ecosystems over time. In natural ecosystems, gene frequencies and community assembly respond to local biotic and abiotic selection pressures, and the fitness of plants and fungi are reciprocally influenced by the performance of locally adapted AM symbioses (**Figure 2**). This reciprocity does not occur in high-input agriculture

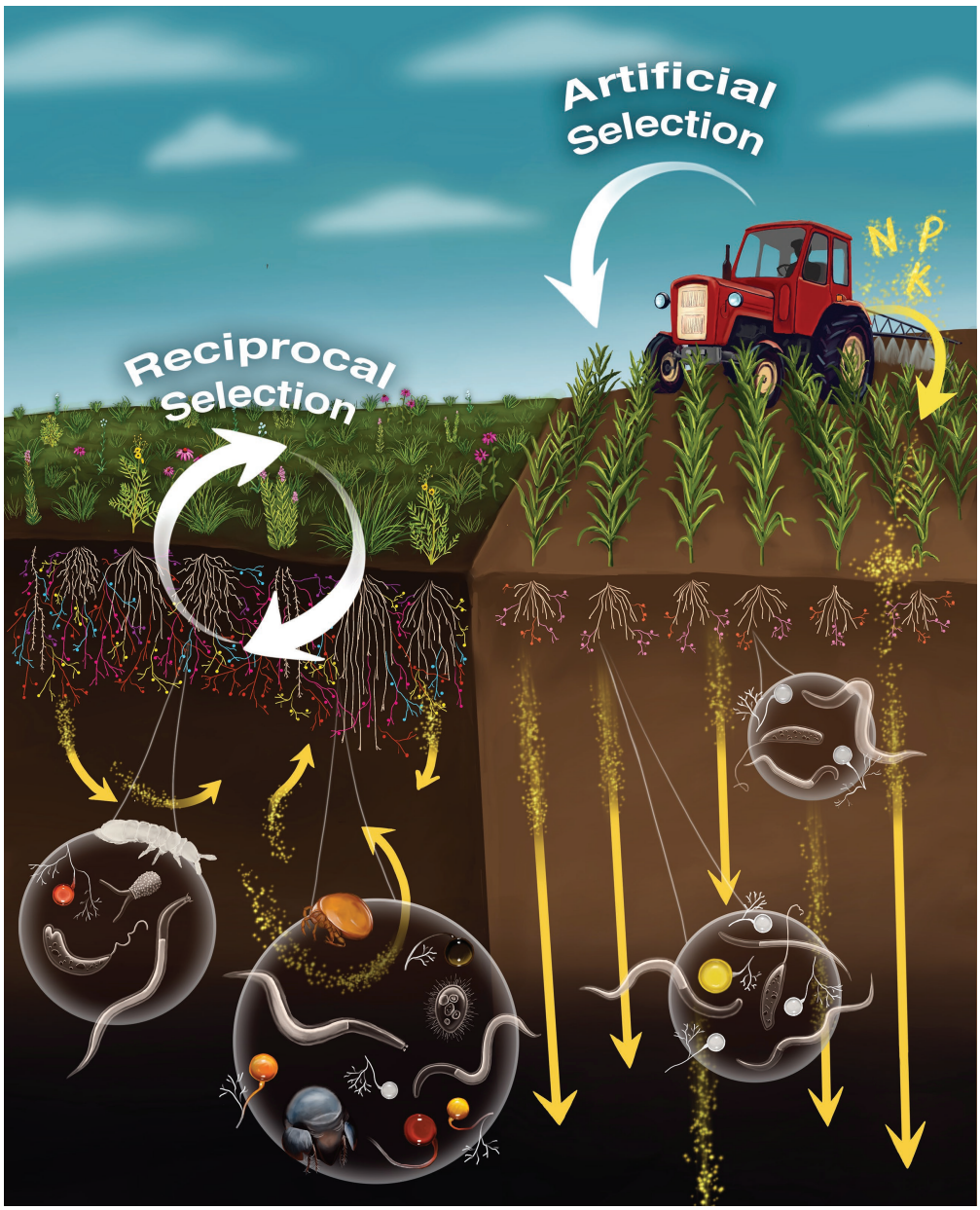

FIGURE 2 | The flow of matter and energy (yellow arrows) and genetic information (white arrows) through mycorrhizae differs in natural (left) and high-input agricultural (right) ecosystems. Abundance and diversity of arbuscular mycorrhizal (AM) fungi tend to be greater in natural compared to high-input agricultural ecosystems because plants allocate more photosynthate to mycorrhizae when they are nutrient and/or water limited. Communities of soil organisms are generally more diverse in natural ecosystems. Intact networks of AM fungal hyphae and complex foodwebs help conserve nutrients in natural ecosystems, and tillage and fallow periods reduce these networks in agroecosystems so that more nutrients are lost through leaching (yellow arrows). Evolutionary feedback occurs in natural ecosystems because genotypes of plants and AM fungi are selected through reciprocal adaptation to each other and to the environment. This evolutionary feedback is not possible if farmers select plant genotypes without consideration of the indigenous AM fungi and associated microbiome. Illustration by Kara Skye Gibson.

because artificial selection interrupts the feedback of information between plant and AM fungal communities. Genetic feedback is uncoupled because the farmer selects crop cultivars based on economic criteria, and the performance of AM symbioses is generally not considered. The exception to this is when traditional farmers maintain on-going selection of crops to ever changing local conditions (biotic and abiotic) by choosing the best performing phenotypes each year for the next year's seed stock. Their choice integrates plant-soil feedback in the selection process and generates locally adapted crops and AM symbioses (Martinez and Johnson, 2010).

REALIZATION OF MULTILEVEL SELECTION IN AGRICULTURE

Mycorrhizae in natural ecosystems are complex adaptive systems with feedback between communities of plants and soil organisms, but high-input agricultural management has inadvertently removed these feedbacks. Mycorrhizae in high-input agriculture are not complex adaptive systems because: (1) fertilization, irrigation, and pesticides remove resource limitation and stress, (2) AM fungi are not selected by plants to enhance cooperative traits like nutrient uptake or pathogen resistance, and instead, AM fungi are selected for selfish traits that enhance their own survival in an environment that does not favor mycotrophy, and (3) crop traits are completely controlled by the farmer's choice of seed, not through evolutionary feedbacks from the environment and soil biota.

A better understanding of how multilevel selection controls mycorrhizal functioning in natural ecosystems may provide insights into ways to effectively harness mycorrhizal benefits in modern agroecosystems. The sustainable agriculture movement aims to enhance environmental quality, maintain soil fertility, reduce erosion, and sustain the economic viability of farm operations (USDA, 2007). Managing for mycorrhizae may help achieve these goals. Management practices that maximize mycorrhizal benefits include planting cover crops and minimizing use of tillage and fertilizers (Bowles et al., 2017; Banerjee et al., 2019). Locally adapted communities of AM fungi and other soil microbes may help protect crops from site-specific stresses (Johnson et al., 2010; Remke et al., 2020); and maintaining intact networks of AM fungal mycelium may increase soil organic matter and improve nutrient retention, soil stability, and drought tolerance (Zhu and Miller, 2003; Gosling et al., 2006; Jia et al., 2020).

Darwinian Agriculture in the 21st Century

The traditional narrow focus on individuals as the only unit of selection (Williams, 1966) has been shaken by the discovery that individual organisms are not individuals, but really complex communities of bacteria, archaea, viruses, fungi, and other organisms. Furthermore, horizontal gene transfer allows adaptive genes to be readily exchanged among unrelated organisms. The asexual fungi that form AM symbioses carry variable nuclei in their hyphae and spores such that nucleotypes within the same fungal clone may be differentially selected by fine-scale heterogeneity in environmental conditions (Rosendahl, 2008; Kokkoris et al., 2020b). So, what is the unit of selection? Does selection occur at a highly localized scale with certain genes increasing in abundance in the specific locations where they are the most adaptive? Do organisms actively select teams of nucleotypes of microorganisms to help them adapt to stresses in their local environment? Darwin developed the theory of evolution through natural selection without a knowledge of genetics or microbiomes. Julian Huxley merged genetics with Darwin's theory in his 1942 book *Evolution: The Modern Synthesis*. It is time to update the modern synthesis to include microbiomes.

How Can Agricultural Management Maximize Beneficial Mycorrhizae?

Mechanistic insights informing the development of sustainable agricultural systems may be gained through systematic testing of hypotheses related to the mycorrhizal phenotype model (**Figure 1**). The focus should be on generating conditions that favor cooperative traits and minimize selfish traits in both plant and fungal partners. In addition to judicious management of fertilizers, crop breeders should identify particular physiological and morphological traits that influence the degree to which crops utilize AM symbioses. This would allow farmers to select crop cultivars that vary in their level of mycotrophy to account for the nutrient and water availability in their fields. When soil resources are in ample supply, it may be best to grow cultivars that minimize AM colonization, but when nutrients and water are limited, farmers should select cultivars that leverage symbioses with indigenous AM fungi that are best adapted to the local stresses. Prior research suggests the following list of hypothesized principles for *in situ* management of mycorrhizas in agroecosystems:

1. To reduce fertilizer inputs, develop mycotrophic crop varieties that substitute symbiotic uptake of nutrients for fertilizer supplements.
2. Maintain soil P at levels that encourage selection of mutualistic mycorrhizae which optimize trading of minerals for photosynthate but do not limit crop yield.
3. Provide sufficient N to prevent N-competition between plants and AM fungi.
4. Maintain soil water availability so that mycorrhizal enhancement of drought tolerance can be manifested, but not so low that AM fungi are chronically water limited.
5. Reduce tillage to maintain the fine-scale spatial structure necessary for intact hyphal networks to evolve over time through multilevel selection.
6. Maintain host plant continuity through planting mycotrophic cover crops or perennial crop varieties.
7. Add spatial or temporal diversity through crop rotation, intercropping or other polyculture practices.

Field-based studies in many different environments are necessary to sufficiently test whether or not these hypothesized

principles maximize mycorrhizal mutualism and to develop strategies to enhance their value for crop production and other ecosystem services.

Managing biological soil fertility in general and mycorrhizal symbioses in particular is environmentally sustainable, but it may not be economically sustainable under current market conditions (Gowdy and Baveye, 2019). Many conventional agricultural practices such as application of inorganic fertilizers, irrigation, and selection of non-mycotrophic cultivars make the management of mycorrhizal symbioses superfluous for optimizing crop performance (Ryan and Graham, 2018). These high-input agricultural practices are perpetuated by economic forces that do not account for externalities such as greenhouse gas emissions, groundwater contamination, and topsoil erosion and degradation. Managing mycorrhizae may only make economic sense if all of the environmental costs of agriculture are monetized and accounted for in farmers' profits. National and international policies are necessary to combat climate change and protect common pool resources such as topsoil, ground water, and mineral P stocks. Awareness of the potential benefits of locally adapted mycorrhizal symbioses for their many ecosystem services is an important step toward the design and implementation of truly sustainable agriculture.

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

NJ drafted the initial version of the manuscript. KG contributed critical ideas and created **Figure 2**. All authors contributed to the article and approved the submitted version.

ACKNOWLEDGMENTS

Thanks to Karin Hage-Ahmed and the students in the May, 2018 class on *Mycorrhizas in Natural and Agroecosystems* at the University of Natural Resources and Life Sciences (BOKU), Vienna, Austria for their inspiring conversations and for rekindling my interest in this important topic. Thanks to a Bullard Fellowship from Harvard Forest and the US Department of Energy program in *Systems Biology Research to Advance Sustainable Bioenergy Crop Development* (DE-FOA-0002214) for financial support.

REFERENCES

Bahar, S. (2018). *The essential tension: Competition, cooperation and multilevel selection in evolution. 1st Edn*. Netherlands: Springer, 377.

Bakker, P. A. H. M., Pieterse, C. M. J., de Jonge, R., and Berendsen, R. L. (2018). The soil-borne legacy. *Cell* 172, 1178–1180. doi: 10.1016/j.cell.2018.02.024

Banerjee, S., Walder, F., Büchi, L., Meyer, M., Held, A. Y., Gattinger, A., et al. (2019). Agricultural intensification reduces microbial network complexity and the abundance of keystone taxa in roots. *ISME J.* 13, 1722–1736. doi: 10.1038/s41396-019-0383-2

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

Bowles, T. M., Jackson, L. E., Loeher, M., and Cavagnaro, T. R. (2017). Ecological intensification and arbuscular mycorrhizas: a meta-analysis of tillage and cover crop effects. *J. Appl. Ecol.* 54, 1785–1793. doi: 10.1111/1365-2664.12815

Darwin, C. (1859). *The origin of species: By means of natural selection, or the preservation of favoured races in the struggle for life. 1st Edn*. New York: D. Appleton and Company.

De Long, J. R., Fry, E. L., Veen, G. F., and Kardol, P. (2019). Why are plant–soil feedbacks so unpredictable, and what to do about it? *Funct. Ecol.* 33, 118–128. doi: 10.1111/1365-2435.13232

Frank, A. B. (1885). Ueber die auf Wurzelsymbiose beruhende Ernährung gewisser Bäume durch unterirdische Pilze. *Ber Dtsch Bot Ges* 3, 128–145 [English translation (2005), *Mycorrhiza* 15, 267-275]. doi: 10.1007/s00572-004-0329-y

Gosling, P., Hodge, A., Goodlass, G., and Bending, G. D. (2006). Arbuscular mycorrhizal fungi and organic farming. *Agric. Ecosyst. Environ.* 113, 17–35. doi: 10.1016/j.agee.2005.09.009

Gowdy, J., and Baveye, P. (2019). "An evolutionary perspective on industrial and sustainable agriculture" in *Agroecosystem diversity: Reconciling contemporary agriculture and environmental quality*. ed. G. Lemaire (Amsterdam: Elsevier), 433–435.

Hammer, E. C., Pallon, J., Wallander, H., and Olsson, P. A. (2011). Tit for tat? A mycorrhizal fungus accumulates phosphorus under low plant carbon availability. *FEMS Microbiol. Ecol.* 76, 236–244. doi: 10.1111/j.1574-6941.2011.01043.x

Hart, M. M., Antunes, P. M., Chaudhary, V. B., and Abbott, L. K. (2018). Fungal inoculants in the field: is the reward greater than the risk? *Funct. Ecol.* 32, 126–135. doi: 10.1111/1365-2435.12976

Hoeksema, J. D., Chaudhary, V. B., Gehring, C. A., Johnson, N. C., Karst, J., Koide, R. T., et al. (2010). A meta-analysis of context-dependency in plant response to inoculation with mycorrhizal fungi. *Ecol. Lett.* 13, 394–407. doi: 10.1111/j.1461-0248.2009.01430.x

Janoušková, M., Krak, K., Wagg, C., Štorchová, H., Caklová, P., and Vosátka, M. (2013). Effects of inoculum additions in the presence of a preestablished arbuscular mycorrhizal fungal community. *Appl. Environ. Microbiol.* 79, 6507–6515. doi: 10.1128/AEM.02135-13

Jia, Y., van der Heijden, M. G. A., Wagg, C., Feng, G., and Walder, F. (2020). Symbiotic soil fungi enhance resistance and resilience of an experimental grassland to drought and nitrogen deposition. *J. Ecol.* doi: 10.1111/1365-2745.13521

Johnson, N. C. (2010). Resource stoichiometry elucidates the structure and function of arbuscular mycorrhizas across scales. *New Phytol.* 185, 631–647. doi: 10.1111/j.1469-8137.2009.03110.x

Johnson, N. C., Copeland, P. J., Crookston, R. K., and Pfleger, F. L. (1992). Mycorrhizae: possible explanation for yield decline with continuous corn and soybean. *Agron. J.* 84, 387–390. doi: 10.2134/agronj1992.00021962008400030007x

Johnson, N. C., Graham, J. H., and Smith, F. A. (1997). Functioning of mycorrhizal associations along the mutualism-parasitism continuum. *New Phytol.* 135, 575–585. doi: 10.1046/j.1469-8137.1997.00729.x

Johnson, N. C., and Wedin, D. A. (1997). Soil carbon, nutrients, and mycorrhizae during conversion of dry tropical forest to grassland. *Ecol. Appl.* 7, 171–182. doi: 10.1890/1051-0761(1997)007[0171:SCNAMD]2.0.CO;2

Johnson, N. C., Wilson, G. W. T., Bowker, M. A., Wilson, J. A., and Miller, R. M. (2010). Resource limitation is a driver of local adaptation in mycorrhizal symbioses. *Proc. Natl. Acad. Sci. U. S. A.* 107, 2093–2098. doi: 10.1073/pnas.0906710107

Johnson, N. C., Wilson, G. W. T., Wilson, J. A., Miller, R. M., and Bowker, M. A. (2015). Mycorrhizal phenotypes and the law of the minimum. *New Phytol.* 205, 1473–1484. doi: 10.1111/nph.13172

Kiers, E. T., Duhamel, M., Beesetty, Y., Mensah, J. A., Franken, O., Verbruggen, E., et al. (2011). Reciprocal rewards stabilize cooperation in the mycorrhizal symbiosis. *Science* 333, 880–882. doi: 10.1126/science.1208473

Klironomos, J. N. (2003). Variation in plant response to native and exotic arbuscular mycorrhizal fungi. *Ecology* 84, 2292–2301. doi: 10.1890/02-0413

Kokkoris, V., Lekberg, Y., Antunes, P. M., Fahey, C., Fordyce, J. A., Kivlin, S. N., et al. (2020a). Codependency between plant and arbuscular mycorrhizal fungal communities: what is the evidence? *New Phytol.* 228, 828–838. doi: 10.1111/nph.16676

Kokkoris, V., Stefani, F., Dalpé, Y., Dettman, J., and Corradi, N. (2020b). Nuclear dynamics in the arbuscular mycorrhizal fungi. *Trends Plant Sci.* 25, 765–778. doi: 10.1016/j.tplants.2020.05.002

Lekberg, Y., Schnoor, T., Kjøller, R., Gibbons, S. M., Hansen, L. H., Al-Soud, W. A., et al. (2012). 454-sequencing reveals stochastic local reassembly and high disturbance tolerance within arbuscular mycorrhizal fungal communities. *J. Ecol.* 100, 151–160. doi: 10.1111/j.1365-2745.2011.01894.x

Mariotte, P., Mehrabi, Z., Bezemer, T. M., De Deyn, G. B., Kulmatiski, A., Drigo, B., et al. (2018). Plant–soil feedback: bridging natural and agricultural sciences. *Trends Ecol. Evol.* 33, 129–142. doi: 10.1016/j.tree.2017.11.005

Martinez, T. N., and Johnson, N. C. (2010). Agricultural management influences propagule densities and functioning of arbuscular mycorrhizas in low- and high-input agroecosystems in arid environments. *Appl. Soil Ecol.* 46, 300–306. doi: 10.1016/j.apsoil.2010.07.001

Modjo, H. S., Hendrix, J. W., and Nesmith, W. C. (1987). Mycorrhizal fungi in relation to control of tobacco stunt disease with soil fumigants. *Soil Biol. Biochem.* 19, 289–295. doi: 10.1016/0038-0717(87)90011-3

Moora, M., Davison, J., Öpik, M., Metsis, M., Saks, Ü., Jairus, T., et al. (2014). Anthropogenic land use shapes the composition and phylogenetic structure of soil arbuscular mycorrhizal fungal communities. *FEMS Microbiol. Ecol.* 90, 609–621. doi: 10.1111/1574-6941.12420

Pärtel, M., Öpik, M., Moora, M., Tedersoo, L., Szava-Kovats, R., Rosendahl, S., et al. (2017). Historical biome distribution and recent human distrubance shape the diversity of arbuscular mycorrhizal fungi. *New Phytol.* 216, 227–238. doi: 10.1111/nph.14695

Pérez-Jaramillo, J. E., Mendes, R., and Raaijmakers, J. M. (2016). Impact of plant domestication on rhizosphere microbiome assembly and functions. *Plant Mol. Biol.* 90, 635–644. doi: 10.1007/s11103-015-0337-7

Raya-Hernández, A. I., Jaramillo-López, P. F., López-Carmona, D. A., Díaz, T., Carrera-Valtierra, J. A., and Larsen, J. (2020). Field evidence for maize-mycorrhiza interactions in agroecosystems with low and high P soils under mineral and organic fertilization. *Appl. Soil Ecol.* 149:103511. doi: 10.1016/j.apsoil.2020.103511

Remke, M., Johnson, N. C., Wright, J., Williamson, M., and Bowker, M. (2020). Sympatric pairings of dryland grass populations, mycorrhizal fungi, and associated soil biota enhane mutualism and ameliorate drought stress. *J. Ecol.* doi: 10.1111/1365-2745.13546

Revillini, D., Gehring, C. A., and Johnson, N. C. (2016). The role of locally adapted mycorrhizas and rhizobacteria in plant–soil feedback systems. *Funct. Ecol.* 30, 1086–1098. doi: 10.1111/1365-2435.12668

Rillig, M. C., Aguilar-Trigueros, C. A., Camenzind, T., Cavagnaro, T. R., Degrune, F., Hohmann, P., et al. (2019). Why farmers should manage the arbuscular mycorrhizal symbiosis. *New Phytol.* 222, 1171–1175. doi: 10.1111/nph.15602

Rosendahl, S. (2008). Communities, populations and individuals of arbuscular mycorrhizal fungi. *New Phytol.* 178, 253–266. doi: 10.1111/j.1469-8137.2008.02378.x

Rúa, M. A., Antoninka, A., Antunes, P. M., Chaudhary, V. B., Gehring, C., Lamit, L. J., et al. (2016). Home-field advantage? Evidence of local adaptation among plants, soil, and arbuscular mycorrhizal fungi through meta-analysis. *BMC Evol. Biol.* 16:122. doi: 10.1186/s12862-016-0698-9

Ryan, M. H., and Graham, J. H. (2018). Little evidence that farmers should consider abundance or diversity of arbuscular mycorrhizal fungi when managing crops. *New Phytol.* 220, 1092–1107. doi: 10.1111/nph.15308

Smith, S. E., and Read, D. J. (2008). *Mycorrhizal symbiosis. 3rd Edn*. Cambridge, Massachusetts: Academic Press.

Trappe, J. M. (2005). A.B. Frank and mycorrhizae: the challenge to evolutionary and ecologic theory. *Mycorrhiza* 15, 277–281. doi: 10.1007/s00572-004-0330-5

USDA (2007). National Agriculture Library. Special Reference Briefs Series no. SRB 99-02 Update SRB 94-05 Sustainable Agriculture: Definitions and Terms. Available at: https://www.nal.usda.gov/afsic/sustainable-agriculture-definitions-and-terms (Accessed December 12, 2020).

Vandenkoornhuyse, P., Quaiser, A., Duhamel, M., Le Van, A., and Dufresne, A. (2015). The importance of the microbiome of the plant holobiont. *New Phytol.* 206, 1196–1206. doi: 10.1111/nph.13312

Verbruggen, E., and Kiers, E. T. (2010). Evolutionary ecology of mycorrhizal functional diversity in agricultural systems. *Evol. Appl.* 3, 547–560. doi: 10.1111/j.1752-4571.2010.00145.x

Verbruggen, E., van der Heijden, M. G. A., Rillig, M. C., and Kiers, E. T. (2013). Mycorrhizal fungal establishment in agricultural soils: factors determining inoculation success. *New Phytol.* 197, 1104–1109. doi: 10.1111/j.1469-8137.2012.04348.x

Whiteside, M. D., Werner, G. D. A., Caldas, V. E. A., van't Padje, A., Dupin, S. E., Elbers, B., et al. (2019). Mycorrhizal fungi respond to resource inequality by moving phosphorus from rich to poor patches across networks. *Curr. Biol.* 29, 2043.e8–2050.e8. doi: 10.1016/j.cub.2019.04.061

Whitham, T. G., Allan, G. J., Cooper, H. F., and Shuster, S. M. (2020). Intraspecific genetic variation and species interactions contribute to community evolution. *Annu. Rev. Ecol. Evol. Syst.* 51, 587–612. doi: 10.1146/annurev-ecolsys-011720-123655

Williams, G. C. (1966). *Adaptation and natural selection*. Princeton, New Jersey: Princeton University Press.

Wilson, D. S. (2019). *This view of life: Completing the Darwinian revolution. 1st Edn*. New York: Pantheon Books, 288.

Wilson, G. W. T., and Hartnett, D. C. (1998). Interspecific variation in plant responses to mycorrhizal colonization in tallgrass prairie. *Am. J. Bot.* 85, 1732–1738. doi: 10.2307/2446507

Zhu, Y. G., and Miller, R. M. (2003). Carbon cycling by arbuscular mycorrhizal fungi in soil-plant systems. *Trends Plant Sci.* 8, 407–409. doi: 10.1016/S1360-1385(03)00184-5

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

frontiers in Plant Science

PERSPECTIVE
published: 01 February 2021
doi: 10.3389/fpls.2021.626709

Potential Effects of Microplastic on Arbuscular Mycorrhizal Fungi

Eva F. Leifheit[1,2], Anika Lehmann[1,2] and Matthias C. Rillig[1,2]*

[1]*Institut für Biologie, Freie Universität Berlin, Berlin, Germany,* [2]*Berlin-Brandenburg Institute of Advanced Biodiversity Research, Berlin, Germany*

Microplastics (MPs) are ubiquitously found in terrestrial ecosystems and are increasingly recognized as a factor of global change (GCF). Current research shows that MP can alter plant growth, soil inherent properties, and the composition and activity of microbial communities. However, knowledge about how microplastic affects arbuscular mycorrhizal fungi (AMF) is scarce. For plants it has been shown that microplastic can both increase and decrease the aboveground biomass and reduce the root diameter, which could indirectly cause a change in AMF abundance and activity. One of the main direct effects of microplastic is the reduction of the soil bulk density, which translates to an altered soil pore structure and water transport. Moreover, especially fibers can have considerable impacts on soil structure, namely the size distribution and stability of soil aggregates. Therefore, microplastic alters a number of soil parameters that determine habitat space and conditions for AMF. We expect that this will influence functions mediated by AMF, such as soil aggregation, water and nutrient transport. We discuss how the impacts of microplastic on AMF could alter how plants deal with other GCFs in the context of sustainable food production. The co-occurrence of several GCFs, e.g., elevated temperature, drought, pesticides, and microplastic could modify the impact of microplastic on AMF. Furthermore, the ubiquitous presence of microplastic also relates to earth system processes, e.g., net primary production (NPP), carbon and nitrogen cycling, which involve AMF as key soil organisms. For future research, we outline which experiments should be prioritized.

Keywords: microplastic, global change, earth system processes, pollution, arbuscular mycorrhizal fungi

OPEN ACCESS

Edited by:
Jan Jansa,
Academy of Sciences of the Czech Republic (ASCR), Czechia

Reviewed by:
Mohamed Hijri,
Université de Montréal, Canada
Qiang-Sheng Wu,
Yangtze University, China
Sidney Sturmer,
Regional University of Blumenau, Brazil

****Correspondence:***
Eva F. Leifheit
e.leifheit@fu-berlin.de

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

Received: *06 November 2020*
Accepted: *05 January 2021*
Published: *01 February 2021*

Citation:
Leifheit EF, Lehmann A and Rillig MC (2021) Potential Effects of Microplastic on Arbuscular Mycorrhizal Fungi. Front. Plant Sci. 12:626709. doi: 10.3389/fpls.2021.626709

INTRODUCTION

Microplastics (MPs) are ubiquitously found around the globe and are increasingly recognized as a factor of global change (GCF; Rillig and Lehmann, 2020). While MP research has focused on marine and freshwater ecosystems for a long time, recently attention has shifted to terrestrial ecosystems. MP is expected to enter soil ecosystems predominantly in agricultural fields through fertilization with sewage sludge and compost (Nizzetto et al., 2016; van den Berg et al., 2020). But MP emissions also reach soils *via* atmospheric deposition, runoff or aerial transport from

nearby roads, where particles and fragments are generated through tire and road wear abrasion or from sports grounds with artificial substrates, through the addition of polymer coated fertilizer, littering, flooding events, or irrigation with wastewater (Piehl et al., 2018; Bergmann et al., 2019; Baensch-Baltruschat et al., 2020; Brahney et al., 2020). However, direct analysis of the concentration of MP in the soil is problematic due to analytical difficulties. MP is quickly incorporated into the soil matrix where it "disappears," and thus cannot be easily distinguished from the soil organic matter without dedicated extraction and analytical protocols.

Arbuscular mycorrhizal fungi (AMF) are a key member of terrestrial ecosystems: by entangling soil aggregates with their hyphae, they improve soil structure and potentially stabilize carbon in soil aggregates (Rillig and Mummey, 2006; Verbruggen et al., 2016). They ramify throughout the soil to access nutrients, which they supply to their symbiotic host partner (Parniske, 2008). In exchange for these nutrients, plants provide carbohydrates and fatty acids to the fungus inside root cells where the fungus forms arbuscules for this exchange (Parniske, 2008; Keymer et al., 2017). Arbuscules or other structures such as hyphae and vesicles inside the roots are intraradical measures for activity and abundance, whereas soil mycelial length, spore numbers, and phosphatase activity are used as extraradical abundance and activity measures, which are complemented by molecular techniques such as qPCR (Thonar et al., 2012). As ubiquitous members of the soil microbial community, AMF face a variety of adverse conditions and likely multiple co-occurring GCFs, including novel pollution agents such as microplastic.

Most plastic types are persistent in the environment and are expected to accumulate in the soil, which likely leads to continuously increasing concentrations of MPs in soils (Rillig, 2012). Current research shows that microplastic can alter plant performance, soil properties, and the composition and activity of microbial communities (e.g., Machado et al., 2018; Boots et al., 2019). Some of the soil biota, e.g., nematodes and Rotifera, can be very sensitive to MP and show alterations of the gut microbiome, reproduction rate, motility and life span, show stress reactions, and malfunctioning metabolism in response to different types of MP (Büks et al., 2020). Effects on AMF can equally be expected, but specific knowledge on MP impacts on AMF is scarce. We expect a number of direct effects of MP on AMF, such as the toxicity of some plastic components (additives), as well as indirect effects of MP on AMF *via* altered plant performance and soil properties. We elaborate these effects in the following sections, followed by a discussion on potential interactive effects of MP with other GCFs and feedbacks from earth system processes and we finish with an outline of future research priorities.

HYPOTHESIZED DIRECT EFFECTS OF MICROPLASTIC ON AMF

Microplastic can have direct harmful effects on soil biota as they contain a variety of additives that can be toxic (Kim et al., 2020). The authors of this study demonstrated that the acute toxicity of the MP on nematodes disappeared when the additives were extracted before exposure. Additionally, there is an increasing body of literature showing that organic pollutants (e.g., polycyclic aromatic hydrocarbons or organochlorines such as DDT), polychlorinated biphenyls, antibiotics, herbicides, pesticides, and trace metals can absorb to plastic surfaces (Wang et al., 2019). Especially small MP particles or high concentrations can induce stress reactions, alter metabolic processes, reproduction, and mortality (Büks et al., 2020). MP can directly alter the composition of the bacterial community in soil and for aquatic systems this has been shown for fungal communities (Kettner et al., 2017; Fei et al., 2020). AMF, like other soil biota, can be negatively affected by pollutants, e.g., heavy metals or hydrocarbons (Cabello, 1997; Joner and Leyval, 2003; Wang et al., 2020). We thus expect direct effects of the MP additives or the pollutants absorbed on the MP surface, which will eventually be released upon degradation. Although AMF exhibit a certain tolerance to heavy metals and hydrocarbons, and can even assist in reducing their toxicity to plants, they are negatively affected at high concentrations (Cabello, 1997; Ferrol et al., 2016). Typical reactions of AMF to soil pollutants include reduced root colonization and infectivity, reduced arbuscule and spore numbers or cell damage (Cabello, 1997; Leyval et al., 1997; Desalme et al., 2012; Ferrol et al., 2016). Pharmaceuticals like antibiotics can exhibit mycotoxicity for AMF as well, reducing hyphal length and spore numbers (Hillis et al., 2008). Additionally, changes in AMF community structure can occur with soil pollution: e.g., in soils with high lead contamination the abundance of *Acaulosporaceae* and *Glomeraceae* decreased, while the relative abundance of *Paraglomeraceae* increased (Faggioli et al., 2019). The binding of (toxic) pollutants to persistent MP would lead to their accumulation in the soil and over time, the pollution load for AMF will be further augmented, considering the projected increases in use of plastic, antibiotics, and other human activities that release pollutants (Tilman et al., 2002; Lebreton and Andrady, 2019; Roberts and Zembower, 2020). At the moment, it is difficult to estimate a realistic time scale for MP degradation and release of components, because there is not enough information on degradation processes in the soil and the diversity of plastic types and sizes is too large.

Microplastics can furthermore directly influence AMF *via* breakdown products. Especially biodegradable plastics produce dodecanal, which can be accumulated in the rhizosphere and is known to negatively affect plant and fungal growth (Qi et al., 2020). One recent study showed a strong change in AMF community composition and diversity under MP pollution (Wang et al., 2020). The authors found that the relative abundance of AMF taxa depended on type and concentration of MP: e.g., *Glomeraceae* were reduced in treatments with biodegradable polylactic acid (PLA) compared to the control and treatments with polyethylene (PE); OTU numbers of *Ambispora* and *Archaeosporaceae* increased at higher application rates of MP (10% addition compared to 1% addition) under PLA and PE for *Ambispora* and only under PLA for *Archaeosporaceae*.

AMF diversity was found to be highest under 10% PLA addition and, overall, the biodegradable PLA had a larger influence on AMF diversity compared to PE.

HYPOTHESIZED INDIRECT EFFECTS OF MICROPLASTIC ON PLANT HOST, EXTRARADICAL SOIL ENVIRONMENT AND MICROBIAL COMMUNITIES

The largest influence of MPs on soil is the change in bulk density, which often has positive consequences for plant growth as this reduces the root penetration resistance, improves the water holding capacity (WHC; see Machado et al., 2019) and is often accompanied by a better aeration (Niu et al., 2012). These conditions favor nutrient and water supply to plants, leading to increased root and shoot biomass, but also to altered root traits (Materechera et al., 1992; Machado et al., 2019; Rillig et al., 2019b). This has been demonstrated in recent studies, where MP reduced the root diameter or increased the fine root length (Machado et al., 2019; Lehmann et al., 2020a). However, from a mere change in diameter or length of fine roots, conclusions on AMF reactions cannot be drawn as there are important functional differences within fine roots: First to third order fine roots are the absorptive part of the root system, where most of the AMF structures are usually found, whereas higher order fine roots are more active in transport and less likely to host AMF (McCormack et al., 2015). Additionally, thin diameter fine roots have a higher absorptive surface area compared to thicker fine roots and can show lower colonization rates by AMF because they do not need to rely on a mycorrhizal partner for nutrient uptake (Eissenstat et al., 2015). Fine root traits differ substantially between plant types, e.g., the average of absorptive fine roots is 33% for woody plants and 81% for herbaceous plants (McCormack et al., 2015). Although general predictions of the effect of altered root traits on AMF colonization is difficult with the current literature, it is fair to assume that altered root traits will likely lead to changes in intraradical and extraradical fungal traits, such as root colonization, hyphal extension, abundance, and branching behavior (Rillig et al., 2015; Cockerton et al., 2020). Two studies have found an increase in root colonization by 8 and 22%, respectively, with polyester fiber addition, supporting this postulation (Machado et al., 2019; Lehmann et al., 2020a). However, other plastic types (PA, PEHD, PET, PP, and PS) have not induced an increase in colonization (Machado et al., 2019). Therefore, the effect predominantly depends on the MP parameters (concentration, type, shape, and additives), but also on the specific host-symbiont-relationship: A mycorrhizal symbiosis with a highly dependent plant host might be more strongly influenced by MP-induced alterations compared to a symbiosis in which the plant host is not as dependent on AMF; mycorrhizal dependent plants might profit more from increased AMF activity, i.e., receive more nutrients or water, supporting plant growth. Additionally, there will certainly be differences in the reactions to MP between single AMF species, i.e., some species might be more susceptible to adverse soil conditions (see section "Hypothesized direct effects of microplastic on AMF").

In some cases, MP has reduced plant growth (Qi et al., 2018; Wang et al., 2020), which could limit the C allocation to AMF, and thus reduce AMF abundance and activity, including the supply of nutrients and water to the plant.

Microplastic additions to soil clearly influence soil structure: Laboratory studies found positive and negative effects, but reductions of aggregate stability and aggregate size have been observed more frequently (Lehmann et al., 2020b). Especially fibers with their linear shape can reduce soil aggregate stability and mean weight diameter (by currently unknown mechanisms; Lehmann et al., 2020b), and thus pore size distribution. Smaller pores and improved oxygen availability could change hyphal ramification (Crawford, 1992; Drew et al., 2003). In relation with a reduced bulk density of the soil through MP addition it can be expected that hyphae, like roots, experience a reduced penetration resistance and will thus be able to explore more soil space. This assumption is supported by several studies that show increased root growth due to decreased soil bulk density, leading to improved root colonization with AMF, which facilitated nutrient uptake by the plants (Nadian et al., 1996; Entry et al., 2002). Assuming a reduced bulk density and smaller mean weight diameter of soil aggregates, associated with smaller soil pores, changes in water transport and WHC are expected. Soil water is usually only available to plants in soil pores >5 µm (Weil and Brady, 2017). These smaller soil pores can easily be penetrated by AM hyphae, which can have diameters as small as 1.2 µm (Dodd et al., 2012). The creation of more smaller pores by MP could thus foster the AMF assisted water supply to plants.

Another soil property that can be changed by MP is the soil pH. Depending on polymer type and chemistry, pH can be increased or decreased: in a recent study non-biodegradable PE decreased the soil pH, while biodegradable PLA increased the soil pH and such effects on soil pH can affect AMF community composition (Wang et al., 2020); however, in another study, high density polyethylene decreased the soil pH but PLA had no effect (Boots et al., 2019). Soil pH plays a crucial role for the composition of microbial communities in general and of AMF communities in particular (Porter et al., 1987; Aciego Pietri and Brookes, 2009). Thus, Wang et al. (2020) suggest that the observed effects of MP on the AMF community are mediated by soil pH.

Microplastic additions to soil have varying effects on the overall microbial community composition and activity, likely as a function of concentration and chemical composition of MP (Liu et al., 2017; Machado et al., 2018, 2019; Yu et al., 2020). The underlying mechanisms can be assumed to be in the change of soil properties, especially bulk density, which improves the aeration of the soil and could thus stimulate aerobic microorganisms; or more generally speaking MP induces a shift in the microbial community composition (Liu et al., 2017). Ren et al. (2020) studied effects of two different MP particle sizes (<150 and <13 µm) on microbial communities and found increases and decreases of richness and diversity, depending on MP particle size, with smaller particles tending

to increase these parameters. When MP was added to the soil, the microbial community structure changed, e.g., *Actinobacteria* increased in soils with MP, whereas other groups such as *Proteobacteria* or *Acidobacteria*, and for smaller particles also some fungal groups, e.g., *Basidiomycota* and *Chytridiomycota* were reduced in MP treatments (Ren et al., 2020). Similar observations, i.e., dominating *Actinobacteria* and reduced *Proteobacteria*, have been made by other authors (Huang et al., 2019; Zhang et al., 2019). The soil microbial community composition can have a strong influence on AMF: for example, there are "mycorrhiza helper bacteria" (e.g., *Pseudomonas* sp., *Burkholderia* sp.) that facilitate root colonization or hyphal growth from spores (Frey-Klett et al., 2007; Viollet et al., 2017) and nitrogen-fixing bacteria can help AMF to maximize nutrient acquisition in the host (van der Heijden et al., 2016). *Proteobacteria*, which can be reduced with MP pollution (Ren et al., 2020), can have interactions with AMF: their presence can alter the structure of AMF assemblages and in contaminated soils it can increase root colonization (Dagher et al., 2020).

In addition to microbial community composition the microbial activity can be altered by MP (Liang et al., 2019). One of the mechanisms that can be responsible for this alteration is the addition of an energy resource through MP, as MP itself represents organic carbon (Rillig, 2018). Although generally inert, MPs are mainly composed of carbon, which can partly leach as dissolved organic carbon before fragmentation occurs (Romera-Castillo et al., 2018). The degradation of thermoplastics in soil varies among plastic types and largely depends on the presence of UV-light (Scalenghe, 2018; Chamas et al., 2020). Elastomers such as tire particles can have a rather short half-life of only 16 months (Baensch-Baltruschat et al., 2020). This introduces an artificial resource into the soil that potentially changes the activity of the natural microbial community. This effect has been observed in several studies: e.g., Machado et al. (2019) found an increase in fluorescein diacetate hydrolase (FDA) by several MP types (polyamid beads, polyester fibers, and pellets of high density polyethylene); Liu et al. (2017) found an increase in FDA and phenol oxidase by polypropylene-microparticles. In a plastic-free soil, it could be shown that there are strong synergistic interactions between *Rhizophagus irregularis* and other soil microbes: more than half of the N that the AMF provided to the plant could be related to a synergistic interaction with the host and the soil microbial community (Hestrin et al., 2019). The MP-induced increase in microbial activity could have an indirect effect on AMF: it could trigger similar synergies between AMF and other soil microbes, leading to increased nutrient uptake by plants.

On the other hand, there are studies showing negative effects of MP on enzyme activities, e.g., polyethylene mulching film reduced catalase, laccase, and phenol oxidase (Yu et al., 2020). Differences among studies can be explained by use of different MP types, concentrations, and experiment durations. Some MP types might have toxic effects (additives, absorbed pollutants, see section "Hypothesized direct effects of microplastic on AMF") or alter the soil properties in a way which is disadvantageous for some species, thus reducing overall activity and enzyme production (Yu et al., 2020). These MP-induced reduced microbial activities could also have an indirect effect on AMF: it could prevent synergistic interactions between AMF and other microbes or reduce positive interactions between AMF and "mycorrhiza helper bacteria" if their activity is decreased as a consequence to MP pollution.

EFFECTS OF MICROPLASTIC ON AMF IN A PERSPECTIVE OF GLOBAL CHANGE

Long-term consequences of MP effects cannot currently be foreseen, as effects are only studied in the short-term and there are no realistic estimates of MP accumulation in soils. But long-term predictions are necessary as deposited MP will interact with other environmental impacts in the future. In fact, MP is increasingly recognized as a GCF (Rillig and Lehmann, 2020) and should thus be studied in the context of other GCFs, instead of being regarded in isolation.

As MP is now found in almost every ecosystem around the globe, multiple GCFs will likely occur in combination. The co-occurrence of several GCFs could intensify the impact of each single factor (Rillig et al., 2019d). For grassland ecosystems, in which the AM symbiosis is the dominant mycorrhizal type, the occurrence of GCFs such as elevated temperature, elevated CO_2, drought, pesticides, heavy metals, and MP is likely. MP research in general has been heavily focused on agricultural systems; this means, we know little about effects in other ecosystem types (such as tropical forests or Mediterranean woodlands), and the same applies to effects on AMF. Elevated CO_2 has been shown to stimulate AMF activity (Sanders et al., 1998; Drigo et al., 2010), like MP did for AMF colonization (Machado et al., 2019; Lehmann et al., 2020a). AMF have repeatedly been proposed as an important contributor for sustainable agriculture, where they contribute to resistance and resilience against GCFs such as drought, salinity, but also against pathogens (Veresoglou and Rillig, 2012; Rillig et al., 2016, 2019a; Begum et al., 2019). This role, however, can be challenged if AMF are affected by MP. If MP alters soil porosity and water transport of the soil, AMF-mediated supply with water and nutrients to plants could be assigned a different relevance in both directions. In a recent study, it could not be shown that AMF helped mitigate negative effects of the GCFs MP and drought (Lehmann et al., 2020a), which would represent a diminished role for AMF in ecosystem resilience. It is possible that a negative impact of MP prevented the plant protection that AMF usually provide. If AMF are directly affected by MP, e.g., its toxic components, their ability to protect plants from pathogens or co-occurring GCFs could be changed. We think that this is an important question highlighting the role of AMF for sustainable agriculture in times of global change and for the production of healthy, nutritional food.

Agricultural soils are among the most strongly MP-polluted terrestrial ecosystems because they receive organic fertilizers (e.g., compost, sewage sludge, and biowaste fermentation

digestates) that can contain high numbers of MP particles (Mahon et al., 2017; Weithmann et al., 2018) and additional sources of MP such as fragments from mulching films that can be extensive (Zhang et al., 2019). Many crops form AM-symbioses. The association of a crop-host with AMF can have positive effects on nutrient uptake and biomass yield, can enhance drought-, metal-, and salinity-tolerance, reduce nutrient leaching, improve soil structure, and increase plant biodiversity (Thirkell et al., 2017). AMF have been proposed to assist in stabilizing sustainable forest and agricultural productivity in the struggle with increasing impact of GCFs (Solaiman et al., 2014; Sosa-Hernández et al., 2019), which now include MPs. However, the colonization by AMF can also reduce yields, depending on the crop species identity and soil nutrient status (Hoeksema et al., 2010). Many of the well-recognized AMF functions (especially improvement of soil structure and N retention) are connected to key ecosystem services that are important for soil health and eventually for human health, as humans depend on the production of healthy food from soils and the filtration of water for potable groundwater (Lehmann et al., 2020c). MP has the potential to interfere with these important ecosystem functions, as outlined in the sections above, consequently affecting nutrient cycling, i.e., nutrient supply and release of N, with spillover effects on net primary production (NPP). AMF often increase the nutrient status of crops, thereby improving food quality (Thirkell et al., 2017). MP often increases plant growth, but if this positive effect is supported by increased nutrient uptake through an association with AMF is currently not known.

The current state-of-the-art only shows us pieces of a larger puzzle, in which we know that AMF, MP, and other GCFs all contribute to soil and plant biodiversity (Lozano and Rillig, 2020), which are also key for soil (and human) health. But how these factors interact and what the outcome of their interactions are, is difficult to predict (Rillig et al., 2019d).

POTENTIAL EARTH SYSTEM FEEDBACKS AND THE ROLE OF AMF

Accumulation of MP in the soil has the potential to interfere with earth system processes such as NPP (Rillig and Lehmann, 2020). Enhanced NPP, i.e., enhanced plant growth, will lead to a change in root exudation quantity and quality. Increased C allocation to roots will likely also alter C allocation to AMF and subsequently process rates of P and N cycling, for which AMF play a major role (van der Heijden et al., 2008). Furthermore, AMF play a role in carbon cycling in the soil (Cheng et al., 2012; Averill et al., 2014; Leifheit et al., 2015). It has been postulated that during the decomposition of labile litter AMF stimulate other microbes that increase decomposition (Cheng et al., 2012) and thus soil respiration, with more C loss as CO_2. MP-induced changes in AMF activity would thus create a feedback loop to the atmosphere, where increased levels of CO_2 foster NPP that is again influenced by MP, while at the same time plant productivity is the main resource for AMF determining their activity.

Another earth system feedback loop might occur with nitrogen. Nutrient cycles around the globe are changing, mainly the N cycle due to ubiquitous N deposition. N inputs introduce nutrient imbalances in the soil leading to altered microbial activity. MP induced increase in microbial activity (and mobilization of N) and N inputs could lead to more N_2O emissions from soil. Increased N release in the soil plus atmospheric N deposition could reduce AMF performance, possibly affecting AMF's potential to reduce N emissions from soil (Asghari and Cavagnaro, 2012; Storer et al., 2018; Sosa-Hernández et al., 2019), thus fostering greenhouse gas emissions from the soil to the atmosphere. MP thus has the potential to alter the nitrogen cycle and the role of AMF in the cycle, finally leading to increased turnover rates and reduced AMF activity.

FUTURE RESEARCH PRIORITIES

At the moment, we cannot fully assess the relevance of MP for AMF. We here propose research areas at individual scales, the corresponding research topics for microplastic effects on AMF and give a conceptual summary of these ideas in **Figure 1**.

As AMF are obligate symbionts, it will be difficult to disentangle direct impacts of MP on AMF and indirect effects *via* plants, soil properties, or the microbial community. Therefore, basic ecotoxicological research is needed for AMF, how MP affects the AMF community composition, the diversity and their functioning. For these studies, MP with known or without additives is needed, to differentiate between effects from chemistry and plastic traits such as shape or size. In order to draw generalizations for MP effects on this key symbiont, studies should then use different MP (with known chemistry) and soil types. Such experiments with a focus on the fungal part of the symbiosis would need to use compartmentalized designs, in which the fungal extraradical mycelium in the soil is physically separated from the root system, using mesh impenetrable to roots. The addition of MP would then be in these fungal compartments to prioritize effects on the mycobionts. More controlled studies could also be carried out using soil-free *in vitro* culture systems; here, also the fungal mycelium could be separated from the root.

Once this baseline is established, the focus should move to the study of interactions of MP with other GCFs and their effects on AMF. For this question, factorial experiments with other key factors are needed, or new types of designs that can simultaneously take into account a larger number of factors (e.g., Rillig et al., 2019d) should be pursued. Such experiments should focus on the entire plant-AM symbiotic system and its responses.

Research on MP in soils has emerged only a few years ago and is still based on a number of assumptions, because of the lack of systematic MP quantification in soils, in part due to the absence of suitable high-throughput analytical methods. Hence, our knowledge about MP concentrations in the environment is limited. Additionally, concentrations will differ by orders of magnitude according to the distance from a point source of emission. Current laboratory research thus uses

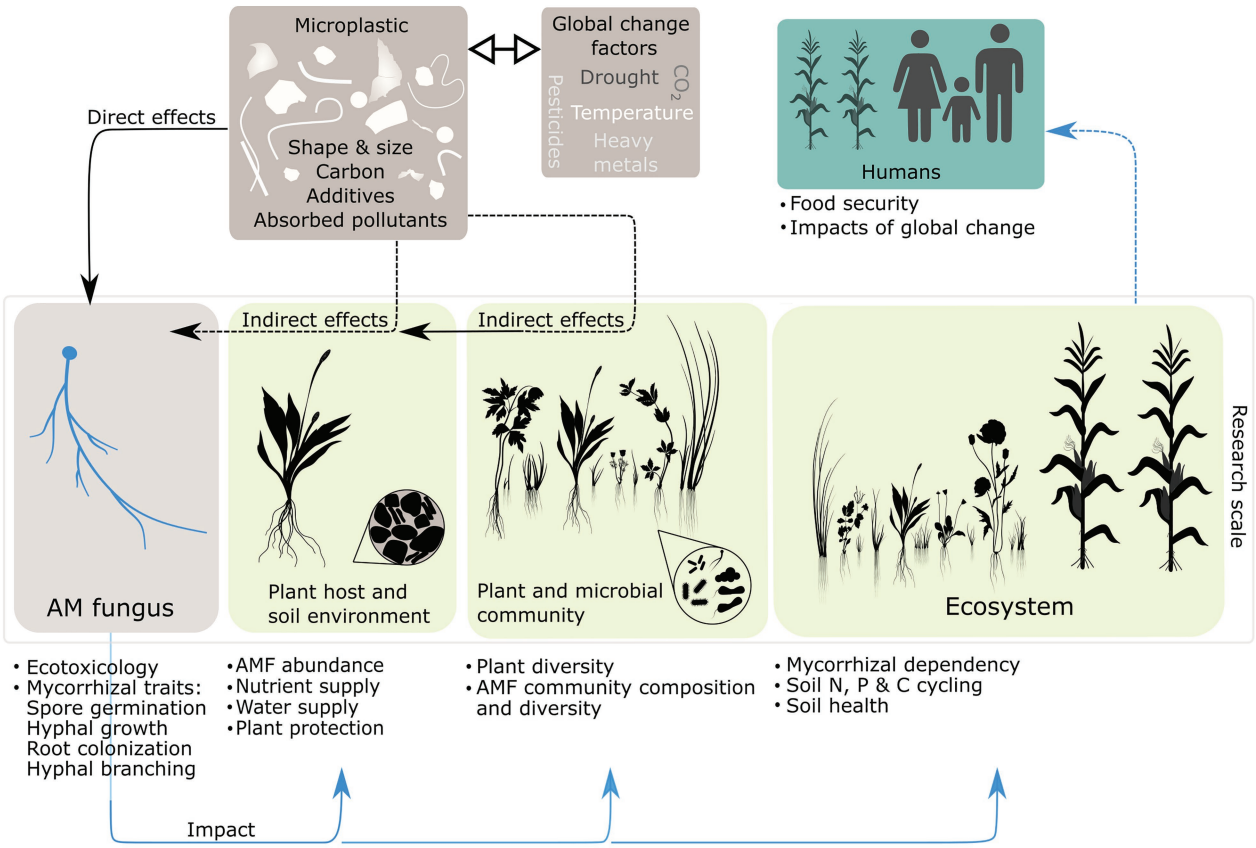

FIGURE 1 | Proposed research scales for microplastic (MP) effects on arbuscular mycorrhizal fungi (AMF) and the corresponding research topics for AMF (text fields). Dashed lines represent indirect effects and solid lines represent direct effects. Black arrows represent the potential impact of microplastic and blue arrows represent the potential impact of microplastic-induced changes in the arbuscular mycorrhiza (AM) symbiosis. The ↔ symbol indicates potential interactions of microplastic with other factors of global change (GCFs).

concentrations that might not be representative of the situation in the field. Moreover, effects of MP depend on polymer type, its additives and shape (Rillig et al., 2019c), and current analytical methods do not capture this level of detail. Due to these issues (uncertain concentration and chemistry), it is difficult to perform observational studies in the field with native AMF communities under realistic conditions.

Finally, research also needs to move to the plant community level, to more fully explore how AMF and a host community respond to MP. This type of experiment also opens the door to an ecosystem-level assessment (Rillig and Lehmann, 2020), and could include treatments in which mycorrhizal fungi have either been added or not; this way, we will learn how a plant community-level or ecosystem-level response to MP will depend on mycorrhiza.

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

EL wrote the first draft of the paper. EL and AL created the artwork. All authors contributed to the article and approved the submitted version.

FUNDING

EL acknowledges funding from Deutsche Forschungsgemeinschaft (LE 3859/1-1). MR acknowledges funding from an ERC Advanced Grant (694368) and from the Federal Ministry of Education and Research (BMBF) for the projects BIBS and µPlastic.

ACKNOWLEDGMENTS

We acknowledge support by the Open Access Publication Initiative of Freie Universität Berlin.

REFERENCES

Asghari, H. R., and Cavagnaro, T. R. (2012). Arbuscular mycorrhizas reduce nitrogen loss via leaching. *PLoS One* 7:e29825. doi: 10.1371/journal.pone.0029825

Averill, C., Turner, B. L., and Finzi, A. C. (2014). Mycorrhiza-mediated competition between plants and decomposers drives soil carbon storage. *Nature* 505, 543–545. doi: 10.1038/nature12901

Baensch-Baltruschat, B., Kocher, B., Stock, F., and Reifferscheid, G. (2020). Tyre and road wear particles (TRWP)-A review of generation, properties, emissions, human health risk, ecotoxicity, and fate in the environment. *Sci. Total Environ.* 733:137823. doi: 10.1016/j.scitotenv.2020.137823

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

Bergmann, M., Mützel, S., Primpke, S., Tekman, M. B., Trachsel, J., and Gerdts, G. (2019). White and wonderful? Microplastics prevail in snow from the Alps to the Arctic. *Sci. Adv.* 5:eaax1157. doi: 10.1126/sciadv.aax1157

Boots, B., Russell, C. W., and Green, D. S. (2019). Effects of microplastics in soil ecosystems: above and below ground. *Environ. Sci. Technol.* 53, 11496–11506. doi: 10.1021/acs.est.9b03304

Brahney, J., Hallerud, M., Heim, E., Hahnenberger, M., and Sukumaran, S. (2020). Plastic rain in protected areas of the United States. *Science* 368, 1257–1260. doi: 10.1126/science.aaz5819

Büks, F., Schaik, N. L. V., and Kaupenjohann, M. (2020). What do we know about how the terrestrial multicellular soil fauna reacts to microplastic? *SOIL* 6, 245–267. doi: 10.5194/soil-6-245-2020

Cabello, M. N. (1997). Hydrocarbon pollution: its effect on native arbuscular mycorrhizal fungi (AMF). *FEMS Microbiol. Ecol.* 22, 233–236. doi: 10.1111/j.1574-6941.1997.tb00375.x

Chamas, A., Moon, H., Zheng, J., Qiu, Y., Tabassum, T., Jang, J. H., et al. (2020). Degradation rates of plastics in the environment. *ACS Sustain. Chem. Eng.* 8, 3494–3511. doi: 10.1021/acssuschemeng.9b06635

Cheng, L., Booker, F. L., Tu, C., Burkey, K. O., Zhou, L. S., Shew, H. D., et al. (2012). Arbuscular mycorrhizal fungi increase organic carbon decomposition under elevated CO2. *Science* 337, 1084–1087. doi: 10.1126/science.1224304

Cockerton, H. M., Li, B., Stavridou, E., Johnson, A., Karlström, A., Armitage, A. D., et al. (2020). Genetic and phenotypic associations between root architecture, arbuscular mycorrhizal fungi colonisation and low phosphate tolerance in strawberry (Fragaria × ananassa). *BMC Plant Biol.* 20:154. doi: 10.1186/s12870-020-02347-x

Crawford, R. M. M. (1992). "Oxygen availability as an ecological limit to plant distribution" in *Advances in ecological research*. eds. M. Begon and A. H. Fitter (London and San Diego, CA: Academic Press), 93–185.

Dagher, D. J., de la Providencia, I. E., Pitre, F. E., St-Arnaud, M., and Hijri, M. (2020). Arbuscular mycorrhizal fungal assemblages significantly shifted upon bacterial inoculation in non-contaminated and petroleum-contaminated environments. *Microorganisms* 8:602. doi: 10.3390/microorganisms8040602

Desalme, D., Chiapusio, G., Bernard, N., Gilbert, D., Toussaint, M. L., and Binet, P. (2012). Arbuscular mycorrhizal fungal infectivity in two soils as affected by atmospheric phenanthrene pollution. *Water Air Soil Pollut.* 223, 3295–3305. doi: 10.1007/s11270-012-1110-z

Dodd, J., Boddington, C., Rodriguez, A., Chavez, M. D. C. A. G., and Mansur, I. (2012). Mycelium of arbuscular mycorrhizal fungi (AMF) from different genera: form, function and detection. *Plant Soil* 226, 131–151. doi: 10.1023/A:1026574828169

Drew, E. A., Murray, R. S., Smith, S. E., and Jakobsen, I. (2003). Beyond the rhizosphere: growth and function of arbuscular mycorrhizal external hyphae in sands of varying pore sizes. *Plant Soil* 251, 105–114. doi: 10.1023/A:1022932414788

Drigo, B., Pijl, A. S., Duyts, H., Kielak, A. M., Gamper, H. A., Houtekamer, M. J., et al. (2010). Shifting carbon flow from roots into associated microbial communities in response to elevated atmospheric CO_2. *Proc. Natl. Acad. Sci. U. S. A.* 107, 10938–10942. doi: 10.1073/pnas.0912421107

Eissenstat, D. M., Kucharski, J. M., Zadworny, M., Adams, T. S., and Koide, R. T. (2015). Linking root traits to nutrient foraging in arbuscular mycorrhizal trees in a temperate forest. *New Phytol.* 208, 114–124. doi: 10.1111/nph.13451

Entry, J. A., Rygiewicz, P. T., Watrud, L. S., and Donnelly, P. K. (2002). Influence of adverse soil conditions on the formation and function of arbuscular mycorrhizas. *Adv. Environ. Res.* 7, 123–138. doi: 10.1016/S1093-0191(01)00109-5

Faggioli, V., Menoyo, E., Geml, J., Kemppainen, M., Pardo, A., Salazar, M. J., et al. (2019). Soil lead pollution modifies the structure of arbuscular mycorrhizal fungal communities. *Mycorrhiza* 29, 363–373. doi: 10.1007/s00572-019-00895-1

Fei, Y., Huang, S., Zhang, H., Tong, Y., Wen, D., Xia, X., et al. (2020). Response of soil enzyme activities and bacterial communities to the accumulation of microplastics in an acid cropped soil. *Sci. Total Environ.* 707:135634. doi: 10.1016/j.scitotenv.2019.135634

Ferrol, N., Tamayo, E., and Vargas, P. (2016). The heavy metal paradox in arbuscular mycorrhizas: from mechanisms to biotechnological applications. *J. Exp. Bot.* 67, 6253–6265. doi: 10.1093/jxb/erw403

Frey-Klett, P., Garbaye, J., and Tarkka, M. (2007). The mycorrhiza helper bacteria revisited. *New Phytol.* 176, 22–36. doi: 10.1111/j.1469-8137.2007.02191.x

Hestrin, R., Hammer, E. C., Mueller, C. W., and Lehmann, J. (2019). Synergies between mycorrhizal fungi and soil microbial communities increase plant nitrogen acquisition. *Commun. Biol.* 2:233. doi: 10.1038/s42003-019-0481-8

Hillis, D. G., Antunes, P., Sibley, P. K., Klironomos, J. N., and Solomon, K. R. (2008). Structural responses of *Daucus carota* root-organ cultures and the arbuscular mycorrhizal fungus, glomus intraradices, to 12 pharmaceuticals. *Chemosphere* 73, 344–352. doi: 10.1016/j.chemosphere.2008.05.063

Hoeksema, J. D., Chaudhary, V. B., Gehring, C. A., Johnson, N. C., Karst, J., Koide, R. T., et al. (2010). A meta-analysis of context-dependency in plant response to inoculation with mycorrhizal fungi. *Ecol. Lett.* 13, 394–407. doi: 10.1111/j.1461-0248.2009.01430.x

Huang, Y., Zhao, Y., Wang, J., Zhang, M., Jia, W., and Qin, X. (2019). LDPE microplastic films alter microbial community composition and enzymatic activities in soil. *Environ. Pollut.* 254:112983. doi: 10.1016/j.envpol.2019.112983

Joner, E. J., and Leyval, C. (2003). Rhizosphere gradients of polycyclic aromatic hydrocarbon (PAH) dissipation in two industrial soils and the impact of arbuscular mycorrhiza. *Environ. Sci. Technol.* 37, 2371–2375. doi: 10.1021/es020196y

Kettner, M. T., Rojas-Jimenez, K., Oberbeckmann, S., Labrenz, M., and Grossart, H. -P. (2017). Microplastics alter composition of fungal communities in aquatic ecosystems. *Environ. Microbiol.* 19, 4447–4459. doi: 10.1111/1462-2920.13891

Keymer, A., Pimprikar, P., Wewer, V., Huber, C., Brands, M., Bucerius, S. L., et al. (2017). Lipid transfer from plants to arbuscular mycorrhiza fungi. *Elife* 6:e29107. doi: 10.7554/eLife.29107

Kim, S. W., Waldman, W. R., Kim, T. -Y., and Rillig, M. C. (2020). Effects of different microplastics on nematodes in the soil environment: tracking the extractable additives using an ecotoxicological approach. *Environ. Sci. Technol.* 54, 13868–13878. doi: 10.1021/acs.est.0c04641

Lebreton, L., and Andrady, A. (2019). Future scenarios of global plastic waste generation and disposal. *Palgrave Commun.* 5:11. doi: 10.1057/s41599-018-0212-7

Lehmann, J., Bossio, D. A., Kögel-Knabner, I., and Rillig, M. C. (2020c). The concept and future prospects of soil health. *Nat. Rev. Earth Environ.* 1, 544–553. doi: 10.1038/s43017-020-0080-8

Lehmann, A., Leifheit, E. F., Feng, L., Bergmann, J., Wulf, A., and Rillig, M. C. (2020a). Microplastic fiber and drought effects on plants and soil are only slightly modified by arbuscular mycorrhizal fungi. *Soil Ecol. Lett.* doi: 10.1007/s42832-020-0060-4

Lehmann, A., Leifheit, E. F., Gerdawischke, M., and Rillig, M. C. (2020b). Microplastics have shape- and polymer-dependent effects on soil processes. bioRxiv [Preprint]. doi: 10.1101/2020.06.02.130054

Leifheit, E. F., Verbruggen, E., and Rillig, M. C. (2015). Arbuscular mycorrhizal fungi reduce decomposition of woody plant litter while increasing soil aggregation. *Soil Biol. Biochem.* 81, 323–328. doi: 10.1016/j.soilbio.2014.12.003

Leyval, C., Turnau, K., and Haselwandter, K. (1997). Effect of heavy metal pollution on mycorrhizal colonization and function: physiological, ecological and applied aspects. *Mycorrhiza* 7, 139–153. doi: 10.1007/s005720050174

Liang, Y., Lehmann, A., Ballhausen, M. -B., Muller, L., and Rillig, M. C. (2019). Increasing temperature and microplastic fibers jointly influence soil aggregation by saprobic fungi. *Front. Microbiol.* 10:2018. doi: 10.3389/fmicb.2019.02018

Liu, H., Yang, X., Liu, G., Liang, C., Xue, S., Chen, H., et al. (2017). Response of soil dissolved organic matter to microplastic addition in Chinese loess soil. *Chemosphere* 185, 907–917. doi: 10.1016/j.chemosphere.2017.07.064

Lozano, Y. M., and Rillig, M. C. (2020). Effects of microplastic fibers and drought on plant communities. *Environ. Sci. Technol.* 54, 6166–6173. doi: 10.1021/acs.est.0c01051

Machado, A. A. S., Lau, C. W., Kloas, W., Bergmann, J., Bachelier, J. B., Faltin, E., et al. (2019). Microplastics can change soil properties and affect plant performance. *Environ. Sci. Technol.* 53, 6044–6052. doi: 10.1021/acs.est.9b01339

Machado, A. A. S., Lau, C. W., Till, J., Kloas, W., Lehmann, A., Becker, R., et al. (2018). Impacts of microplastics on the soil biophysical environment. *Environ. Sci. Technol.* 52, 9656–9665. doi: 10.1021/acs.est.8b02212

Mahon, A. M., O'Connell, B., Healy, M. G., O'Connor, I., Officer, R., Nash, R., et al. (2017). Microplastics in sewage sludge: effects of treatment. *Environ. Sci. Technol.* 51, 810–818. doi: 10.1021/acs.est.6b04048

Materechera, S. A., Alston, A. M., Kirby, J. M., and Dexter, A. R. (1992). Influence of root diameter on the penetration of seminal roots into a compacted subsoil. *Plant Soil* 144, 297–303. doi: 10.1007/BF00012888

McCormack, M. L., Dickie, I. A., Eissenstat, D. M., Fahey, T. J., Fernandez, C. W., Guo, D., et al. (2015). Redefining fine roots improves understanding of below-ground contributions to terrestrial biosphere processes. *New Phytol.* 207, 505–518. doi: 10.1111/nph.13363

Nadian, H., Smith, S. E., Alston, A. M., and Murray, R. S. (1996). The effect of soil compaction on growth and P uptake by *Trifolium subterraneum*: interactions with mycorrhizal colonisation. *Plant Soil* 182, 39–49. doi: 10.1007/BF00010993

Niu, W., Guo, Q., Zhou, X., and Helmers, M. J. (2012). Effect of aeration and soil water redistribution on the air permeability under subsurface drip irrigation. *Soil Sci. Soc. Am. J.* 76, 815–820. doi: 10.2136/sssaj2011.0329

Nizzetto, L., Futter, M., and Langaas, S. (2016). Are agricultural soils dumps for microplastics of urban origin? *Environ. Sci. Technol.* 50, 10777–10779. doi: 10.1021/acs.est.6b04140

Parniske, M. (2008). Arbuscular mycorrhiza: the mother of plant root endosymbioses. *Nat. Rev. Microbiol.* 6, 763–775. doi: 10.1038/nrmicro1987

Piehl, S., Leibner, A., Löder, M. G. J., Dris, R., Bogner, C., and Laforsch, C. (2018). Identification and quantification of macro- and microplastics on an agricultural farmland. *Sci. Rep.* 8:17950. doi: 10.1038/s41598-018-36172-y

Pietri, J. C. A., and Brookes, P. C. (2009). Substrate inputs and pH as factors controlling microbial biomass, activity and community structure in an arable soil. *Soil Biol. Biochem.* 41, 1396–1405. doi: 10.1016/j.soilbio.2009.03.017

Porter, W. M., Robson, A. D., and Abbott, L. K. (1987). Field survey of the distribution of vesicular-arbuscular mycorrhizal fungi in relation to soil pH. *J. Appl. Ecol.* 24, 659–662. doi: 10.2307/2403900

Qi, Y., Ossowicki, A., Yang, X., Lwanga, E. H., Dini-Andreote, F., Geissen, V., et al. (2020). Effects of plastic mulch film residues on wheat rhizosphere and soil properties. *J. Hazard. Mater.* 387:121711. doi: 10.1016/j.jhazmat.2019.121711

Qi, Y., Yang, X., Pelaez, A. M., Lwanga, E. H., Beriot, N., Gertsen, H., et al. (2018). Macro- and micro- plastics in soil-plant system: effects of plastic mulch film residues on wheat (*Triticum aestivum*) growth. *Sci. Total Environ.* 645, 1048–1056. doi: 10.1016/j.scitotenv.2018.07.229

Ren, X., Tang, J., Liu, X., and Liu, Q. (2020). Effects of microplastics on greenhouse gas emissions and the microbial community in fertilized soil. *Environ. Pollut.* 256:113347. doi: 10.1016/j.envpol.2019.113347

Rillig, M. C. (2012). Microplastic in terrestrial ecosystems and the soil? *Environ. Sci. Technol.* 46, 6453–6454. doi: 10.1021/es302011r

Rillig, M. C. (2018). Microplastic disguising as soil carbon storage. *Environ. Sci. Technol.* 52, 6079–6080. doi: 10.1021/acs.est.8b02338

Rillig, M. C., Aguilar-Trigueros, C. A., Bergmann, J., Verbruggen, E., Veresoglou, S. D., and Lehmann, A. (2015). Plant root and mycorrhizal fungal traits for understanding soil aggregation. *New Phytol.* 205, 1385–1388. doi: 10.1111/nph.13045

Rillig, M. C., Aguilar-Trigueros, C. A., Camenzind, T., Cavagnaro, T. R., Degrune, F., Hohmann, P., et al. (2019a). Why farmers should manage the arbuscular mycorrhizal symbiosis. *New Phytol.* 222, 1171–1175. doi: 10.1111/nph.15602

Rillig, M. C., and Lehmann, A. (2020). Microplastic in terrestrial ecosystems. *Science* 368, 1430–1431. doi: 10.1126/science.abb5979

Rillig, M. C., Lehmann, A., Machado, A. A. d. S., and Yang, G. (2019b). Microplastic effects on plants. *New Phytol.* 223, 1066–1070. doi: 10.1111/nph.15794

Rillig, M. C., Lehmann, A., Ryo, M., and Bergmann, J. (2019c). Shaping up: toward considering the shape and form of pollutants. *Environ. Sci. Technol.* 53, 7925–7926. doi: 10.1021/acs.est.9b03520

Rillig, M. C., and Mummey, D. L. (2006). Mycorrhizas and soil structure. *New Phytol.* 171, 41–53. doi: 10.1111/j.1469-8137.2006.01750.x

Rillig, M. C., Ryo, M., Lehmann, A., Aguilar-Trigueros, C. A., Buchert, S., Wulf, A., et al. (2019d). The role of multiple global change factors in driving soil functions and microbial biodiversity. *Science* 366, 886–890. doi: 10.1126/science.aay2832

Rillig, M. C., Sosa-Hernández, M. A., Roy, J., Aguilar-Trigueros, C. A., Vályi, K., and Lehmann, A. (2016). Towards an integrated mycorrhizal technology: harnessing mycorrhiza for sustainable intensification in agriculture. *Front. Plant Sci.* 7:1625. doi: 10.3389/fpls.2016.01625

Roberts, S. C., and Zembower, T. R. (2020). Global increases in antibiotic consumption: a concerning trend for WHO targets. *Lancet Infect. Dis.* 21, 10–11. doi: 10.1016/S1473-3099(20)30456-4

Romera-Castillo, C., Pinto, M., Langer, T. M., Álvarez-Salgado, X. A., and Herndl, G. J. (2018). Dissolved organic carbon leaching from plastics stimulates microbial activity in the ocean. *Nat. Commun.* 9:1430. doi: 10.1038/s41467-018-03798-5

Sanders, I. R., Streitwolf-Engel, R., van der Heijden, M. G. A., Boller, T., and Wiemken, A. (1998). Increased allocation to external hyphae of arbuscular mycorrhizal fungi under CO_2 enrichment. *Oecologia* 117, 496–503. doi: 10.1007/s004420050685

Scalenghe, R. (2018). Resource or waste? A perspective of plastics degradation in soil with a focus on end-of-life options. *Heliyon* 4:e00941. doi: 10.1016/j.heliyon.2018.e00941

Solaiman, Z. M., Abbott, L. K., and Varma, A. (2014). *Mycorrhizal fungi: Use in sustainable agriculture and land restoration*. Berlin: Springer.

Sosa-Hernández, M. A., Leifheit, E. F., Ingraffia, R., and Rillig, M. C. (2019). Subsoil arbuscular mycorrhizal fungi for sustainability and climate-smart agriculture: a solution right under our feet? *Front. Microbiol.* 10:744. doi: 10.3389/fmicb.2019.00744

Storer, K., Coggan, A., Ineson, P., and Hodge, A. (2018). Arbuscular mycorrhizal fungi reduce nitrous oxide emissions from N_2O hotspots. *New Phytol.* 220, 1285–1295. doi: 10.1111/nph.14931

Thirkell, T. J., Charters, M. D., Elliott, A. J., Sait, S. M., and Field, K. J. (2017). Are mycorrhizal fungi our sustainable saviours? Considerations for achieving food security. *J. Ecol.* 105, 921–929. doi: 10.1111/1365-2745.12788

Thonar, C., Erb, A., and Jansa, J. (2012). Real-time PCR to quantify composition of arbuscular mycorrhizal fungal communitiesumarker design, verification, calibration and field validation. *Mol. Ecol. Resour.* 12, 219–232. doi: 10.1111/j.1755-0998.2011.03086.x

Tilman, D., Cassman, K. G., Matson, P. A., Naylor, R., and Polasky, S. (2002). Agricultural sustainability and intensive production practices. *Nature* 418, 671–677. doi: 10.1038/nature01014

van den Berg, P., Huerta-Lwanga, E., Corradini, F., and Geissen, V. (2020). Sewage sludge application as a vehicle for microplastics in eastern Spanish agricultural soils. *Environ. Pollut.* 261:114198. doi: 10.1016/j.envpol.2020.114198

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

van der Heijden, M. G. A., Bruin, S. D., and Luckerhoff, L., van Logtestijn, R. S. P., and Schlaeppi, K. (2016). A widespread plant-fungal-bacterial symbiosis promotes plant biodiversity, plant nutrition and seedling recruitment. *ISME J.* 10, 389–399. doi: 10.1038/ismej.2015.120

Verbruggen, E., Jansa, J., Hammer, E. C., and Rillig, M. C. (2016). Do arbuscular mycorrhizal fungi stabilize litter-derived carbon in soil? *J. Ecol.* 104, 261–269. doi: 10.1111/1365-2745.12496

Veresoglou, S. D., and Rillig, M. C. (2012). Suppression of fungal and nematode plant pathogens through arbuscular mycorrhizal fungi. *Biol. Lett.* 8, 214–217. doi: 10.1098/rsbl.2011.0874

Viollet, A., Pivato, B., Mougel, C., Cleyet-Marel, J. C., Gubry-Rangin, C., Lemanceau, P., et al. (2017). *Pseudomonas fluorescens* C7R12 type III secretion system impacts mycorrhization of medicago truncatula and associated microbial communities. *Mycorrhiza* 27, 23–33. doi: 10.1007/s00572-016-0730-3

Wang, J., Liu, X., Li, Y., Powell, T., Wang, X., Wang, G., et al. (2019). Microplastics as contaminants in the soil environment: a mini-review. *Sci. Total Environ.* 691, 848–857. doi: 10.1016/j.scitotenv.2019.07.209

Wang, F., Zhang, X., Zhang, S., Zhang, S., and Sun, Y. (2020). Interactions of microplastics and cadmium on plant growth and arbuscular mycorrhizal fungal communities in an agricultural soil. *Chemosphere* 254:126791. doi: 10.1016/j.chemosphere.2020.126791

Weil, R. R., and Brady, N. C. (2017). *The nature and properties of soils. 15th Edn.* London: Pearson Education Limited.

Weithmann, N., Möller, J. N., Löder, M. G. J., Piehl, S., Laforsch, C., and Freitag, R. (2018). Organic fertilizer as a vehicle for the entry of microplastic into the environment. *Sci. Adv.* 4:eaap8060. doi: 10.1126/sciadv.aap8060

Yu, H., Fan, P., Hou, J., Dang, Q., Cui, D., Xi, B., et al. (2020). Inhibitory effect of microplastics on soil extracellular enzymatic activities by changing soil properties and direct adsorption: an investigation at the aggregate-fraction level. *Environ. Pollut.* 267:115544. doi: 10.1016/j.envpol.2020.115544

Zhang, M., Zhao, Y., Qin, X., Jia, W., Chai, L., Huang, M., et al. (2019). Microplastics from mulching film is a distinct habitat for bacteria in farmland soil. *Sci. Total Environ.* 688, 470–478. doi: 10.1016/j.scitotenv.2019.06.108

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

frontiers in Plant Science

ORIGINAL RESEARCH
published: 22 June 2021
doi: 10.3389/fpls.2021.693037

The Phosphate Inhibition Paradigm: Host and Fungal Genotypes Determine Arbuscular Mycorrhizal Fungal Colonization and Responsiveness to Inoculation in Cassava With Increasing Phosphorus Supply

Ricardo Alexander Peña Venegas[1†§], Soon-Jae Lee[1†], Moses Thuita[2], Deusdedit Peter Mlay[3], Cargele Masso[4], Bernard Vanlauwe[2], Alia Rodriguez[5‡] and Ian R. Sanders[1‡]*

[1]Department of Ecology and Evolution, University of Lausanne, Lausanne, Switzerland, [2]International Institute for Tropical Agriculture (IITA) Kenya, Nairobi, Kenya, [3]Tanzania Agricultural Research Institute, Mwanza, Tanzania, [4]International Institute for Tropical Agriculture (IITA) Cameroon, Yaoundé, Cameroon, [5]Department of Biology, National University of Colombia, Bogotá, Colombia

A vast majority of terrestrial plants are dependent on arbuscular mycorrhizal fungi (AMF) for their nutrient acquisition. AMF act as an extension of the root system helping phosphate uptake. In agriculture, harnessing the symbiosis can potentially increase plant growth. Application of the AMF *Rhizophagus irregularis* has been demonstrated to increase the yields of various crops. However, there is a paradigm that AMF colonization of roots, as well as the plant benefits afforded by inoculation with AMF, decreases with increasing phosphorus (P) supply in the soil. The paradigm suggests that when fertilized with sufficient P, inoculation of crops would not be beneficial. However, the majority of experiments demonstrating the paradigm were conducted in sterile conditions without a background AMF or soil microbial community. Interestingly, intraspecific variation in *R. irregularis* can greatly alter the yield of cassava even at a full application of the recommended P dose. Cassava is a globally important crop, feeding 800 million people worldwide, and a crop that is highly dependent on AMF for P uptake. In this study, field trials were conducted at three locations in Kenya and Tanzania using different AMF and cassava varieties under different P fertilization levels to test if the paradigm occurs in tropical field conditions. We found that AMF colonization and inoculation responsiveness of cassava does not always decrease with an increased P supply as expected by the paradigm. The obtained results demonstrate that maximizing the inoculation responsiveness of cassava is not necessarily only in conditions of low P availability, but that this is dependent on cassava and fungal genotypes. Thus, the modeling of plant symbiosis with AMF under different P levels in nature should be considered with caution.

Keywords: *Rhizophagus irregularis*, intraspecific variation, *Manihot esculenta* Cranz., phosphorus fertilization, phosphate, mycorrhizal symbiosis, inoculation responsiveness, phosphate inhibition

OPEN ACCESS

Edited by:
Jan Jansa,
Academy of Sciences of the Czech Republic (ASCR), Czechia

Reviewed by:
Yoshihiro Kobae,
Rakuno Gakuen University, Japan
Sidney Luiz Stürmer,
Regional University of Blumenau, Brazil
Cecile Thonar,
University of Liege, Belgium

***Correspondence:**
Ian R. Sanders
ian.sanders@unil.ch

[†]These authors have contributed equally to this work

[‡]These authors share senior authorship

[§]Present address:
Ricardo Alexander Peña Venegas, Faculty of Agricultural Sciences, La Salle University, Bogotá, Colombia

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

Received: *09 April 2021*
Accepted: *20 May 2021*
Published: *22 June 2021*

Citation:
Peña Venegas RA, Lee S-J, Thuita M, Mlay DP, Masso C, Vanlauwe B, Rodriguez A and Sanders IR (2021) The Phosphate Inhibition Paradigm: Host and Fungal Genotypes Determine Arbuscular Mycorrhizal Fungal Colonization and Responsiveness to Inoculation in Cassava With Increasing Phosphorus Supply. Front. Plant Sci. 12:693037. doi: 10.3389/fpls.2021.693037

INTRODUCTION

Phosphorus (P) is an essential nutrient for plants that affect the productivity of crops (Karandashov and Bucher, 2005). Unfortunately, P cannot diffuse rapidly in the soil and depletion zones form around root hairs (Schachtman et al., 1998; Vance, 2001). Thus, many plants have difficulty obtaining enough P. Worldwide, 67% of the total farmed land is considered P deficient (Cakmak, 2002). While P limits plant growth in soils globally, this is especially true in the tropics, due to the additional problem of P fixation by aluminum and iron oxides common in tropical acidic soils (Rahman et al., 2018). Consequently, P fertilization is essential in agriculture to support high crop yields.

In nature, plants interact with diverse microorganisms to improve P uptake (Sharma et al., 2013). One of the most important and ubiquitous interactions is the mutualism between plants and arbuscular mycorrhizal fungi (AMF), which occurs in 72% of land plant species (Brundrett and Tedersoo, 2018). In AM symbiosis, the plant provides AMF with sugars and lipids. After the colonization of the roots, the fungus forms an extra-radical mycelial network that extends beyond the P depletion zone and exploits a greater volume of soil than that possible by roots alone (Jansa et al., 2011; Smith and Smith, 2011). The fungi can efficiently take up P from the soil and transfer it to the plant, thus increasing plant productivity (Smith and Read, 2008). The symbiosis occurs in almost all economically important crops worldwide. Because of the capacity of AMF to improve plant P acquisition, there is much interest in applying AMF in agriculture to increase crop yields while simultaneously reducing the amount of P fertilizer applied to crops. This is especially so in the tropics because of the severe difficulties for crops to obtain enough P in much of the agricultural soils.

Based on the results of many experimental studies in the laboratory and greenhouse, it is widely accepted that the development of AMF inside plant roots is regulated by P availability to the plant. *In vitro* experiments have demonstrated that under P limitation, plant roots increase the production of signal molecules stimulating the establishment of the symbiosis (Nagahashi et al., 1996; Nagahashi and Douds, 2000). When P is applied to plants, the plant regulates colonization by AMF such that colonization decreases with increasing available P (Mosse, 1973; Thomson et al., 1986; Smith and Read, 2008; Balzergue et al., 2010; Breuillin et al., 2010). The molecular basis in plants for this P-induced suppression of AMF colonization has also been experimentally demonstrated (Breuillin et al., 2010; Salvioli di Fossalunga and Novero, 2019) as well as how the supply of inorganic P influences the expression of genes regulating P homeostasis in the fungus itself (Ezawa and Saito, 2018). Additionally, studies have demonstrated that applying more P reduces the benefit to plant growth afforded by inoculating the plants with AMF (Bruce et al., 1994; Valentine et al., 2001; Xu et al., 2014). The ability of plants to limit AMF colonization under high P supply is considered a strategy to limit carbohydrate consumption by AMF when the fungi are less needed for P uptake from soil. The negative relationship between P supply and both reduction in colonization and plant benefit has mostly been demonstrated in one-to-one models (one plant cultivar inoculated with one AM fungus isolate), but the same relationship has been observed with a very large variety of different plants and different AMF species and isolates (Mosse, 1973; Bruce et al., 1994; Balzergue et al., 2010; Breuillin et al., 2010). The relationship between increasing P application and decreasing AMF colonization and plant responsiveness to inoculation with AMF is so well-demonstrated experimentally that we refer to it here as the P inhibition paradigm.

Because of the P inhibition paradigm, there is an overriding assumption that if the crop is sufficiently well-fertilized with P and other nutrients, then it will not benefit from inoculation with AMF. However, the markedly phytocentric view in most experimental studies regarding this topic is possibly a serious oversimplification (Smith and Read, 2008) and possibly does not hold true in field situations. Under field conditions, responses to increasing P supply are potentially more complex. Several field trials have reported decreases in AMF colonization of roots and/or mycorrhizal benefit in crops with P fertilization (Thingstrup et al., 1998; Kahiluoto et al., 2001; Al-Karaki, 2006; Verbruggen et al., 2012). However, very low P availability (such as the levels common in some tropical soils) can inhibit AMF colonization so that additions of P are required to stimulate colonization (Bolan et al., 1984; de Miranda and Harris, 1994). More recently, Higo et al. (2020) reported that AMF colonization of tomato roots does not always decrease with increasing P supply in the field in temperate soil, although in a short-term (7 weeks) experiment. At present, the current P inhibition paradigm is yet to be generalized in the field. Moreover, to the knowledge of the authors, there are no published studies testing the P paradigm in globally important crops in tropical soils.

Cassava (*Manihot esculenta* Crantz.) is known to be one of the crops that are most responsive to AMF inoculation (Howeler et al., 1982). This tropical root crop is an important source of carbohydrates for almost 800 million people living in tropical and sub-tropical regions of the world (El-Sharkawy, 2006; FAO, 2013). P availability is a crucial factor determining cassava productivity (Howeler et al., 1982; Smith et al., 2011), and P fertilization is considered essential to improving cassava yields. Pioneering field experiments in Colombia showed that cassava is strongly influenced by AMF inoculation for growth and nutrition in low-P soils (Howeler, 1981; Howeler et al., 1982; Howeler and Sieverding, 1983; Sieverding and Howeler, 1985). The AMF species *Rhizophagus irregularis* is a promising candidate for the inoculation of cassava because of its easy cultivation *in vitro* in a sterile medium and its very wide niche, occurring in agricultural soils on several continents (Ceballos et al., 2013; Savary et al., 2018). The application of *in vitro*-produced *R. irregularis* was shown to improve the productivity of cassava (Ceballos et al., 2013; Aliyu et al., 2019).

Field trials in Kenya and Tanzania have demonstrated that inoculation with different *R. irregularis* isolates, or offspring cultures of those isolates, influenced cassava root productivity enormously by up to a factor of 300% (Ceballos et al., 2019). Remarkably, the plants in those experiments were all fertilized with what is considered an optimal amount of nitrogen (N), P,

and potassium (K). These results are inconsistent with the P inhibition paradigm where the supply of sufficient P to cassava would have inhibited AMF colonization and reduced the effects of AMF inoculation on cassava growth to a negligible level. In those experiments, large differences in cassava responsiveness to AMF inoculation were observed among locations and cassava varieties at optimal fertilization levels.

In the present study, we investigated whether the application of different amounts of P fertilizer altered the responsiveness of cassava to inoculation by different *in vitro*-produced *R. irregularis* isolates and their offspring and influenced the colonization of cassava roots by AMF. We conducted field trials at three locations (one in Kenya and two in Tanzania), using a landrace and an improved cassava variety at each location. The soils at the three locations were considered very-low to medium-low P content soils (according to threshold values for cassava growth reported by Howeler, 2012). We hypothesized that (a) following the P inhibition paradigm, the benefit cassava receives from inoculation with AMF decreases with increasing P supply and that AMF colonization will also decrease irrespective of the identity of the AMF identity or cassava variety; (b) genetically different AMF isolates will not have the same effect among cassava varieties and locations under different P fertilization levels. To verify the hypotheses, we performed an experiment at three locations in Africa (**Supplementary Figure S1**), where a cassava growth response to P fertilization was expected due to the defined low P content of the soils.

MATERIALS AND METHODS

Field Sites

The experiments were established at three different locations: (1) Ukwala-Kawayo, Siaya County, Kenya (00° 15' 12.1" N; 34° 10' 32.7" E); (2) Kayenze, Biharamulo district, Tanzania (03° 12' 3.33" S; 31° 26' 37.18" E); and (3) Kijuka, Sengerema district, Tanzania (02° 35' 20.41" S; 32°35'42.79" E; **Supplementary Figure S1**). Climatic and soil physical and chemical characteristics of the three locations are shown in **Supplementary Tables S1**, **S2**. The soil P content at each of the three locations in this study spanned the range from very low to medium-low according to the specific cassava threshold values (low: 2–4 mg.kg^{-1} and medium: 4–15 mg.kg^{-1}) reported by Howeler (2012).

Plant and Fungal Material

At each location, we planted improved cassava (*M. esculenta*) variety that had been bred for disease resistance and a locally grown landrace (**Supplementary Table S3**). The choice of which improved variety was planted at each location was based on recommendations by the International Institute of Tropical Agriculture (IITA). The choice of landrace at each location was based on which variety the local farmers frequently cultivated, and the landrace was different at each location. Inoculum of the AMF species *R. irregularis* was produced in an *in vitro* culture system by Symbiom s.r.o. (Lanskroun, Czech Republic) and mixed with a sterile inert carrier (calcified diatomite). The culture conditions, including the type of medium and temperature for incubation, were previously described by Rosikiewicz et al. (2017). Six *R. irregularis* cultures were chosen based on preliminary results of a previous field trial conducted at Ukwala-Kawayo, Kenya that was performed with a greater number of fungal cultures and the cassava landrace Fumba chai (Ceballos et al., 2019). The three *R. irregularis* cultures that resulted in the greatest cassava productivity in that experiment, namely C3.14, C3.16, and C3.22, and the three cultures that gave the lowest cassava productivity, namely C2, C3, and A5.8, were chosen for this study. *Rhizophagus irregularis* C2 and C3 are isolates that originated from the field at Hausweid, Tänikon, Switzerland (Koch et al., 2004). C3.14, C3.16, and C3.22 are progeny cultures of parental isolate C3, each initiated from a single spore of the parental isolate (Ceballos et al., 2019). A5.8 is a progeny culture of parental isolate A5 that was isolated at the same time and from the same field as C2 and C3. We refer to these as progeny or single spore lines (SSLs). The parental isolates have been maintained as *in vitro* cultures in identical conditions at the University of Lausanne since 2000, and the SSL cultures were established *in vitro* in 2015 in the same conditions.

Design and Establishment of the Field Experiments

Cassava was planted and inoculated in a randomized block design with eight blocks at each location. The experiment comprised eight inoculation treatments with the six *R. irregularis* cultures, as well as two control treatments: no inoculation and inoculation with the carrier but with no fungus. There were two cassava varieties, namely one landrace and one improved variety, and three locations. There were three levels of P fertilization, namely 0, 50, and 100%, where 100% represented the recommended dose for optimal cassava growth (**Supplementary Note S1**), based on soil chemical analyses at each location. Thus, there were 48 treatment combinations and each of the eight blocks contained all the 48 treatment combinations (**Supplementary Figure S2**). Plots containing nine plants represented one experimental unit (or replicate) that received one of the 48 treatment combinations, and each plot was surrounded by 16 non-inoculated plants (**Supplementary Figure S2**). The experiment at each location covered an approximate area of 1 ha with a planting density of 10,000 plants ha^{-1}.

Cassava stem cuttings (30 cm long) were inoculated with 1 g of diatomite carrier containing 1,000 fungal spores of a given fungal parental isolate or SSL, and this was placed around the stem of the cassava at planting. Fertilizer was applied between 30 and 45 days after planting (DAP). The amount of fertilizer applied was determined by the initial soil nutrient content, nutritional requirements of cassava, and fertilizer efficiency (**Supplementary Note S1**). The nutritional requirements, proposed by the IITA, were as follows: 150 kg ha^{-1} N, 40 kg ha^{-1} P, and 180 kg ha^{-1} K (Ezui et al., 2016). The sources of fertilizer applied were urea, triple super phosphate (TSP), and muriate of potash. Cassava plants at different

locations received the appropriate amount of P so that at each location, plants in the 100% P treatment were exposed to 40 kg ha^{-1} available P. This was calculated separately for each site using the values of available P obtained in the soil analyses (**Supplementary Table S2**). The amount of P fertilizer added to the different treatments was adjusted by the dosage of TSP.

In Kayenze and Kijuka (Tz), the trials were established in January 2018 and harvested a year later. In Ukwala-Kawayo (Ke), the trial was sown in March 2018 and also harvested a year later.

Plant and Fungal Growth Measurements

The colonization of AMF in roots (% colonized root length) and root fresh weight (kg $plant^{-1}$) was measured at harvest time. Cassava fine roots were collected at a depth of 0–30 cm. Plants were uprooted by removing the soil around the stem and starchy roots with a shovel, and then manually pulling them out to assure that many of the fine roots came out with the main starchy root. Then, 3 g of fine roots were collected from the main roots after pulling out the plant from each cassava plant and stained following the method of Koske and Gemma (1989), except trypan blue was replaced by acid fuchsin 0.01%, and checked for AMF colonization using the grid-line intersection method (Giovanetti and Mosse, 1980). Previous studies have shown that fresh and dry root weights are the best traits explaining the cassava responsiveness to inoculation with AMF (Ceballos et al., 2013, 2019). In this study, root fresh weight (kg $plant^{-1}$) showed a strong linear correlation ($R^2 > 0.83$, $p < 0.001$) with root dry weight (kg $plant^{-1}$) regardless of the cassava variety (**Supplementary Figure S3**). Therefore, here, we only report further analyses concerning root fresh weight at harvest as it is a good estimator of root biomass production. In addition, we only report on this variable because fresh cassava roots are the edible part of the plant, which directly corresponds to the yield of the crop. Additionally, to have a standardized measure of mycorrhizal effects on plants inoculated with different inoculation treatments, we calculated the direction of the productivity response of plants to inoculation with a given *R. irregularis* culture compared to both non-inoculated plants and plants inoculated with the carrier but without fungus by a modification of mycorrhizal responsiveness (measured as the biomass difference between inoculated plants and non-inoculated controls; Janos, 2007). In this study, we refer to this as inoculation responsiveness. We only discuss the results of inoculation responsiveness where it was observed that both calculations of responsiveness, using either the non-inoculated plants or the carrier without fungus, gave the same result.

Statistical Analyses

Statistical analyses were conducted with R (R Development Core Team, 2019; v3.5.3) and JMP® v14.2.0 (SAS Institute Inc.). A linear correlation analysis was performed on fresh and dry root weight. Before ANOVA, the data were tested for normality using a Shapiro–Wilk test and for homoscedasticity using Bartlett's and Levene's tests. Root fresh weight and AMF colonization (%) were normally distributed but with unequal variance, thus violating the assumptions of conventional ANOVA. Therefore, Welch's ANOVA was performed, followed by a Games-Howell *post hoc* test.

In this study, there were four main factors: location, cassava variety, P fertilization level, and AMF inoculum. However, because different cassava varieties were planted among locations, and locations differ in soil characteristics (**Supplementary Tables S1**, **S2**, **S3**), the data revealed a significant interaction effect between the two main factors: location and cassava variety. The nature of the data greatly hindered the statistical power of a generalized model-based analysis even with using random factor assumption. Therefore, following the testing of the P fertilization effect on root fresh weight and mycorrhizal colonization rate in a global analysis, we divided the data by location and by cassava variety for further testing the effect of P fertilization and AMF inoculation. The first hypothesis we tested was that AMF colonization and the benefit cassava receive from AMF inoculation decreases with increasing P supply. For this, we first tested if there is any difference in cassava root fresh weight or AMF colonization in both cases of with and without inoculation and by the level of P fertilization in the global dataset. After this, we divided the dataset by location and cassava variety to test the same question. We further calculated the inoculation responsiveness and tested if this was affected by P fertilization in each cassava variety and at each location separately. There were two different inoculation responsiveness measurements calculated using two different controls: non-inoculated plants and plants inoculated with the carrier but without fungus. Therefore, statistical tests were conducted independently for each of the two different calculations of inoculation responsiveness. The second hypothesis was that genetically different AMF isolates will not induce the same inoculation responsiveness among cassava varieties and locations under different P fertilization levels. To test this hypothesis, we compared the inoculation responsiveness of different AMF isolates at each level of P, with each cassava variety, and at each location.

RESULTS

Effects of Location, Cassava Variety, and P Fertilization on Root Fresh Weight and AMF Colonization

We first analyzed the effects of the main factors on cassava root fresh weight or AMF colonization in both cases, with and without inoculation. A global analysis of the whole dataset (including all the data from the three locations, all cassava varieties, and AMF treatments) was conducted first to assess the general effect of each of the main factors on root fresh weight (**Supplementary Table S4**). The results showed that cassava root fresh weight significantly differed among locations, cassava variety, and P treatment. Although cassava root fresh weight in the whole dataset was significantly greater when plants were fertilized with the 100% P compared to the 0% P treatment, the differences in root weight were extremely small (**Figure 1**; **Supplementary Table S4**). There was no

significant difference in root weight between the 50 and 100% P treatments. We also performed the same analysis on AMF colonization to see if there were differences among P treatments. However, even though, a mean colonization of over 30% was observed in all P treatments [0% P = 32.93 (mean) ± 1.71 (SE), 50% P = 35.50 ± 1.67, and 100% P = 32.18 ± 1.54], there were no significant differences among the treatments.

Because there were large variations in cassava root weight among locations and cassava varieties, and because the cassava varieties were not always the same at each location, an overall analysis considering locations and cassava varieties as random effects resulted in the loss of statistical power. Therefore, we separated the dataset by location and cassava variety to further investigate the effect of P treatment on cassava root fresh weight and AMF colonization. This analysis revealed location-specific and variety-specific responses to P fertilization, which were masked in the analysis of the whole dataset (**Figure 2**). P fertilization significantly increased root fresh weight in both cassava varieties in Kayenze (**Figure 2A**). Root fresh weight of the landrace Mwanaminzi variety significantly increased with increasing P fertilization in Kijuka but the improved variety (Mkombozi) was unresponsive (**Figure 2A**). There was no effect of P fertilization on root fresh weight in Ukwala-Kawayo in either variety.

The fertilization of cassava with different levels of P did not affect the AMF colonization of cassava roots in Kayenze in either variety (**Figure 2B**). In Kijuka, the colonization of cassava roots by AMF in the improved variety (Mkombozi) was unaffected by P fertilization. Colonization of the roots by AMF in the landrace Mwanaminzi variety was significantly lower at 100% P compared to 50% P fertilization (**Figure 2B**). There was a significant effect of P fertilization on the AMF colonization in both cassava varieties in Ukwala-Kawayo, where higher P application resulted in higher AMF colonization.

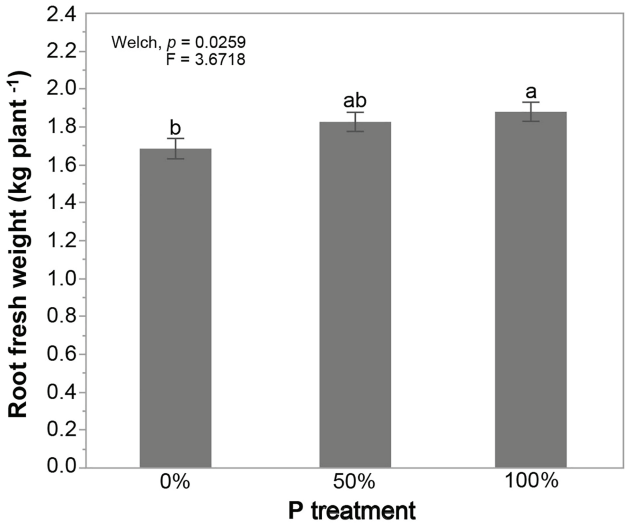

FIGURE 1 | Effects of phosphorus (P) fertilization on cassava root fresh weight averaged over the three locations, all cassava varieties, and all arbuscular mycorrhizal fungi (AMF) treatments. Error bars represent ±SE. Means with different letters are significantly different at $p < 0.05$ according to a Games-Howell *post hoc* test.

Even though in this study, we were not able to distinguish root colonization of the inoculated AMF from that of the native AMF community, there was no significant difference in root fresh weight and AMF colonization between the non-inoculated controls and inoculation treatments at different levels of P supply, in each plant variety and at each location. In general, there was no significant difference in root fresh weight or AMF colonization observed in non-inoculated controls following different levels of P application (**Supplementary Figure S4**). Only one case of an increase in root fresh weight at 50 and 100% P compared to 0% P was observed (Mkombozi variety in Kayenze; **Supplementary Figure S4**). However, the same result was also observed in plants inoculated with AMF (**Figure 2**; **Supplementary Figure S4**). At the same time, the effect of P fertilization on AMF colonization was not significant between cassava varieties at any of the locations (**Supplementary Figure S4**).

Effect of P Fertilization on Inoculation Responsiveness of Cassava

To focus on the effect of AMF inoculum, we next tested whether the inoculation responsiveness of cassava decreased with increasing levels of P fertilization. Inoculation responsiveness of cassava was, indeed, significantly affected by the level of P fertilization, but this effect was not the same in each location and each of the two cassava varieties at each location (**Figure 3**). Inoculation responsiveness decreased with increasing P fertilization in Kayenze, supporting the hypothesis of the P inhibition paradigm (**Figures 3A,B**). However, different effects of P fertilization on inoculation responsiveness were observed in the other two locations. In Kijuka, the highest inoculation responsiveness was observed in the 100% P fertilization treatment in the cassava landrace Mwanaminzi (**Figures 3A,B**). In the improved variety Mkombozi, the highest mycorrhizal responsiveness was observed at 50 (**Figure 3A**) and 0% P (**Figure 3B**) according to which the control treatment was used to calculate responsiveness to inoculation and did not differ significantly from those in the 100% P treatment. In Ukwala-Kawayo, plants receiving 50% P treatment exhibited greater inoculation responsiveness than plants in the 0 and 100% P treatments (**Figures 3A,B**).

Effect of Host and Fungal Identity on Inoculation Responsiveness of Cassava

To investigate the effects of P fertilization on the inoculation responsiveness of cassava to the different AMF inoculation treatments, we further analyzed inoculation responsiveness to P fertilization in each cassava variety and each AMF inoculation treatment. Out of 36 tests, eight tests revealed that inoculation responsiveness of a cassava variety differed among P treatments but depended on the fungus the plants were inoculated with **Supplementary Table S5**. For example, in Kijuka, the improved cassava variety Mkombozi was significantly more responsive to inoculation with A5.8 at 50% P application, compared to either 0 or 100% P. In the other landrace (Mwanaminzi), the

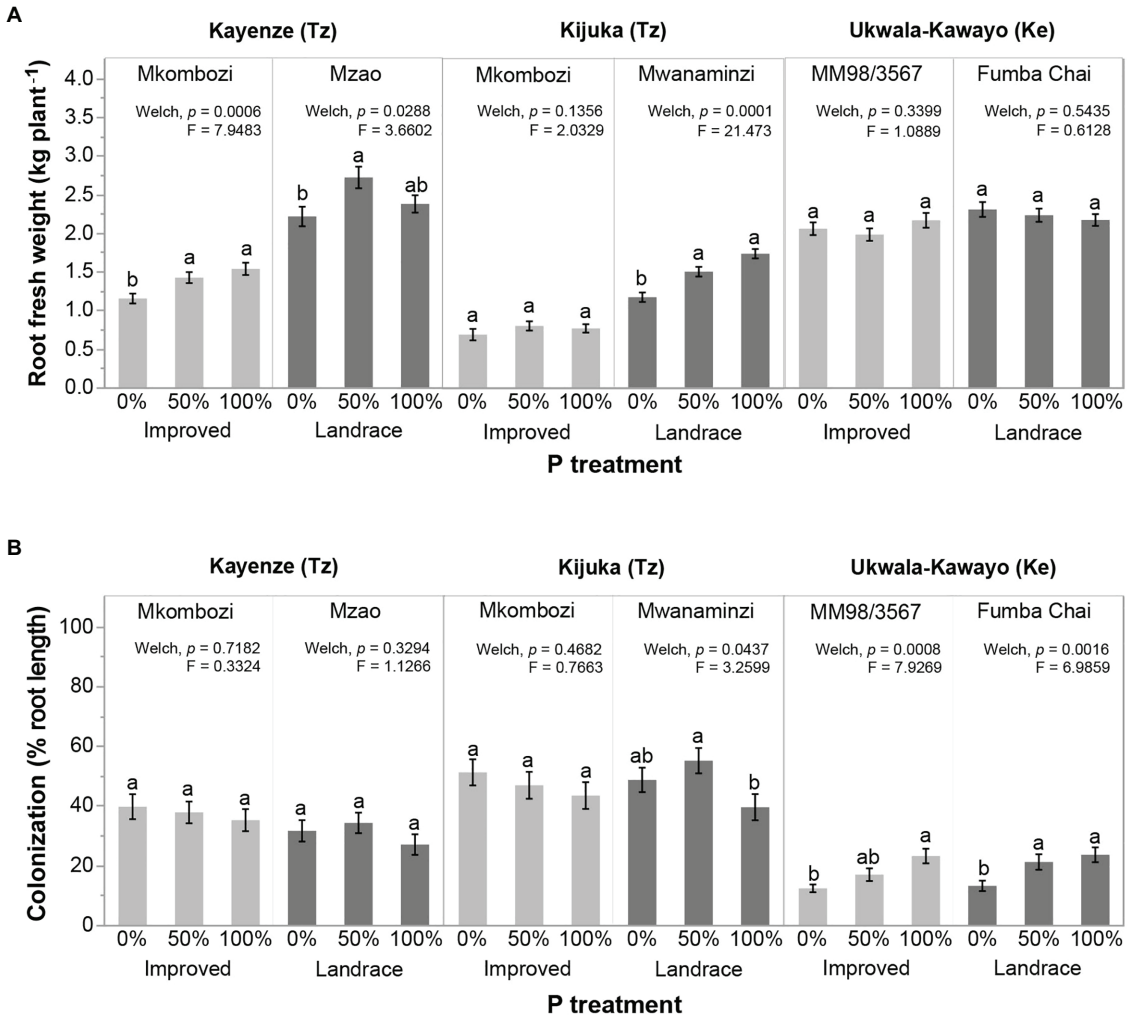

FIGURE 2 | Effects of P fertilization on **(A)** cassava root fresh weight (kg plant^{-1}) and **(B)** AMF colonization (% root length) of AMF inoculated treatments at each of the three locations and in the two cassava varieties grown at each location. Error bars represent ±SE. Means with different letters are significantly different at $p < 0.05$ according to a Games-Howell *post hoc* test. The statistical test represents a comparison among P treatments within each cassava variety at each location. Tz, Tanzania; Ke, Kenya.

responsiveness to inoculation with A5.8 was significantly lower in the 50% P treatment compared to 0 and 100% P (**Figure 4A**). At the same location, the responsiveness of the improved variety Mkombozi to inoculation with C3.16 was significantly higher at 50% P than at 0% P. The landrace Mwanaminzi was more responsive to inoculation with C3.16 at 100% P than at 50% P (**Figure 4B**).

We tested whether the inoculation responsiveness of cassava to different inoculation treatments was the same among cassava varieties and locations under different P fertilization treatments. We observed that inoculation responsiveness to the different AMF treatments, at a given P treatment, was sometimes completely opposing, depending on the cassava variety. For example, the inoculation responsiveness of the Mkombozi variety in Kijuka at 0% P, when inoculated with C2, was positive, while that of plants inoculated with C3.16 showed negative responsiveness (**Figure 5**). However, the opposite pattern was detected at the same location in the Mwanaminzi variety when plants were fertilized with 100% P (**Figure 5**). The same pattern of inoculation responsiveness was observed when calculated by comparing inoculated plants to the carrier without fungus treatment (data not shown).

DISCUSSION

The P inhibition paradigm, where increased P supply suppresses AMF colonization, as well as plant growth responsiveness to the symbiosis, has been fundamental in choosing where the application of AMF is considered appropriate. This study showed that in tropical soils, AMF colonization and responsiveness to inoculation with increasing application of P fertilizer did not follow the trajectory expected from the P inhibition paradigm. Therefore, the first hypothesis of the P inhibition paradigm

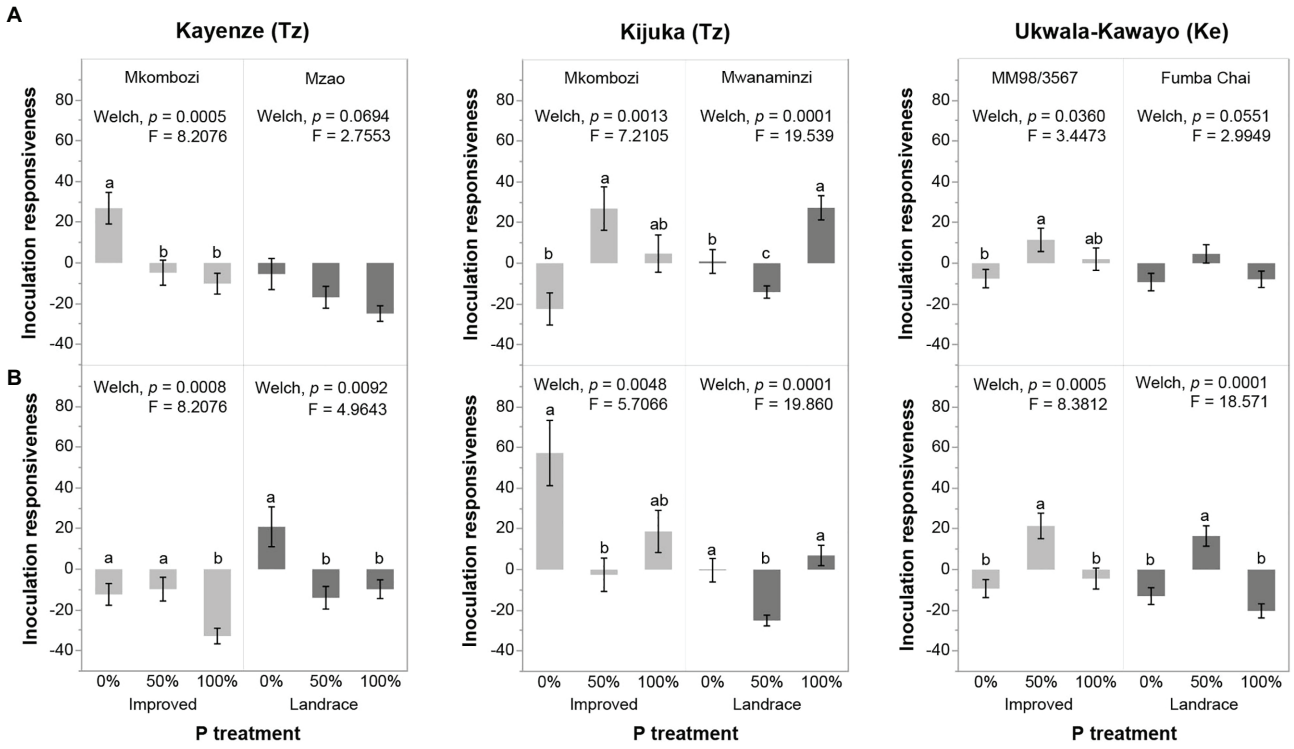

FIGURE 3 | Inoculation responsiveness of cassava to AMF inoculation at the three different P fertilization levels, in two cassava varieties at each of three locations. Inoculation responsiveness was calculated based on cassava root fresh weight response to AMF inoculation compared to **(A)** the non-inoculated treatment and **(B)** the carrier without fungus treatment. Error bars represent ±SE. Means with different letters are significantly different at $p < 0.05$ according to a Games-Howell *post hoc* test. *Post hoc* comparisons were among P treatments within each cassava variety at each location separately. Tz, Tanzania; Ke, Kenya.

is rejected. We found that AMF colonization and responsiveness to inoculation, indeed, differ with increasing P application but not in a way that is predicted by the paradigm. Effects of location, cassava variety, and AMF isolate all play a significant role in determining AMF development in cassava roots and plant responsiveness to inoculation at different levels of P application. The results of this study supported the second hypothesis that genetically different AMF isolates do not have the same effect among cassava varieties and locations under different P fertilization levels, even though the AMF isolate identity was not the sole determinant of plant responsiveness to AMF inoculation under different P supply. We discuss these in more detail below and point out where these results help to suggest where research should be focused on to make successful AMF applications more predictive in agriculture.

Site-Specific Effects

We observed that the way cassava root weight, AMF colonization, and responsiveness to inoculation responded to increasing P application was strongly influenced by location. Despite the soils in these locations being very deficient to moderately deficient in available P, increased P application only induced greater cassava productivity in three of the six cases surveyed in the present study: two varieties at one location, Kayenze, and one variety at Kijuka. However, in these cases, AMF colonization was either unaffected (in Kayenze) or higher in the 50% P treatment than in the 100% P treatment (Kijuka). In Kayenze, responsiveness to inoculation was suppressed with increasing P treatments, but in Kijuka, responsiveness to inoculation was significantly lower in the 50% P treatment compared to both of the other treatments, thus not following the hypothesized pattern. In Ukwala-Kawayo, P application had the complete opposite effect on AMF colonization to that predicted by the P inhibition paradigm, with AMF colonization increasing with increasing P application in both cassava varieties. In these cases, responsiveness to inoculation also did not follow that expected by the P inhibition paradigm.

Cassava Variety and AMF Identity Effects

Interestingly, the variety of cassava did not play a very important role in determining the AMF colonization responses to increasing P application but greatly affected responsiveness to inoculation. In Kijuka, responsiveness to inoculation in the two varieties was opposite, with responsiveness being highest in one variety at 50% P application and lowest in this treatment in the other variety. Again, these results are not consistent with the P inhibition paradigm. This was especially true for certain fungal treatments where responsiveness to inoculation differed among P treatments but in an opposite way in the two cassava varieties (**Figure 5**). Responsiveness to inoculation also differed significantly according to AMF identity, but again, which fungus induced the most responsiveness to inoculation differed markedly

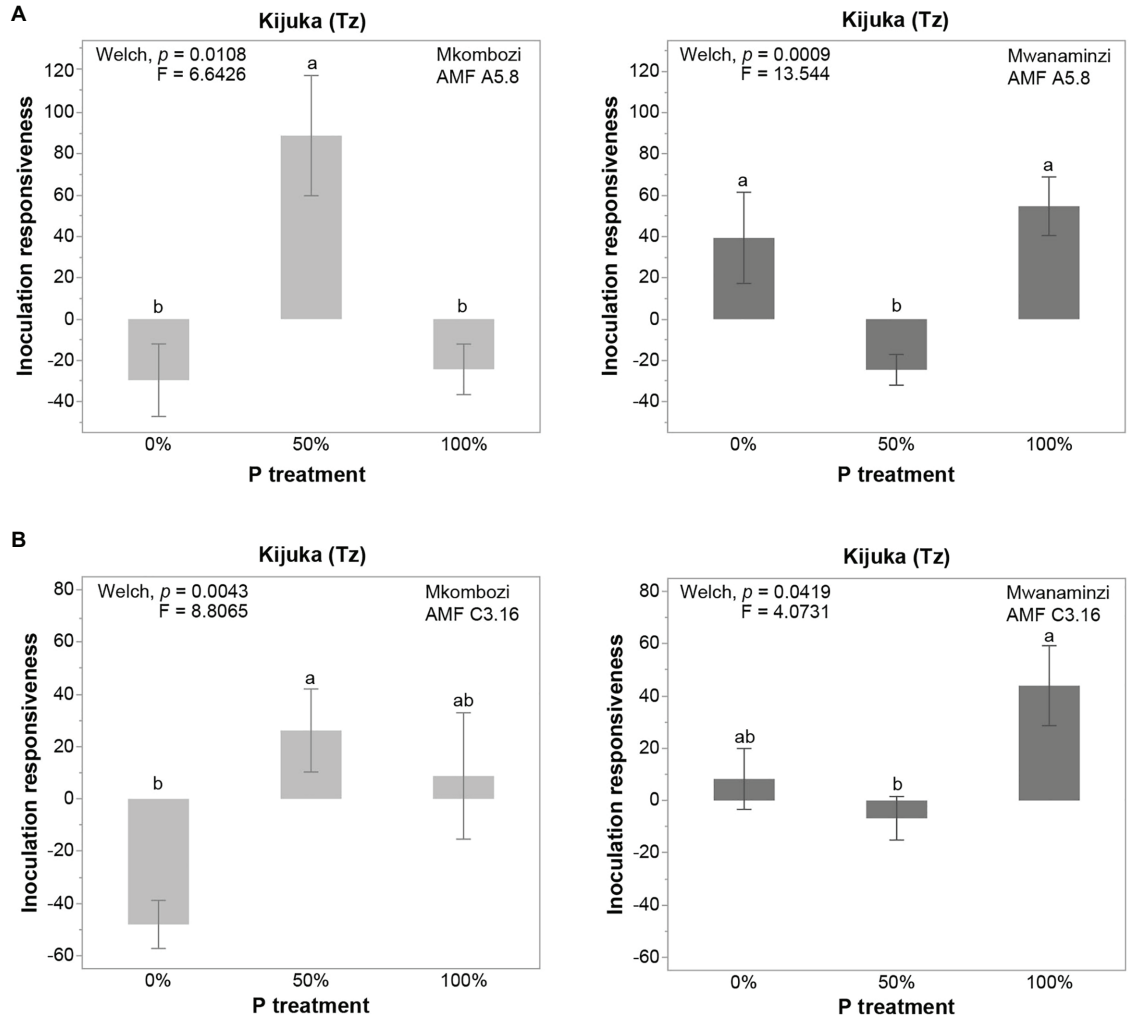

FIGURE 4 | Inoculation responsiveness of Mkombozi and Mwanaminzi varieties to inoculation with *Rhizophagus irregularis* **(A)** culture A5.8 and **(B)** culture C3.16, subjected to different P fertilization treatments at Kijuka. Error bars represent ±SE. Means with different letters are significantly different at $p < 0.05$ according to a Games-Howell *post hoc* test. Tz, Tanzania. Inoculation responsiveness was calculated by comparing inoculated plants to non-inoculated plants.

between no P application and 100% P application. Again, this would not be predicted by the P inhibition paradigm.

Explanations for the Lack of P-Induced Mycorrhizal Suppression

One possible explanation for the lack of P-induced mycorrhizal suppression is that added P fertilizer was not available to the plants because of P fixation in these soils. Although P availability after the application was not measured, this appears highly unlikely because cassava root weight was P responsive in some cases regardless of location. Furthermore, a significant P application effect on the responsiveness to inoculation, with maximum responsiveness at 50 or 100% P application, is not consistent with the hypothesis that the added P had been fixed in the soil rendering it unavailable. The suppressive effect of P application is known to be attenuated by limited N availability (Nouri et al., 2014), but in these experiments, cassava was supplied with sufficient N.

Among-location differences in P responsiveness may reflect differences in the physical or chemical characteristics of the soils. Such characteristics have previously been shown to affect the AMF-plant symbiosis and alter P uptake and exchange of other nutrients between partners (Liu et al., 2000; Entry et al., 2002; Carrenho et al., 2007; Zaller et al., 2011; Howeler, 2012). However, it cannot explain within-location differences in how cassava varieties or AMF identity influenced plant responsiveness to inoculation at different levels of P fertilization, which were not consistent with that expected by the P inhibition paradigm.

Arbuscular Mycorrhizal Fungi Community Responses to Changes in P Availability

One large difference between many published experiments that demonstrate the existence of the P inhibition paradigm and these field trials is that most of the studies have been conducted in sterile soil where plants were inoculated with one AMF

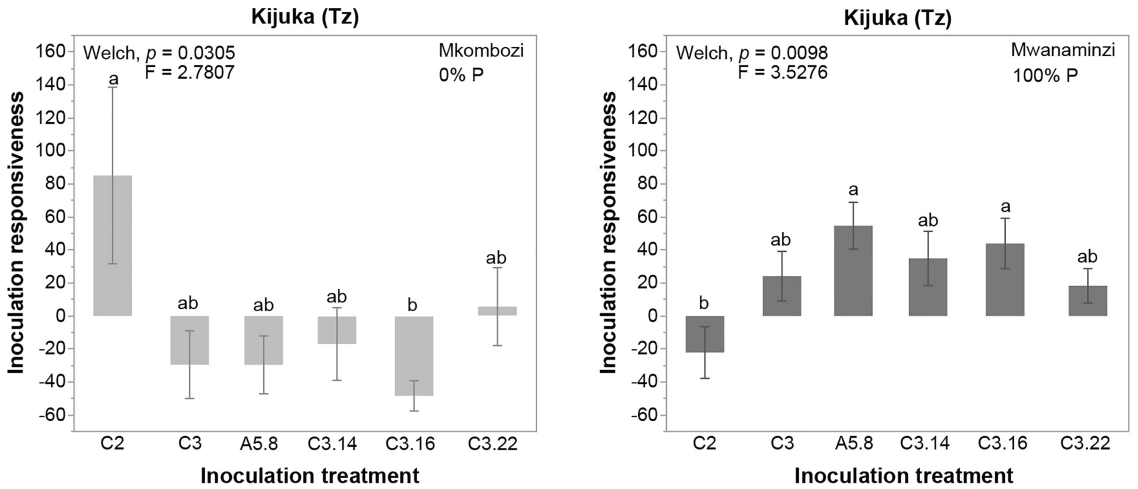

FIGURE 5 | Responsiveness of Mkombozi and Mwanaminzi varieties to inoculation with six *R. irregularis* inoculation treatments and at three levels of P fertilization. Error bars represent ±SE. Means with different letters are significantly different at $p < 0.05$ according to a Games-Howell *post hoc* test. Tz, Tanzania. Inoculation responsiveness was calculated by comparing inoculated plants to non-inoculated plants.

isolate. In agricultural soils, AMF is almost always present. Thus, many previous studies have assessed how colonization by one AMF taxon, in the absence of other microbes, responds to the effects of P application. In infield trials, AMF colonization represents the response of the pre-existing AMF community, plus the introduced taxa, to different levels of P supply. In several cases, P supply did not suppress mycorrhizal colonization and even had the opposite effect. This indicates that either the taxa we added and/or all or some of the components of the AMF community were not adversely affected by increased P supply. Indeed, the AMF community structure in both the soil and the root can be altered by P supply (Verbruggen et al., 2012; Xu et al., 2018). Thus, we conclude that in the soils where we conducted this study, AMF taxa existed that is not adversely affected by the presence of increased P concentrations.

Because of the pre-existing AMF community and microbiome in agricultural soils, measuring the responsiveness to inoculation in field trials represents the response of plants to the inoculant, in the presence of the AMF community and other components of the microbiome, compared to the effect of the pre-existing AMF community and microbiome alone. This is very different from pot experiments comparing an inoculated plant to a non-mycorrhizal control. In several cases, in this study, we report responsiveness to inoculation that is inconsistent with the P inhibition paradigm. Phosphate availability to roots can be dynamic in time as there should be a gap between the timing of P depletion near AMF hyphae in soil, the growth of new foraging hyphae, and plant uptake of soil P. Consequently, this can affect the temporal patterns of plant P nutrition that can further modulate the AMF community colonizing roots and affect plant responsiveness to inoculation. However, the inoculation responsiveness was measured 1 year after inoculation and is, therefore, based on the accumulation of all possible temporal changes in plant P nutrition and the associated AMF community until the harvest. Therefore, the accumulated effect can help the understanding of the overall impact of inoculation under different P supply. We see the addition of the introduced mycorrhizal taxa as a potential perturbation of the community and that change in the existing microbial community. While we did not measure the effects of inoculation on the AMF community at different levels of P application, it is already known that adding AMF can alter the structure of existing experimental AMF communities (Janoušková et al., 2013). Indeed, adding different *R. irregularis* isolates or their offspring to cassava in tropical soils has been shown to alter both the alpha and beta diversity of AMF communities (Ordoñez et al., 2020). Therefore, we think the effects observed in this study are most likely to be due to mediation by the pre-existing AMF community and the microbiome.

Conclusion: Challenging the P Inhibition Paradigm

Given the overwhelming number of experimental studies, we do not put into question the existence of the P inhibition paradigm and the mechanism afforded by plants to limit AMF colonization. However, the results of this study strongly suggest that the paradigm cannot be used as a valid generalization for whether or not inoculation will be effective in tropical soils or that AMF should only be applied in P-deficient soils in the absence of P fertilization. The fact that the location, plant variety, and AMF identity all play a role in how P fertilization affects responsiveness to inoculation means that we do not currently have a way of predicting where and in what management conditions, AMF inoculation will be effective. Despite this, the fact that inoculation with different *R. irregularis* strains, in the presence of a pre-existing AMF community and with 100% P fertilization, can alter cassava productivity by up to 300% in these soils (Ceballos et al., 2019) shows that finding the predictors of mycorrhizal responsiveness is a highly pertinent

and valuable research pursuit. We propose that this could be addressed by measuring responsiveness to inoculation and screening AMF communities in roots in a large-scale set of field trials, replicated across large edaphic gradients and environmental conditions, as well as with different cassava varieties. A metadata set generated from such trials would allow researchers to search for associations among responsiveness to inoculation, the identity of the fungal inoculant, cassava variety, and climatic and edaphic variables, and above all, the composition of the soil microbial community. We propose that this would allow researchers to predict which combination of factors will give optimal responsiveness to AMF inoculation in a given location.

DATA AVAILABILITY STATEMENT

The original data collected in the present study are included in the article/**Supplementary Material**. Further inquiries can be directed to the corresponding author.

AUTHOR CONTRIBUTIONS

RAPV conceived and conducted the experiments, analyzed the data, interpreted the data, and wrote the manuscript. S-JL analyzed and interpreted the data, and wrote the manuscript. MT conceived and conducted the experiments. DM conducted the experiments in Tanzania. CM and BV conceived the experiments. AR and IS conceived the experiments, interpreted the data, and wrote the manuscript. All authors contributed to the article and approved the submitted version.

FUNDING

This research was funded by the Swiss National Science Foundation (Grant number: CR31l3-159665) and by a Swiss Government Excellence Scholarship to RAPV.

ACKNOWLEDGMENTS

We thank Aleš Látr at Symbiom s.r.a. for the large-scale *in vitro* production of the AMF inocula and Miroslav Vosatka for advice. We thank the farmers in Western Kenya and Northern Tanzania for allowing us to perform the field trials. We also thank Jeremy Bonvin, Diego Camilo Peña, Elias Mwangi, Robert S. Ngomuo, Joseph J. Simuda, Celvin Tibihenda, Paul Kibinza, and Dr. Simon C. Jeremiah. We thank three reviewers for their valuable comments that helped improve the manuscript.

SUPPLEMENTARY MATERIAL

The Supplementary Material for this article can be found online at: https://www.frontiersin.org/articles/10.3389/fpls.2021.693037/full#supplementary-material

REFERENCES

Aliyu, I. A., Yusuf, A. A., Uyovbisere, E. O., Masso, C., and Sanders, I. R. (2019). Effect of co-application of phosphorus fertilizer and in vitro-produced mycorrhizal fungal inoculants on yield and leaf nutrient concentration of cassava. *PLoS One* 14:e0218969. doi: 10.1371/journal. pone.0218969

Al-Karaki, G. N. (2006). Field response of garilic inoculated with arbuscular mycorrhizal fungi to phosphorus fertilization. *J. Plant Nutr.* 25, 747–756. doi: 10.1081/PLN-120002956

Balzergue, C., Puech-Pagès, V., Bécard, G., and Rochange, S. F. (2010). The regulation of arbuscular mycorrhizal symbiosis by phosphate in pea involves early and systemic signalling events. *J. Exp. Bot.* 62, 1049–1060. doi: 10.1093/jxb/erq335

Bolan, N. S., Robson, A. D., and Barrow, N. J. (1984). Increasing phosphorus supply can increase the infection of plant roots by vesicular-arbuscular mycorrhizal fungi. *Soil Biol. Biochem.* 16, 419–420.

Breuillin, F., Schramm, J., Hajirezaei, M., Ahkami, A., Favre, P., Druege, U., et al. (2010). Phosphate systemically inhibits development of arbuscular mycorrhiza in Petunia hybrida and represses genes involved in mycorrhizal functioning. *Plant J.* 64, 1002–1017. doi: 10.1111/j.1365-313X.2010.04385.x

Bruce, A., Smith, S. E., and Tester, M. (1994). The development of mycorrhizal infection in cucumber: effects of P supply on root growth, formation of entry points and growth of infection units. *New Phytol.* 127, 507–514. doi: 10.1111/j.1469-8137.1994.tb03968.x

Brundrett, M., and Tedersoo, L. (2018). Evolutionary history of mycorrhizal symbioses and global host plant diversity. *New Phytol.* 220, 1108–1115. doi: 10.1111/nph.14976

Cakmak, I. (2002). Plant nutrition research: priorities to meet human needs for food in sustainable ways. *Plant Soil* 247, 3–24. doi: 10.1023/A:1021194511492

Carrenho, R., Trufem, S. F. B., Bononi, V. L. R., and Silva, E. S. (2007). The effect of different soil properties on arbuscular mycorrhizal colonization of peanuts, sorghum and maize. *Acta Bot. Bras.* 21, 723–730. doi: 10.1590/S0102-33062007000300018

Ceballos, I., Mateus, I. D., Peña, R., Peña-Quemba, D. C., Robbins, C., Ordoñez, Y. M., et al. (2019). Using variation in arbuscular mycorrhizal fungi to drive the productivity of the food security crop cassava. bioRxiv [Preprint]. doi: 10.1101/830547

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

de Miranda, J. C. C., and Harris, P. J. (1994). The effect of soil phosphorus on the external mycelium growth of arbuscular mycorrhizal fungi during the early stages of mycorrhiza formation. *Plant. Soil.* 166, 271–280. doi: 10.1007/BF00008340

El-Sharkawy, M. A. (2006). International research on cassava photosynthesis, productivity, eco-physiology, and responses to environmental stresses in the tropics. *Photosynthetica* 44, 481–512. doi: 10.1007/s11099-006-0063-0

Entry, J. A., Rygiewicz, P. T., Watrud, L. S., and Donnelly, P. K. (2002). Influence of adverse soil conditions on the formation and function of Arbuscular mycorrhizas. *Adv. Environ. Res.* 7, 123–138. doi: 10.1016/S1093-0191(01)00109-5

Ezawa, T., and Saito, K. (2018). How do arbuscular mycorrhizal fungi handle phosphate? New insight into fine-tuning of phosphate metabolism. *New Phytol.* 220, 1116–1121. doi: 10.1111/nph.15187

Ezui, K. S., Franke, A. C., Mando, A., Ahiabor, B. D. K., Tetteh, F. M., Sogbedji, J., et al. (2016). Fertiliser requirements for balanced nutrition of cassava across eight locations in West Africa. *Field Crop Res.* 185, 69–78. doi: 10.1016/j.fcr.2015.10.005

FAO (2013). *Save and Grow: Cassava. A Guide to Sustainable Production Intensification*. Rome, Italy: FAO Publications: Food and Agriculture Organization of the United Nations.

Giovanetti, M., and Mosse, B. (1980). An evaluation of techniques for measuring vesicular mycorrhizal infection in roots. *New Phytol.* 97, 447–453. doi: 10.1111/j.1469-8137.1980.tb04556.x

Higo, M., Azuma, M., Kamiyoshihara, Y., Kanda, A., Tatewaki, Y., and Isobe, K. (2020). Impact of phosphorus fertilization on tomato growth and arbuscular mycorrhizal fungal communities. *Microorganisms* 8:178. doi: 10.3390/microorganisms8020178

Howeler, R. H. (1981). *Mineral Nutrition and Fertilization Of Cassava (Manihot esculenta Crantz)*. Colombia: CIAT (El Centro Internacional de Agricultura Tropical), Cali.

Howeler, R. H. (2012). "Diagnosis of nutritional problems of cassava," in *The cassava handbook: A reference manual based on the asian regional cassava training course, held in Thailand* (Bangkok, TH: Centro Internacional de Agricultura Tropical (CIAT)), 305–320.

Howeler, R. H., Cadavid, L. F., and Burckhardt, E. (1982). Response of cassava to VA mycorrhizal inoculation and phosphorus application in greenhouse and field experiments. *Plant Soil* 69, 327–339. doi: 10.1007/BF02372454

Howeler, R. H., and Sieverding, E. (1983). Potentials and limitations of mycorrhizal inoculation illustrated by experiments with field-grown cassava. *Plant Soil* 75, 245–261. doi: 10.1007/BF02375570

Janos, D. P. (2007). Plant responsiveness to mycorrhizas differs from dependence upon mycorrhizas. *Mycorrhiza* 17, 75–91. doi: 10.1007/s00572-006-0094-1

Janoušková, M., Krak, K., Wagg, C., Štorchová, H., Caklová, P., and Vosátka, M. (2013). Effects of inoculum additions in the presence of a preestablished arbuscular mycorrhizal fungal community. *Appl. Environ. Microbiol.* 79, 6507–6515. doi: 10.1128/AEM.02135-13

Jansa, J., Finlay, R., Wallander, H., Smith, F. A., and Smith, S. E. (2011). "Role of mycorrhizal symbioses in phosphorus cycling," in *Phosphorus in Action. Soil Biology. Vol. 26.* eds. E. Bünemann, A. Oberson and E. Frossard (Berlin, Heidelberg: Springer), 137–168.

Kahiluoto, H., Ketoja, E., Vestberg, M., and Saarela, I. (2001). Promotion of utilization of arbuscular mycorrhiza through reduced P fertilization. 2. Field studies. *Plant Soil* 231, 65–79. doi: 10.1023/A:1010366400009

Karandashov, V., and Bucher, M. (2005). Symbiotic phosphate transport in arbuscular mycorrhizas. *Trends Plant Sci.* 10, 22–29. doi: 10.1016/j.tplants.2004.12.003

Koch, A. M., Kuhn, G., Fontanillas, P., Fumagalli, L., Goudet, J., and Sanders, I. R. (2004). High genetic variability and low local diversity in a population of arbuscular mycorrhizal fungi. *Proc. Natl. Acad. Sci. U. S. A.* 101, 2369–2374. doi: 10.1073/pnas.0306441101

Koske, R. E., and Gemma, J. N. (1989). A modified procedure for staining roots to detect VA mycorrhizas. *Mycol. Res.* 92, 486–488. doi: 10.1016/S0953-7562(89)80195-9

Liu, A., Hamel, C., Hamilton, R. I., Ma, B. L., and Smith, D. L. (2000). Acquisition of Cu, Zn, Mn and Fe by mycorrhizal maize (*Zea mays* L.) grown in soil at different P and micronutrient levels. *Mycorrhiza* 9, 331–336. doi: 10.1007/s005720050277

Mosse, B. (1973). Plant growth responses to vesicular-arbuscular mycorrhiza IV. In soil given additional phosphate. *New Phytol.* 72, 127–136. doi: 10.1111/j.1469-8137.1973.tb02017.x

Nagahashi, G., and Douds, D. D. Jr. (2000). Partial separation of root exudates components and their effects upon the growth of germinated spores of AM fungi. *Mycol. Res.* 104, 1453–1464. doi: 10.1017/S0953756200002860

Nagahashi, G., Douds, D. D. Jr., and Abney, G. D. (1996). Phosphorus amendment inhibits hyphal branching of the VAM fungus Gigaspora margarita directly and indirectly through its effect on root exudation. *Mycorrhiza* 6, 403–408. doi: 10.1007/s005720050139

Nouri, E., Breuillin-Sessoms, F., Feller, U., and Reinhardt, D. (2014). Phosphorus and nitrogen regulate arbuscular mycorrhizal symbiosis in *Petunia hybrida*. *PLoS One* 9:e90841. doi: 10.1371/journal.pone.0090841

Ordoñez, Y. M., Villard, L., Ceballos, I., Masclaux, F. G., Rodriguez, A., and Sanders, I. R. (2020). Inoculation with highly-related mycorrhizal fungal siblings, and their interaction with plant genotypes, strongly shapes tropical mycorrhizal fungal community structure. bioRxiv [Preprint]. doi: 10.1101/2020.07.31.230490

Rahman, M., Lee, S.-H., Ji, H., Kabir, A., Jones, C., and Lee, K.-W. (2018). Importance of mineral nutrition for mitigating aluminum toxicity in plants on acidic soils: current status and opportunities. *Int. J. Mol. Sci.* 19:3073. doi: 10.3390/ijms19103073

R Development Core Team (2019). R: A Language and Environment for Statistical Computing. Vienna, Austria: R Foundation for Statistical Computing.

Rosikiewicz, P., Bonvin, J., and Sanders, I. R. (2017). Cost-efficient production of *in vitro Rhizophagus irregularis*. *Mycorrhiza* 27, 477–486. doi: 10.1007/s00572-017-0763-2

Salvioli di Fossalunga, A., and Novero, M. (2019). To trade in the field: the molecular determinants of arbuscular mycorrhiza nutrient exchange. *Chem. Biol. Technol. Agric.* 6:12. doi: 10.1186/s40538-019-0150-7

Savary, R., Masclaux, F. G., Droh, G., Cruz-Corella, J., Machado, A. P., Morton, J. B., et al. (2018). A population genomics approach shows widespread geographical distribution of cryptic genomic forms of the symbiotic fungus *Rhizophagus irregularis*. *ISME J.* 12, 17–30. doi: 10.1038/ismej.2017.153

Schachtman, D. P., Reid, R. J., and Ayling, S. M. (1998). Phosphorus uptake by plants: from soil to cell. *Plant Physiol.* 116, 447–453. doi: 10.1104/pp.116.2.447

Sharma, S. B., Sayyed, R. Z., Trivedi, M. H., and Gobi, T. A. (2013). Phosphate solubilizing microbes: sustainable approach for managing phosphorus deficiency in agricultural soils. *Springerplus* 2:587. doi: 10.1186/2193-1801-2-587

Sieverding, E., and Howeler, R. H. (1985). Influence of species of VA mycorrhizal fungi on cassava yield response to phosphorus fertilization. *Plant Soil* 88, 213–221. doi: 10.1007/BF02182447

Smith, S. E., Jakobsen, I., Gronlund, M., and Smith, F. A. (2011). Roles of arbuscular mycorrhizas in plant phosphorus nutrition: interactions between pathways of phosphorus uptake in arbuscular mycorrhizal roots have important implications for understanding and manipulating plant phosphorus acquisition. *Plant Physiol.* 156, 1050–1057. doi: 10.1104/pp.111.174581

Smith, S. E., and Read, D. J. (2008). *The mycorrhizal symbiosis*. San Diego, USA: Academic Press.

Smith, S. E., and Smith, F. A. (2011). Roles of arbuscular mycorrhizas in plant nutrition and growth: new paradigms from cellular to ecosystem scales. *Annu. Rev. Plant Biol.* 62, 227–250. doi: 10.1146/annurev-arplant-042110-103846

Thingstrup, I., Rubaek, G., Sibbesen, E., and Jakobsen, I. (1998). Flax (*Linum usitatissimum* L.) depends on arbuscular mycorrhizal fungi for growth and P uptake at intermediate but not high soil P levels in the field. *Plant Soil* 203, 37–46. doi: 10.1023/A:1004362310788

Thomson, B. D., Robson, A. D., and Abbott, L. K. (1986). Effects of phosphorus on the formation of mycorrhizas by Gigaspora calospora and Glomus fasciculatum in relation to root carbohydrates. *New Phytol.* 103, 751–765. doi: 10.1111/j.1469-8137.1990.tb00393.x

Valentine, A. J., Osborne, B. A., and Mitchell, D. T. (2001). Interactions between phosphorus supply and total nutrient availability on mycorrhizal colonization, growth and photosynthesis of cucumber. *Sci. Hortic.* 88, 177–189. doi: 10.1016/S0304-4238(00)00205-3

Vance, C. P. (2001). Symbiotic nitrogen fixation and phosphorus acquisition. Plant nutrition in a world of declining renewable resources. *Plant Physiol.* 127, 390–397. doi: 10.1104/pp.010331

Verbruggen, E., van der Heijden, M. G. A., Rillig, M. C., and Kiers, E. T. (2012). Mycorrhizal fungal establishment in agricultural soils: factors determining inoculation success. *New Phytol.* 197, 1104–1109. doi: 10.1111/j.1469-8137.2012.04348.x

Xu, P., Liang, L. Z., Dong, X. Y., Xu, J., Jiang, P. K., and Shen, R. F. (2014). Response of soil phosphorus required for maximum growth of *Asparagus officinalis* L. to inoculation of arbuscular mycorrhizal fungi. *Pedosphere* 24, 776–782. doi: 10.1016/S1002-0160(14)60064-3

Xu, J., Liu, S., Song, S., Guo, H., Tang, J., Yong, J. W. H., et al. (2018). Arbuscular mycorrhizal fungi influence decomposition and the associated soil microbial community under different soil phosphorus availability. *Soil Biol. Biochem.* 120, 181–190. doi: 10.1016/j.soilbio.2018.02.010

Zaller, J. G., Frank, T., and Drapela, T. (2011). Soil sand content can alter effects of different taxa of mycorrhizal fungi on plant biomass production of grassland species. *Eur. J. Soil Biol.* 47, 175–181. doi: 10.1016/j.ejsobi.2011.03.001

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

frontiers in Plant Science

ORIGINAL RESEARCH
published: 02 July 2021
doi: 10.3389/fpls.2021.628769

Arbuscular Mycorrhizal Fungi (*Rhizophagus clarus*) and Rhizobacteria (*Bacillus subtilis*) Can Improve the Clonal Propagation and Development of Teak for Commercial Plantings

*Flávia Sampaio Alexandre[1], Larissa Venturini Della Flora[1], Ivanildo Guilherme Henrique[1], Daniely Camila da Silva[1], Andreza Pereira Mercedes[1], Aline Cardoso Silva[1], Afonso Silva de Oliveira[1], Mariane Patrícia Bondespacho da Silva[1], Bruna Patrícia Formelh Ronning[1], Douglas Rafael Dreher[1], Bárbara Gionco Cano[2], Matheus Felipe de Lima Andreata[2], Joamir Barbosa Filho[3], Eva Roseane Santos[3], Fausto Hissashi Takisawa[3], Rafael Ferreira Alfenas[4], Galdino Andrade[2] and Martha Viviana Torres Cely[1]**

[1] Laboratorio de Microbiologia do Solo e Biotecnologia Agrícola, Universidade Federal de Mato Grosso, Sinop, Brazil, [2] Laboratório de Ecologia Microbiana, Departamento de Microbiologia, Universidade Estadual de Londrina, Londrina, Brazil, [3] TRC, Teak Resources Company, Cáceres, Brazil, [4] Laboratório de Patologia Florestal, Universidade Federal de Viçosa, Viçosa, Brazil

OPEN ACCESS

Edited by:
Jan Jansa,
Academy of Sciences of the Czech Republic (ASCR), Czechia

Reviewed by:
Jana Rydlová,
Institute of Botany (ASCR), Czechia
Weria Weisany,
Islamic Azad University, Iran

***Correspondence:**
Martha Viviana Torres Cely
vivianatorrescely95@gmail.com

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

Received: 12 November 2020
Accepted: 05 May 2021
Published: 02 July 2021

Citation:
Alexandre FS, Della Flora LV, Henrique IG, da Silva DC, Mercedes AP, Cardoso Silva A, Silva de Oliveira A, Bondespacho da Silva MP, Formelh Ronning BP, Dreher DR, Gionco Cano B, Andreata MFL, Barbosa Filho J, Santos ER, Takisawa FH, Alfenas RF, Andrade G and Cely MVT (2021) Arbuscular Mycorrhizal Fungi (Rhizophagus clarus) and Rhizobacteria (Bacillus subtilis) Can Improve the Clonal Propagation and Development of Teak for Commercial Plantings. Front. Plant Sci. 12:628769. doi: 10.3389/fpls.2021.628769

The *Tectona grandis* L.f. (teak) is an important forest species with high economy value in Asia, Africa, and Latin America. In Latin America, Brazil is one of the countries with the most cultivated areas. The cultivation of teak turns out to be challenging because of its high nutritional demand and the need for seedling production by clonal propagation that includes about 90 days in the nursery phase. The optimization of seedling production is necessary for better results in the nursery and to enhance growth in the field. In this way, the well-known advantage of using microorganisms that promote plant development appears as a potential biotechnological approach to be explored and for the implantation of new areas of wood production. In this study, the inoculation of *Bacillus subtilis* as plant growth-promoting rhizobacteria (PGPR) was evaluated, and *Rhizophagus clarus*, an arbuscular mycorrhizal fungus (AMF), and the co-inoculation of these microorganisms in the teak seedling production phase can improve the development of commercial plantations under field conditions. Experiments were carried out under greenhouse and field conditions to evaluate four treatments based on the substrate inoculation of the seedlings. Treatments consisted of a non-inoculated control, PGPR inoculation, AMF inoculation, and PGPR + AMF inoculation. The results of the biometric evaluation of seedlings in the greenhouse showed that there was a significant difference in AMF inoculation and PGPR + AMF inoculation in terms of the specific root length and root density treatments, there was also a positive correlation between these two treatments and the absorption of some nutrients, such as P, N, K, Mg, Cu, Mn, and Zn. This response led to an increase between 4.75 and 11.04% in the field growth rate.

Keywords: forestry, nursery, soil microbiology, *Tectona grandis*, wood production

INTRODUCTION

The forestry sector has become increasingly important for the Brazilian gross domestic product (GDP), having 1.3% share in 2018, reaching a total revenue of 86.6 billion. The cultivation of trees for industrial purposes is a renewable source of raw material for almost 5,000 products, such as sawn wood, paper, cellulose, floors, panels, and charcoal, being an important weapon in the recovery of degraded areas and protection of habitats (IBÁ, 2019).

Being one of the main nobles manufactured in the world, teak (*Tectona grandis* L.f.) is a tree species in the Lamiaceae family, native to the Asian continent, initiated throughout tropical Asia, as well as tropical Africa, Latin America, and the Caribbean. It is characterized by its soft color, fine grain, and durability, and it is commonly used for products of high commercial value, such as furniture, shipbuilding, and decorative construction components (Pandey and Brown, 2000). In Brazil, ∼93,957 ha of planted areas of this species were registered in 2018 (IBÁ, 2019).

A limiting factor for its cultivation is the low germination rate of seeds and their sensitivity to storage, with genetic improvement being one of the main alternatives to this problem, bringing long-term results (Schuhli and Paludzyszyn Filho, 2010). In this scenario, the clonal production of selected trees has been boosted. The mini-cutting technique can provide, in the short term, homogeneous seedlings with desired characteristics, increase the number of rooted mini-cuttings, and improve the root system, directly influencing the performance of seedlings in the field (Meza et al., 2015).

The inoculation of plant growth-promoting rhizobacteria (PGPR) during seedling production has already proved efficient, generating healthier plants with gains in biomass and nutrient content (Rodrigues et al., 2018; Raghu et al., 2020). Moreover, the response of teak to different and selected microbial consortiums, such as *Ambispora leptoticha*, *Azotobacter chroococcum*, and *Trichoderma harzianum* has already been tested (Raghu et al., 2020). Among the PGPR, *Bacillus* is one of the most important genera in soil, and its ability to generate spores increases its survival and competitiveness in adverse conditions. The genus assists plants in their defense against attacks by pathogens and increases their tolerance to stress (Hashem et al., 2016).

Arbuscular mycorrhizal fungi (AMF) also improve plant growth (Akinrinlola et al., 2018). AMF are able to promote growth, and they are resistant to biotic and abiotic stresses, because of greater absorption of nutrients, with emphasis on phosphorus (P) (Smith and Read, 2008). They increase the formation of soil aggregates through the production of glomalin, and they are also important regulators of soil stability and quality (Rillig and Mummey, 2006). A greenhouse study demonstrated the beneficial effect of AMF inoculation on teak seedlings, with greater growth of aerial parts and roots, content of nutrients, such as potassium (K) and sulfur (S), and better efficiency in the use of nutrients, such as P (Rodrigues et al., 2018). Another study conducted to examine the efficacy of three native species of AMF (*Funneliformis mosseae*, *Glomus intradices*, and *Glomus proliferum*) on the growth and seedling quality of *T grandis* showed that mycorrhizal symbiosis significantly improved seedling growth and physiological parameters, proving that this technology has a potential to reduce the nursery period and that it increases the quality of produced seedlings, resulting in considerable economic gains (Ajeesh et al., 2017).

The inoculation of AMF and *Bacillus* species and the co-inoculation of these microorganisms have already been proven as efficient methods to increase plant growth by several researchers (Medina et al., 2003; Marulanda-Aguirre et al., 2007; Alam et al., 2011; Awasthi et al., 2011). *B subtilis* is sold commercially (Biobaci®), and *R clarus* is in the process of launching the commercial product (Andrade and Cely, 2019), facilitating the implantation of these microorganisms in the operational routine of companies for the production of teak seedlings. Thus, this study aims to demonstrate the effect of AMF *R clarus* and PGPR *B subtilis,* their co-inoculation interaction in the nursery phase, and their potential to improve the growth process of *T grandis* under field conditions.

MATERIALS AND METHODS

Seedling Experiments

The experiment was carried out in the Teak Resources Company (TRC) seedling nursery, located in Jangada city, MT, Brazil from September 2016 to January 2017. The principal commercial clone of the company was multiplied by mini-cutting systems from clonal garden. Microorganisms were incorporated in the substrate of pine bark (Mec Plant - Florestal 2) fertilized with Osmocote Mini Prill-3M (N 19%, P_2O_5 6%, and K_2O 10%) −8 g + PG Mix (N 14%, P_2O_5 16%, and K_2O 18%) −5 g/kg of substrate. Before planting, mini-cuttings were dipped in indole butyric acid (IBA) (500 mg L^{-1}) and then planted in tubes of 53 cm^3. The treatments presented in **Table 1** are considered. Two types of microorganisms were inoculated: AMF *R clarus*, with a concentration of 200 propagules/ml multiplied *in vitro* system according to the patent application: BR 102019008109-0 A2 of 22/04/2019 (Andrade and Cely, 2019) and commercial product Biobaci® containing viable cells (1×10^8 CFU/ml) of PGPR *B subtilis*. In total, 2,112 seedlings were produced (528 seedlings per treatment). A completely randomized design was chosen under greenhouse conditions.

The mini-cuttings were kept in an air-conditioned greenhouse with a transparent polyethylene cover and 50% shadowing fabric for 30 days. Parameters, namely, relative humidity (RH > 80%), temperature (35–40°C), and irrigation were controlled *via* nebulization. In the 1st week, the frequency of irrigation was 10 s

TABLE 1 | Treatments and doses used for incorporation into the substrate of microorganisms *Rhizophagus clarus* and *Bacillus subtilis* in the production of *Tectona grandis* seedlings.

Treatment	Description
T1	Control (substrate without microorganisms)
T2	PGPR (*Bacillus subtilis*)- 50 mL/L of substrate
T3	AMF (*Rhizophagus clarus*) - 15 g/L of substrate
T4	PGPR + AMF(*Bacillus subtilis* - 50 mL + *Rhizophagus clarus* - 15 g/L of substrate)

each 15 min; and in the following weeks, the frequency was 20 s each 40 min (24 L/h/micro sprinkler). Subsequently, they were placed in the shade house with 50% shadowing fabric and 4 h of irrigation for 15 min, for acclimatization until they reached 60 days. During this phase, the seedlings were fertigated once a day with 600 mg. L^{-1} of the following substances: $MgSO_4$ $7H_2O$, $Ca(NO_3)_2$, and NH_4NO_3 each; 800 mg. L^{-1} of $NH_4H_2PO_4$, and 400 mg. L^{-1} of KNO_3. After this period, the seedlings went full

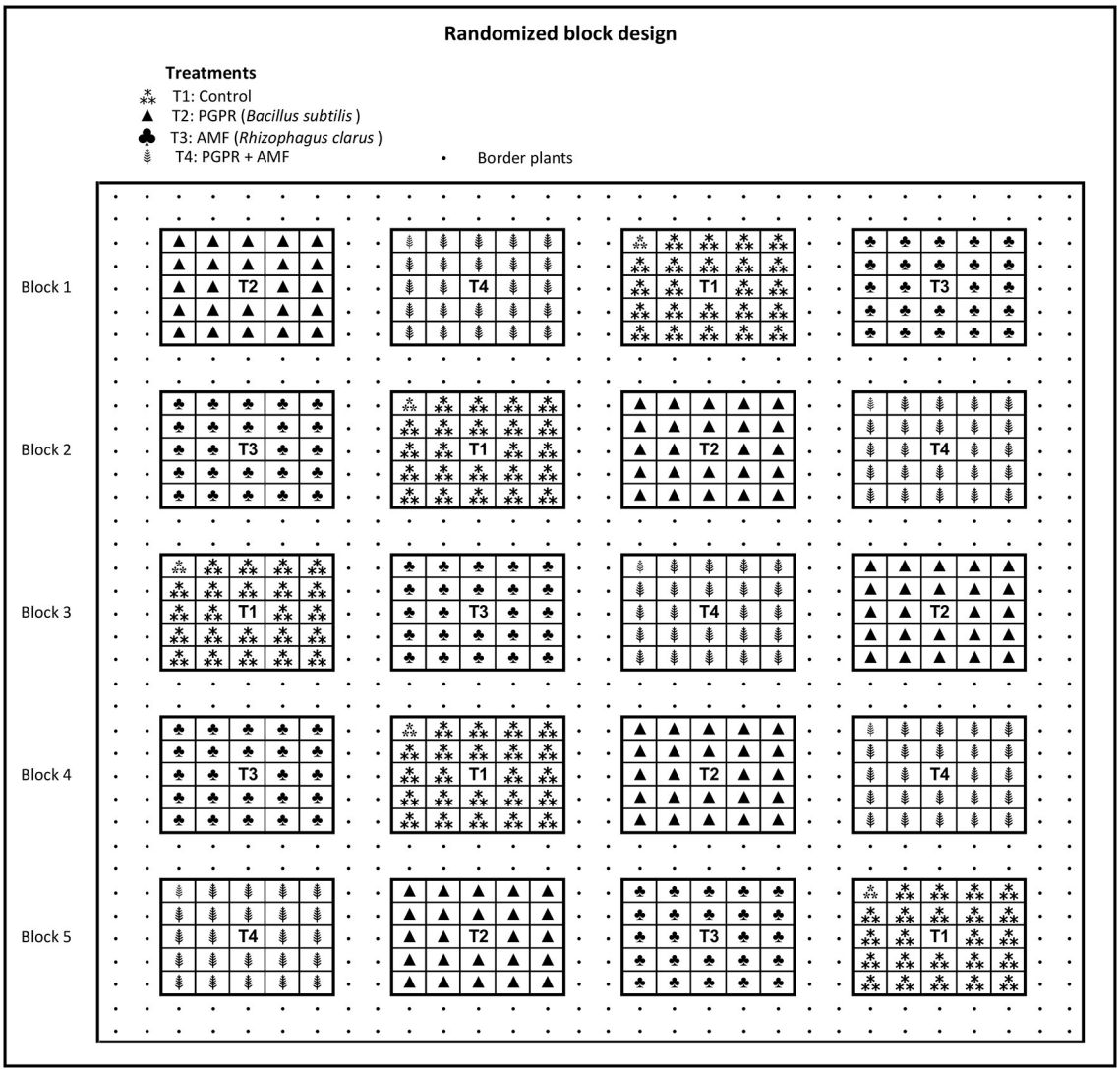

FIGURE 1 | Randomized block design of field experiments with *Tectona grandis* in states Mato Grosso and Pará, Brazil.

TABLE 2 | Summary of the general linear models (GLMs) of *Tectona grandis* seedling survival at 30, 60, and 90 days in nursery.

Predictor	df	Estimate	X_2	*P*	OR	Confidence interval	
						25%	95%
T1	1	1.090	40.75	1.7^{-10*}	2.97	2.12	4.15
T2	1	1.732	73.70	2.2^{-16*}	5.65**	3.80	8.38
T3	1	1.247	58.11	2.4^{-14*}	3.48**	2.52	4.79
T4	1	0.798	19.06	1.2^{-05*}	2.22	1.55	3.18

**Significant predictors at ($p \leq 0.001$).*

***Highest odds ratio for survival.*

sun until they completed 90 days. The frequency of irrigation was every 6 h for 10 min (810 L/h/micro sprinkler), and fertigation was performed twice a week with the same nutrient solution mentioned above.

Data Collected in Nursery Phase

Seedling survival was evaluated 30, 60, and 90 days after planting. Ninety days after planting, 20 seedlings per treatment were collected for biometric analysis in the laboratory. At this time, height = H (cm), stem diameter = SD (mm), dry shoot biomass = SB (g), root biomass = RB (g) and biomass ratio of the root/shoot = RB/SB (g) were evaluated. The remaining seedlings were intended for field experiments.

Ten root seedlings samples per treatment were also collected to evaluate mycorrhizal colonization. The percentage of mycorrhizal colonization was estimated by the grid-line method (Giovannetti and Mosse, 1980) after staining roots with Trypan blue (0.05%) (Phillips and Hayman, 1970). Total root length = TRL (cm), specific root length = SRL (cm g^{-1}), and root density = RD (g cm^{-3}) were also evaluated at this stage (Ryser and Lambers, 1995). Twenty seedling samples per treatment were separated for macro and micronutrient foliar analysis; and for this, the following methods were used sulfuric digestion and quantification by titration after semi-micro Kjeldahl distillation (N), nitric-perchloric digestion (P, K, Ca, Mg, S, Cu, Fe, Mn, Zn, Na, and Ni), incineration (B, Co, and Mo), agitation (Cl). Sample reading was made by inductively coupled plasma-optical emission spectrometry (ICP-OES) with a Thermo Scientific ICAP 7600 spectrometer (Thermo Fisher Scientific, Waltham, Massachusetts, US).

Field Experiments

The field experiments were started in February 2017. Two experimental areas were used. Area I is located in Cáceres city, state of Mato Grosso (MT), Brazil (16°8′1.75″ S and 58°31′1.77″ W) and has tropical savanna climate (Aw) (Köppen, 1936) and soil classification We-Eutric Planosols (FAO, 1994). Area II is located in Santa Maria das Barreiras city, state of Pará (PA), Brazil (8°43′1.11″ S and 50°29′10.76″ W) and has tropical savanna climate (Aw) (Köppen, 1936) and soil classification Ao-Orthic Acrisols (FAO, 1994). Soil samples were collected in Area I and Area II for physicochemical analysis at depths of 0–20 and 20–40 cm. The analysis showed the following results: Area I depth 0–20 cm: pH ($CaCl_2$) 5.1, Al^{+3} 0 $cmol_c$ dm^{-3}, H + Al 2.48 $cmol_c$ dm^{-3}, P 3.5 mg dm^{-3}, K^+ 30.3 mg dm^{-3}; Ca^{+2} 1.5 $cmol_c$ dm^{-3}, and Mg^{+2} 0.58 $cmol_c$ dm^{-3}. Depth 20–40 cm with pH ($CaCl_2$) 4.6, Al^{+3} 0.28 $cmol_c$ dm^{-3}, H + Al 2.25 $cmol_c$ dm^{-3}, P.3 mg dm^{-3}, K^+ 16.2 mg dm^{-3}; Ca^{+2} 0.90 $cmol_c$ dm^{-3}, and Mg^{+2} 0.37 $cmol_c$ dm^{-3}. Area II depth 0–20 cm: pH ($CaCl_2$) 4.5, Al^{+3} 0.2 $cmol_c$ dm^{-3}, H + Al 2.2 $cmol_c$ dm^{-3}, P 1.8 mg dm^{-3}, K^+ 24.6 mg dm^{-3}, Ca^{+2} 1.2 $cmol_c$ dm^{-3}, and Mg^{+2} 0.4 $cmol_c$ dm^{-3}. Depth 20–40 cm with pH ($CaCl_2$) 4.4, Al^{+3} 0.2 $cmol_c$ dm^{-3}, P.5 mg dm^{-3}, K^+ 19.5 mg dm^{-3}, Ca^{+2} 0.80 $cmol_c$ dm^{-3}; and Mg^{+2} 0.3 $cmol_c$ dm^{-3}. pH ($CaCl_2$) was evaluated in a 0.01 M chloride solution, in the proportion 1:2.5 (soil: $CaCl_2$). P and K^+ were extracted with a 0.05 N HCl and 0.025 N H_2SO_4 solution (Mehlich I). Ca^{+2}, Mg^{+2}, and Al^{+3} were extracted with a 1 N potassium chloride solution.

These experiments were carried out in a randomized block design. Five blocks were installed, and within each block four plots of 5 × 5 plants (25 plants per plot) were used. Each plot corresponds to one of the treatments defined in the nursery phase (**Table 1**), separated by two border plants (**Figure 1**). Plant spacing was 3 × 4 m. Total experimental area was 1.33 ha of planting including border plants. For the implantation and conduction of planting, the standard routine of the company was followed.

Data Collected in Field

Plant survival was evaluated 30, 60, and 90 days after planting in the field. Data collection was made 2 and 3 years after planting. The data collected in this time were: height = H (m) and diameter at breast height = DBH (cm), tree volumes = V (dm^3) and were calculated with these data, using the equation [$\pi * (DBH^2/4) * H * Ff$]. The artificial form factor = Ff = 0.63 was used, the average value indicated for young teak plantation, between 1 and 3 years old (Carneiro et al., 2018). Additionally, the mean annual increment = MAI (dm^3 $year^{-1}$) and the current annual increment = CAI (dm3) were estimated.

TABLE 3 | Percentage of mycorrhizal colonization of *Tectona grandis* seedling at 90 days.

Treatment	Mycorrhizal colonization (%)
T1 – Control	0.0[b]
T2 – PGPR	0.0[b]
T3 – AMF	63.4[a]
T4 – PGPR+ AMF	61.8[a]

Treatments with the same letter are not significantly different by Friedman test ($p \leq 0.05$).

TABLE 4 | Analysis of variance (ANOVA) for treatments of seedling growth parameters of *Tectona grandis* at 90 days after mini-cuttings planting.

Variables	df	*F*	*p*-value	Error
ANOVA				
H	3	0.834	0.48000	31.30
SD	3	5.588	0.00162*	0.951
SB	3	2.333	0.08070	0.870
RB	3	3.345	0.02350*	0.228
RB/SB	3	1.974	0.13500	0.096
TRL	3	0.670	0.57600	369.2
SRL	3	3.631	0.02190*	0.002
RD	3	4.520	0.00864*	0.013

H (cm), Height; SD (mm), stem diameter; SB (g), dry shoot biomass; RB (g), root biomass; RB/SB (g), biomass ratio of the root/shoot; TRL (cm), total root length; SRL (cm g^{-1}), specific root length; RD (g cm^{-3}), root density.
**Significant difference ($p \leq 0.05$).*

Statistical Analysis

To evaluate the effects of treatments on seedling survival, we performed generalized linear models (GLMs) analysis. For models, the treatments (T1, T2, T3, and T4) were used as predictors and the time as an independent factor. The statistical significance (p) of each predictor is based on chi-square tests to assess the significance of the change in deviance for each predictor. For model interpretation, the odds ratio (OR) and its confidence interval were calculated.

Statistical analyses of AMF root colonization were performed using the Friedman test at a significance level of $p \leq 0.05$. Plant growth parameters in the seedling experiments were analyzed by analysis of variance (ANOVA) and the Tukey test ($p \leq 0.05$). Principal component analysis (PCA) was also performed for nutrient content. Analyses of field data at 2 and 3 years after planting were performed by factorial ANOVA. Factor A: treatment. Factor B: block. For comparison of means, the Tukey test ($p \leq 0.05$) was performed. All statistical analyses in this research were performed using the R software (R Core Team, 2020).

RESULTS

Seedling Test

The results of GLMs based on the patterns of seedling survival in the nursery phase revealed significant effects of treatments as

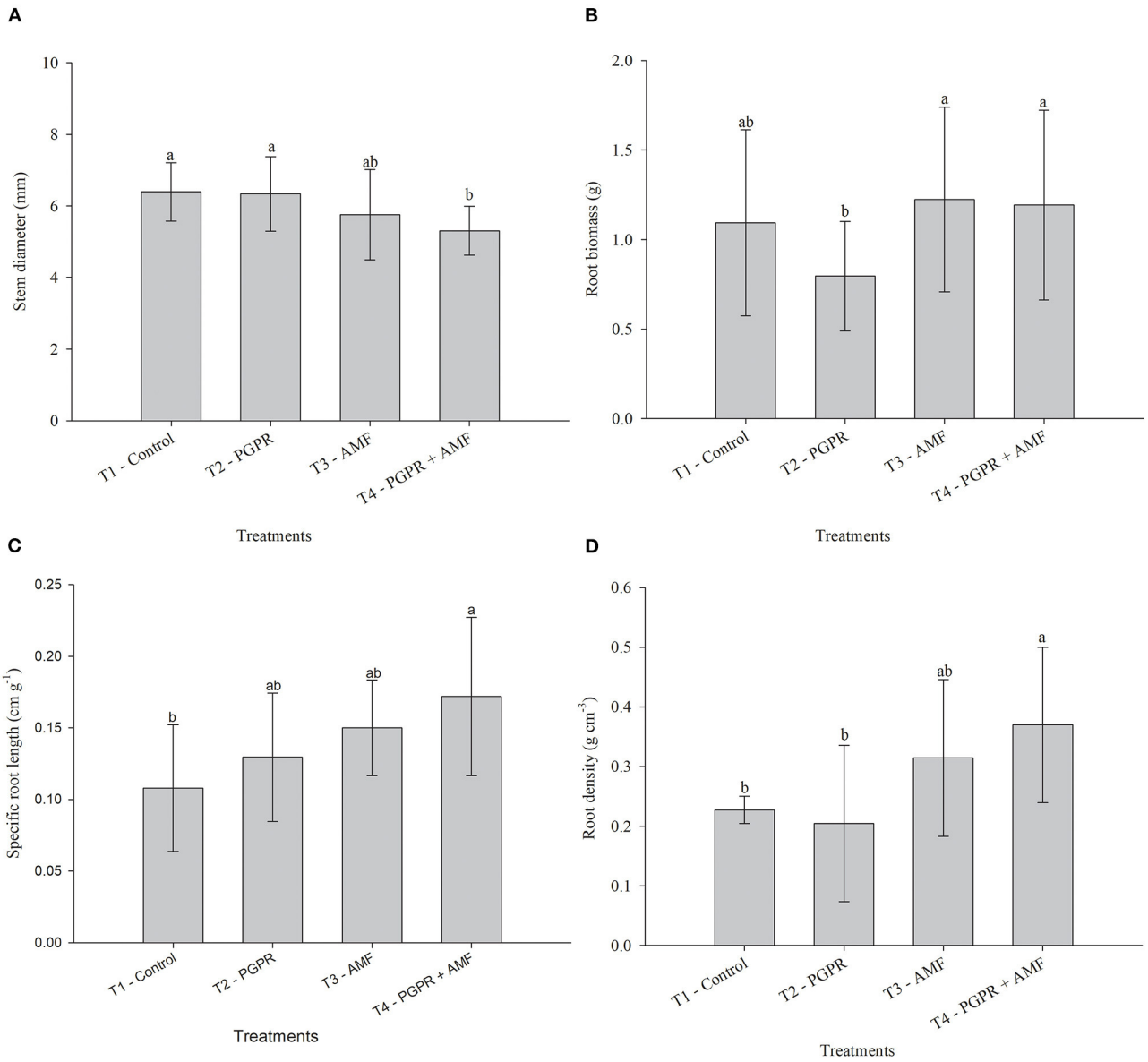

FIGURE 2 | Effect on seedling growth of *Tectona grandis* at 90 days. **(A)** Stem diameter. **(B)** Root biomass. **(C)** Specific root length. **(D)** Root density ($n = 20$). Bars followed by the same letter do not differ by Turkey test ($p \leq 0.05$). Bars represent standard deviation of means.

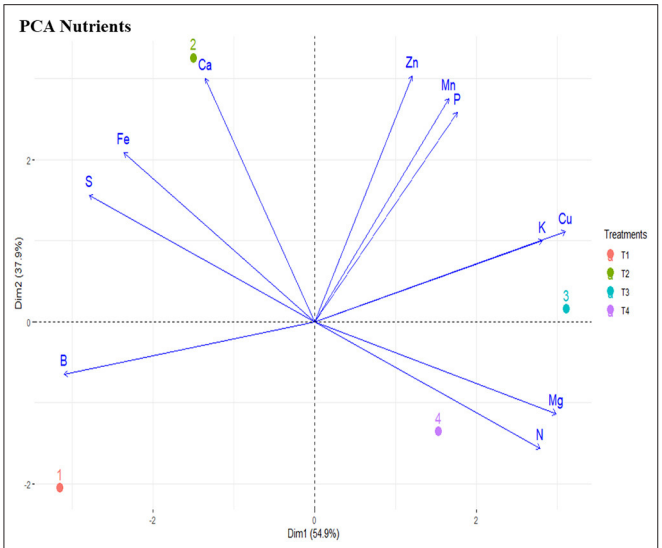

FIGURE 3 | Principal component analysis for nutrients on *Tectona grandis* seedling at 90 days. T1, Control; T2, PGPR *Bacillus subtilis*; T3, AMF *Rhizophagus clarus*; T4, PGPR + AMF.

predictors for this parameter. That is, the treatments can be used to explain the survival pattern in this experiment, being that all models were statistically significant ($p < 0.001$). The OR showed better survival for seedlings of teak when inoculated with PGPR (*B subtilis*) and AMF (*R clarus*) (**Table 2**).

The mycorrhizal colonization in the treatments inoculated with *R clarus* (AMF) was just over 60%. In general, bacterial inoculation with PGPR did not influence the rate of colonization of teak roots by mycorrhiza (**Table 3**).

In the seedling growth evaluation, the treatments have a significant effect on stem diameter, root biomass, specific root length, and root density ($p \leq 0.05$) (**Table 4**). For stem diameter, the treatments with microorganisms PGPR and AMF were statistically equal to the control, only PGPR + AMF was lower than the control treatment (**Figure 2A**). AMF inoculation resulted in a significant decrease in root biomass, while the other two microbial treatments did not affect this variable (**Figure 2B**). The low development of stem diameter in PGPR + AMF was compensated by differentiate response in root system inoculation that showed gains in specific root length and root density (**Figures 2C,D**).

In the PCA, 92.8% of the variability of nutrients data were represented in components one and two. In the first dimension, representing 54.9% of the data variability, macronutrients nitrogen (N), phosphorus (P), and potassium (K) were positively related with treatments T3 and T4 (AMF inoculation and PGPR + AMF inoculation), as well as micronutrients magnesium (Mg), copper (Cu), manganese (Mn), and Zinc (Zn). In the second dimension, representing 37.9% of the data variability, calcium (Ca), iron (Fe), and sulfur (S) data were associated with treatment T2 (PGPR). The control treatment was allocated far from the eigenvalues of the nutrients, showing that the treatment did not interact with the nutritional data (**Figure 3**).

TABLE 5 | Summary of the general linear models (GLMs) of *Tectona grandis* seedling survival at 30, 60, and 90 days in field.

Predictor	df	Estimate	X_2	*p*
Mato Grosso				
T1	1	1.398	1.29	0.2555
T2	1	14.27	7e-04	0.9787
T3	1	14.27	7e-04	0.9787
T4	1	14.27	7e-04	0.9787
Pará				
T1	1	16.31	8^{-04}	0.9781
T2	1	2.53	5.42	0.0198*
T3	1	16.29	5^{-04}	0.9821
T4	1	16.31	8^{-04}	0.9781

*Significant predictors at ($p \leq 0.05$).

Field Test

In the field, seedling survival is not explained by the treatments as predictors in GLM in the Mato Grosso area. On the other hand, in Pará, treatment with *B subtilis* (T2) showed 96% of seedling survival and was considered a predictor for this parameter with GLM statistically significant ($p < 0.05$). This treatment showed an OR of 12.55 (confidence interval of 2.5–95%/1.49–105.45) (**Table 5**).

The tree volume (V) and mean annual increment (MAI) at 2 years after planting in Mato Grosso showed statistical differences between treatments, block, and the interaction between treatment × block. Treatments T1, T2, and T4 were significantly equal, and treatment T3 was statistically lower in both variables analyzed. The plants in block 2 performed better than the plants in blocks 3 and 4. The interaction of the treatment and blocking factors was significant but did not show any clear pattern (**Table 6**).

At 3 years after planting in Mato Grosso, statistical differences between treatments, block, and the interaction between treatment × block for V, MAI, and CAI were observed. The results were similar to those presented at 2 years for V and MAI and for CAI (**Table 7**).

In Pará, the V and MAI showed statistical differences between treatments, block, and the interaction between treatment × block at 2 years. The T2 andT4 treatments presented the best results and were statistically superior to T1 and T3. The plants in blocks 1, 2, and 3 performed better than the plants in blocks 4 and 5. The interaction of the treatment and blocking factors was significant but did not show any clear pattern (**Table 8**).

At 3 years, the V, MAI, and CAI also presented statistical differences between treatments, block, and the interaction between treatment × block. The best treatment was T2, which differed significantly from the control. In the average value of treatments, PGPR (T2) showed an 11.04% increase in V at 3 years followed by PGPR + AMF (T4) with 5.81%, and AMF (T3) with 4.75% compared with the control. Blocks 1, 2, and 3 presented the best results. The interaction of the treatment and blocking factors was significant but did not show any clear pattern. This difference between the blocks may be due to variations in soil conditions, which is due to the large experimental area (**Table 9**).

TABLE 6 | Tree volume and mean annual increment at 2 years old in plants of *Tectona grandis* in Mato Grosso state, Brazil.

Factor		V (dm^3)		MAI (dm^3 $year^{-1}$)	
	df	*F*	*p*-value	*F*	*p*-value
ANOVA - Mato Grosso, 2 years old					
Treatment (A)	3	11.37	0.0000*	11.351	0.0000*
Block (B)	4	6.13	0.0000*	6.131	0.0000*
A × B	12	15.09	0.0000*	15.074	0.0000*
Error		0.224		0.056	

Treatment	V (dm^3)	MAI (dm^3 $year^{-1}$)
Tukey test of treatment		
T1 – Control	0.96 ± 0.46^{a}	0.48 ± 0.23^{a}
T2 – PGPR	0.92 ± 0.55^{a}	0.46 ± 0.27^{a}
T3 – AMF	0.70 ± 0.33^{b}	0.35 ± 0.16^{b}
T4 – PGPR + AMF	1.04 ± 0.79^{a}	0.52 ± 0.40^{a}

Block	V (dm^3)	MAI (dm^3 $year^{-1}$)
Tukey test of block		
1	0.98 ± 0.91^{ab}	0.49 ± 0.45^{ab}
2	1.06 ± 0.58^{a}	0.53 ± 0.29^{a}
3	0.82 ± 0.35^{bc}	0.41 ± 0.18^{bc}
4	0.77 ± 0.35^{c}	0.38 ± 0.17^{c}
5	0.89 ± 0.42^{abc}	0.45 ± 0.21^{abc}

Block	T1 - Control	T2 – PGPR	T3 – AMF	T4 - PGPR + AMF
Tukey test of A × B V (dm^3)				
1	0.86 ± 0.44^{cd}	0.56 ± 0.24^{d}	0.55 ± 0.23^{d}	1.99 ± 1.30^{a}
2	1.16 ± 0.49^{bc}	1.53 ± 0.73^{ab}	0.70 ± 0.29^{cd}	0.85 ± 0.31^{cd}
3	0.73 ± 0.36^{cd}	0.89 ± 0.35^{cd}	0.81 ± 0.32^{cd}	0.85 ± 0.37^{cd}
4	0.92 ± 0.42^{cd}	0.80 ± 0.30^{cd}	0.57 ± 0.25^{d}	0.81 ± 0.33^{cd}
5	1.13 ± 0.48^{bc}	0.80 ± 0.42^{cd}	0.90 ± 0.40^{cd}	0.73 ± 0.24^{cd}
MAI (dm^3 $year^{-1}$)				
1	0.43 ± 0.22^{cd}	0.28 ± 0.12^{d}	0.28 ± 0.12^{d}	0.99 ± 0.65^{a}
2	0.58 ± 0.25^{bc}	0.77 ± 0.37^{ab}	0.35 ± 0.15^{cd}	0.42 ± 0.16^{cd}
3	0.36 ± 0.18^{cd}	0.44 ± 0.18^{cd}	0.41 ± 0.16^{cd}	0.42 ± 0.19^{cd}
4	0.48 ± 0.21^{cd}	0.40 ± 0.15^{cd}	0.28 ± 0.13^{d}	0.40 ± 0.16^{cd}
5	0.57 ± 0.24^{bc}	0.40 ± 0.21^{cd}	0.45 ± 0.20^{cd}	0.36 ± 0.12^{cd}

**Significant difference ($p \leq 0.05$). Same letter does not differ by the Tukey test ($p \leq 0.05$).*

DISCUSSION

The PGPR (*Bacillus subtilis*) inoculation and co-inoculation with AMF caused an increase in seedlings survival. In this period, the survival of seedling is directly related with the increase in cuttings rooting. *B subtilis* can assist in this process through the phytohormone production of indoleacetic acid (IAA) (Radhakrishnan et al., 2017). According to Teixeira et al. (2007), the mini cuttings of eucalypt clones in substrate with inoculation of *B subtilis* shows better root formation, and this result allows optimization of seedling development in clonal nurseries. The presence of rhizobacteria *B. subtilis* can directly influence plant growth not only by phytohormone production (Ali et al., 2009; Galaviz et al., 2018) but also by stress-inhibiting enzymes, siderophores, and indirect P-solubilization (Meng et al., 2016).

Root colonization by AMF *Rhizophagus clarus* was around 60% at 90 days after planting. The AMF efficiency must be evaluated with data of vegetative development, since symbiosis may not be effective, with high colonization rates, depending on the host. Arbuscular mycorrhizal (AM) association can be influenced and differentiated by vegetal species, clones, and cultivars, as well as by inoculated AMF species (Smith and Smith, 2011). In an evaluation of Indian native AMF species (*Funneliformis mosseae*, *Glomus intraradices*, and *Glomus proliferum*), Ajeesh et al. (2017) observed that colonization

TABLE 7 | Tree volume, mean annual increment, and current annual increment at 3 years old in plants of *Tectona grandis* in Mato Grosso state, Brazil.

Factor		V (dm^3)		MAI (dm^3 $year^{-1}$)		CAI (dm^3)	
	df	*F*	*p*-value	*F*	*p*-value	*F*	*p*-value
ANOVA - Mato Grosso, 3 years old							
Treatment (A)	3	13.464	0.0000*	13.466	0.0000*	12.906	0.0000*
Block (B)	4	4.615	0.0012*	4.614	0.0012*	4.411	0.0017*
A × B	12	10.526	0.0000*	10.529	0.0000*	9.790	0.0000*
Error		41.2		4.58		37.4	

Treatment	V (dm^3)	MAI (dm^3 $year^{-1}$)	CAI (dm^3)
Tukey test Treatment			
T1 – Control	23.62 ± 6.80^{a}	7.87 ± 2.27^{a}	22.66 ± 6.44^{a}
T2 – PGPR	22.85 ± 7.82^{a}	7.62 ± 2.61^{a}	21.93 ± 7.40^{a}
T3 – AMF	19.65 ± 5.56^{b}	6.55 ± 1.85^{b}	18.95 ± 5.33^{b}
T4 - PGPR + AMF	24.57 ± 8.40^{a}	8.19 ± 2.80^{a}	23.53 ± 7.85^{a}

Block	V (dm^3)	MAI (dm^3 $year^{-1}$)	CAI (dm^3)
Tukey test of block			
1	23.85 ± 8.92^{ab}	7.95 ± 2.97^{ab}	22.87 ± 8.23^{ab}
2	24.38 ± 8.30^{a}	8.13 ± 2.77^{a}	23.31 ± 7.82^{a}
3	21.27 ± 6.36^{c}	7.09 ± 2.12^{c}	20.45 ± 6.12^{c}
4	22.28 ± 6.86^{abc}	7.43 ± 2.29^{abc}	21.51 ± 6.64^{abc}
5	21.53 ± 5.90^{bc}	7.18 ± 1.97^{bc}	20.64 ± 5.60^{bc}

Block	T1 – Control	T2 – PGPR	T3 – AMF	T4 – PGPR + AMF
Tukey test of A × B V (dm^3)				
1	23.55 ± 6.88^{cde}	20.03 ± 4.28^{de}	18.20 ± 5.80^{e}	34.02 ± 8.61^{a}
2	25.17 ± 6.17^{bcd}	30.85 ± 8.94^{ab}	19.40 ± 5.58^{de}	21.90 ± 7.62^{cde}
3	21.11 ± 6.86^{cde}	22.92 ± 7.42^{cde}	20.66 ± 4.96^{cde}	20.37 ± 5.99^{cde}
4	22.78 ± 7.12^{cde}	20.77 ± 6.29^{cde}	19.06 ± 5.49^{de}	26.85 ± 6.27^{bc}
5	25.38 ± 6.57^{bcd}	19.60 ± 5.61^{de}	20.99 ± 5.87^{cde}	20.09 ± 3.46^{de}
MAI (dm^3 $year^{-1}$)				
1	7.85 ± 2.29^{cde}	6.68 ± 1.43^{de}	6.07 ± 1.83^{e}	11.34 ± 2.87^{a}
2	8.39 ± 2.06^{bcd}	10.28 ± 2.98^{ab}	6.47 ± 1.86^{de}	7.30 ± 2.54^{cde}
3	7.04 ± 2.29^{cde}	7.64 ± 2.47^{cde}	6.89 ± 1.65^{cde}	6.79 ± 2.00^{cde}
4	7.59 ± 2.37^{cde}	6.92 ± 2.10^{cde}	6.35 ± 1.83^{de}	8.95 ± 2.09^{bc}
5	8.46 ± 2.19^{bcd}	6.53 ± 1.87^{de}	7.00 ± 1.96^{cde}	6.70 ± 1.15^{de}
CAI (dm^3)				
1	22.69 ± 6.53^{cde}	19.47 ± 4.27^{de}	17.65 ± 5.69^{e}	32.03 ± 7.71^{a}
2	24.01 ± 5.78^{bcd}	29.32 ± 8.32^{ab}	18.70 ± 5.35^{de}	21.05 ± 7.78^{cde}
3	20.38 ± 6.57^{cde}	22.03 ± 7.15^{cde}	19.85 ± 4.78^{cde}	19.52 ± 5.73^{de}
4	21.86 ± 6.85^{cde}	19.97 ± 6.09^{cde}	18.50 ± 5.39^{de}	26.04 ± 6.12^{abc}
5	24.24 ± 6.19^{bcd}	18.80 ± 5.42^{de}	20.09 ± 5.53^{cde}	19.36 ± 3.33^{de}

*Significant difference ($p \leq 0.05$). Same letter does not differ by the Tukey test ($p \leq 0.05$).

range was from 15 to 36%, and that teak response was better with *G proliferum* than with other mycorrhizal species. In Indonesia, a study on *Acaulospora* sp., *Gigaspora* sp., and mixed *Acaulospora* sp. and *Gigaspora* sp, combined with different doses of compost in soil, showed that seedlings inoculated with *Gigaspora* sp. and 15% of compost increased seedling quality, and the root colonization of teak in this study ranged from 0 to 36% for *Acaulospora* sp and from 32 to 74% for *Gigaspora* sp (Prayudyaningsih and Sari, 2016).

AMF colonization response also can be modulated by the successional status of plant host. *Tectona grandis* has been reported as an early successional species (Chen et al., 2011). Early successional woody species with high metabolic rate dominate the initial stages of succession, occur in highly illuminated

TABLE 8 | Tree volume and mean annual increment at 2 years old in plants of *Tectona grandis* in Pará state, Brazil.

Factor		V (dm^3)		MAI (dm^3 $year^{-1}$)	
	df	*F*	*p*-value	*F*	*p*-value
ANOVA - Pará, 2 years old					
Treatment (A)	3	2.929	0.0334*	2.928	0.0335*
Block (B)	4	36.842	0.0000*	36.843	0.0000*
A × B	12	6.807	0.0000*	6.808	0.0000*
Error		36.4		9.1	

Treatment	V (dm^3)	MAI (dm^3 $year^{-1}$)
Tukey test		
T1 - Control	16.50 ± 7.37^{b}	8.25 ± 3.68^{b}
T2 - PGPR	18.11 ± 7.66^{a}	9.06 ± 3.83^{a}
T3 - AMF	16.14 ± 7.29^{b}	8.07 ± 3.64^{b}
T4 - PGPR + AMF	17.84 ± 7.05^{a}	8.92 ± 3.53^{a}

Block	V (dm^3)	MAI (dm^3 $year^{-1}$)
Tukey test of block		
1	19.85 ± 7.19^{a}	9.92 ± 3.59^{a}
2	21.03 ± 6.96^{ab}	10.52 ± 3.48^{ab}
3	18.50 ± 7.63^{b}	9.25 ± 3.82^{b}
4	13.11 ± 5.10^{c}	6.55 ± 2.55^{c}
5	12.72 ± 5.25^{c}	6.36 ± 2.62^{c}

Block	T1 - Control	T2 – PGPR	T3 – AMF	T4 - PGPR + AMF
Tukey test of A × B V (dm^3)				
1	17.27 ± 4.6^{cdef}	24.92 ± 6.53^{ab}	14.22 ± 6.42^{defgh}	23.07 ± 5.75^{abc}
2	25.74 ± 5.75^{a}	18.87 ± 6.85^{bcde}	21.36 ± 7.27^{abc}	18.55 ± 5.72^{cde}
3	16.63 ± 6.23^{cdefg}	19.79 ± 7.19^{abcd}	18.83 ± 8.63^{bcde}	18.49 ± 8.28^{cde}
4	12.90 ± 4.61^{efgh}	14.57 ± 5.68^{defgh}	10.87 ± 4.49^{gh}	14.51 ± 5.23^{defgh}
5	8.78 ± 2.89^{h}	11.88 ± 4.58^{fgh}	15.12 ± 4.24^{defgh}	14.22 ± 6.42^{defgh}
MAI (dm^3 $year^{-1}$)				
1	8.63 ± 2.31^{cdef}	12.46 ± 3.26^{ab}	7.11 ± 3.21^{defgh}	11.53 ± 2.88^{abc}
2	12.87 ± 2.87^{a}	9.43 ± 3.43^{bcde}	10.68 ± 3.63^{abc}	9.28 ± 2.86^{cde}
3	8.32 ± 3.11^{cdefg}	9.89 ± 3.59^{abcd}	9.41 ± 4.32^{bcde}	9.24 ± 4.14^{cde}
4	6.45 ± 2.30^{efgh}	7.28 ± 2.84^{defgh}	5.43 ± 2.25^{gh}	7.26 ± 2.62^{defgh}
5	4.39 ± 1.45^{h}	5.94 ± 2.29^{fgh}	7.56 ± 2.12^{defgh}	7.11 ± 3.21^{defgh}

**Significant difference ($p \leq 0.05$). Same letter does not differ by the Tukey test ($p \leq 0.05$).*

environments, and usually exhibit high mycorrhizal colonization and responsiveness. In contrast, late-successional woody species with low metabolic rate dominant in mature forest occur in an environment with low light incidence and exhibit low mycorrhizal colonization and responsiveness (Zangaro et al., 2007). High mycorrhizal colonization and responsiveness or early successional species can be modified when these are propagated in fertile substrates, leading to modification of the root architecture by the increase in root density and specific root length.

Biometric evaluation in clonal seedlings showed that *R clarus* colonization induced a differential response in growth parameters. *R clarus* had no negative effect on the growth of aerial parts of teak. It also did not significantly affect biomass and branching of the root system. Stem diameter was decreased, and root branching stimulated the co-inoculated PGPR + AMF treatment (**Figure 3**). One of the factors that lead to this response can be the substrate fertility and container volume used in nurseries. Some authors, such as Zangaro et al. (2015), have described a similar response in seedlings inoculated with AMF in 50 cm^3 tubes and high fertility substrates, where plants showed growth depression. The depression was possibly related to the AMF association cost for the host plant, especially related to carbon; AMF are dependent on organic carbon from plant photosynthates, and the benefits of plant-fungus association can be affected by the decrease in the photosynthetic rate of

TABLE 9 | Tree volume, mean annual increment, and current annual increment at 3 years old in plants of *Tectona grandis* in Pará state, Brazil.

Factor		V (dm^3)		MAI (dm^3 $year^{-1}$)		CAI (dm^3)	
	df	*F*	*p*-value	*F*	*p*-value	*F*	*p*-value
ANOVA - Pará, 3 years old							
Treatment (A)	3	5.237	0.0015*	5.237	0.0015*	5.299	0.0014*
Block (B)	4	27.375	0.0000*	27.378	0.0000*	20.983	0.0000*
A × B	12	7.408	0.0000*	7.409	0.0000*	6.553	0.0000*
Error		903		100.3		712	

Treatment	V (dm^3)	MAI (dm^3 $year^{-1}$)	CAI (dm^3)
Tukey test Treatment			
T1 - Control	128.64 ± 7.37^{b}	42.88 ± 3.68^{b}	112.14 ± 32.33^{b}
T2 - PGPR	144.61 ± 7.66^{a} (↑ 11.04%)	48.20 ± 3.83^{a}	126.50 ± 32.63^{a}
T3 - AMF	135.06 ± 7.29^{ab} (↑ 4.75%)	45.02 ± 3.64^{ab}	118.92 ± 27.56^{ab}
T4 - PGPR + AMF	136.58 ± 7.05^{ab} (↑ 5.81%)	45.53 ± 3.53^{ab}	118.74 ± 30.50^{ab}

Block	V (dm^3)	MAI (dm^3 $year^{-1}$)	CAI (dm^3)
Tukey test of block			
1	152.98 ± 33.19^{a}	50.99 ± 11.06^{a}	133.13 ± 29.78^{a}
2	146.56 ± 36.06^{a}	48.85 ± 12.02^{a}	125.52 ± 31.70^{a}
3	143.68 ± 36.70^{a}	47.89 ± 12.23^{a}	125.18 ± 31.45^{a}
4	117.69 ± 23.26^{b}	39.23 ± 7.75^{b}	104.58 ± 20.78^{b}
5	118.19 ± 33.14^{b}	39.40 ± 11.05^{b}	105.47 ± 29.22^{b}

Block	T1 - Control	T2 – PGPR	T3 – AMF	T4 - PGPR + AMF
Tukey test of A × B V (dm^3)				
1	144.48 ± 19.32^{abcde}	170.65 ± 41.99^{a}	133.10 ± 25.54^{bcdef}	163.96 ± 29.83^{ab}
2	165.48 ± 34.14^{a}	143.06 ± 36.93^{abcde}	154.97 ± 33.49^{abc}	123.48 ± 26.81^{def}
3	129.35 ± 27.49^{cdef}	152.70 ± 39.41^{abcd}	147.54 ± 39.46^{abcde}	143.16 ± 36.65^{abcde}
4	117.26 ± 22.21^{ef}	129.59 ± 25.38^{cdef}	109.18 ± 19.69^{fg}	118.02 ± 23.81^{ef}
5	76.01 ± 20.12^{g}	124.68 ± 20.44^{cdef}	129.30 ± 20.50^{cdef}	132.57 ± 37.17^{cdef}
MAI (dm^3 $year^{-1}$)				
1	48.16 ± 6.44^{abcde}	56.88 ± 14.00^{a}	44.37 ± 8.51^{bcdef}	54.65 ± 9.94^{ab}
2	55.16 ± 11.38^{a}	47.69 ± 12.31^{abcde}	51.66 ± 11.16^{abc}	41.16 ± 8.94^{def}
3	43.12 ± 9.16^{cdef}	50.90 ± 13.14^{abcd}	49.18 ± 13.15^{abcde}	47.72 ± 12.22^{abcde}
4	39.09 ± 7.40^{ef}	43.20 ± 8.46^{cdef}	36.39 ± 6.56^{fg}	39.34 ± 7.94^{ef}
5	25.34 ± 6.71^{g}	41.56 ± 6.81^{cdef}	43.10 ± 6.83^{cdef}	44.19 ± 12.39^{cdef}
CAI (dm^3)				
1	127.22 ± 17.71^{abcde}	145.73 ± 38.71^{a}	$118.88 \pm 25.01^{abcdef}$	140.89 ± 28.35^{ab}
2	139.74 ± 30.14^{abc}	$124.19 \pm 32.95^{abcdef}$	133.61 ± 28.87^{abc}	104.93 ± 24.63^{def}
3	112.72 ± 24.56^{cdef}	132.92 ± 34.26^{abcd}	128.71 ± 33.35^{abcde}	$124.68 \pm 30.60^{abcdef}$
4	104.35 ± 18.84^{ef}	$115.02 \pm 25.78^{abcdef}$	98.31 ± 16.70^{f}	103.51 ± 20.97^{ef}
5	67.23 ± 18.76^{g}	112.80 ± 17.22^{cdef}	114.18 ± 17.75^{bcdef}	$118.36 \pm 31.87^{abcdef}$

**Significant difference ($p \leq 0.05$). Same letter does not differ by the Tukey test ($p \leq 0.05$).*
↑Percentage of gain in relation to the control treatment.

environments with light limitation (Smith and Read, 2008). In this context, the carbon cost of AM overcomes the benefits of mineral absorption for the plant. Therefore, the allocation of resources for the maintenance of AMF can be advantageous for the plant, although it does not result in large accumulation of vegetative biomass, as the nutrients acquired *via* AMF can be allocated to increase the growth capacity of the plants in the field.

The nutrients in the foliar analysis for the nursery phase correlated positively with the treatments with microorganisms, in contrast to the control treatment, which did not present any correlation. The chemical properties of soils can be limiting for

teak growth (Jerez-Rico and Coutinho, 2017). Well-nourished seedlings can be fundamental for good development in the field.

In addition to well-nourished seedlings in the field, the presence of PGPR and AMF can bring several benefits. For example, teak is demanding the availability of nutrients, such as nitrogen, phosphorus, potassium, calcium, and magnesium. Base saturation should be >50%, high pH (>5.5) and low aluminum presence (Matricardi, 1989; Jerez-Rico and Coutinho, 2017). The AMF have been reported to increase the tolerance of teak trees to acidity or high concentrations of Al (Alvarado et al., 2004), and PGPR can be acting in P-solubilization (Meng et al., 2016), helping the plants not to suffer in poor soils such as the soils in the planting areas of this experiment.

The beneficial approach of microorganism inoculation for the development of woody plants in fields has been reported (Siviero et al., 2008; Cely et al., 2016; Duin et al., 2019). Root colonization by AMF in teak (*T grandis* L.f.) has been evaluated by other authors, such as Irianto and Santoso (2005). In this study, the inoculation of *Glomus aggregatum* and a mixture containing *Gigaspora margarita, Glomus manihotis*, *Glomus etunicatum*, and *Acaulospora spinosa* accelerated the height and diameter growth by 61 and 47%, respectively, after 3 months in the field. In our study, in the Pará state, plant growth was positively affected by PGPR in both second and third year and by PGPR + AMF in the third year with the increase in V by 11.04% (PGPR) followed by PGPR + AMF with an increase of 5.81% compared with the control (**Tables 6**, **7**).

The potential of the inoculation of microorganisms for teak development in the nursery and field was recently shown by Raghu et al. (2020), showing that the inoculation of a microbial consortium (*A leptotich*a + *A chroococcum* + *T harzianum*) can improve the growth of plants 289% more than uninoculated plants. These responses of growth promotion can vary depending on the species of microorganisms inoculated, climate characteristics in the field, and genetic profile of teak cultivars, as observed in the evaluations at 2 and 3 years for tree volume (V), mean annual increment (MAI), and current annual increment (CAI).

Thus, a combination of bio-based products in the production of teak seedlings can provide quality seedlings that have good results in the field, even on nutritionally poor soils. The adoption of the use of commercial bio-based products helps to implement this technology in the routine of teak-producing nurseries.

DATA AVAILABILITY STATEMENT

The original contributions presented in the study are included in the article/supplementary materials, further inquiries can be directed to the corresponding author/s.

AUTHOR CONTRIBUTIONS

FS, LV, and IH: coordination, execution, monitoring of experiments, and data processing. DS, AP, MB, BF, DD, AC, and AS: laboratory and field data collection. BG, ML, and GA: arbuscular mycorrhizal inoculum production, scientific support, and manuscript writing. RF and MT: idealization, coordination, and project planning. ES, JB, and FT: coordination, technical, and financial support at the Teak Resources Company (TRC). All authors contributed to the article and approved the submitted version.

FUNDING

The authors acknowledge the Conselho Nacional de Desenvolvimento Científico e Tecnológico (CNPq) and Coordenação de Aperfeiçoamento de Pessoal de Nível Superior (CAPES) for making this study possible via the PIBIC (Institutional Scientific Initiation Scholarship Program) and by providing funds for M.Sc. degrees, the Teak Resources Company (TRC) for economic, technical, and logistic support to the conduct experiments in the nursery and field, and to the Universidade Federal de Mato Grosso/Research Dean PROPeq/PROPG-UFMT, Laboratório de Patologia Florestal of Universidade Federal de Viçosa and the Teak Resources Company (TRC) for economic support for publishing fees. The authors declare the Teak Resources Company (TRC) was not involved in the study design, collection, analysis, interpretation of data, the writing of this article or the decision to submit it for publication.

REFERENCES

Ajeesh, R., Santhoshkuma, A.V., and Go, S. (2017). Screening of selected native arbuscular mycorrhizal fungi at different levels for their symbiotic efficiency with *Tectona grandis* seedlings. *J. Trop. For. Sci.* 29, 395–403. doi: 10.26525/jtfs2017.29.4.395403

Akinrinlola, R. J., Yuen, G. Y., Drijber, R. A., and Adesemoye, A. O. (2018). Evaluation of *Bacillus* strains for plant growth promotion and predictability of efficacy by *in vitro* physiological traits. *Int. J Microb*. 2018:5686874. doi: 10.1155/2018/5686874

Alam, M., Khaliq, A., Sattar, A., Shukla, R.S., Anwar, M., and Seema Dharni, S. (2011). Synergistic effect of arbuscular mycorrhizal fungi and *Bacillus subtilis* on the biomass and essential oil yield of rose-scented geranium (*Pelargonium graveolens*). *Arch. Agron. Soil. Sci.* 57, 889–898. doi: 10.1080/03650340.2010.498013

Ali, B., Sabri, A. N., Ljung, K., and Hasnain, S. (2009). Auxin production by plant associated bacteria: impact on endogenous IAA content and growth of *Triticum aestivum* L. *Lett. Appl. Microbiol.* 48, 542–547. doi: 10.1111/j.1472-765X.2009.02565.x

Alvarado, A., Chavarría, M., Guerrero, R., Boniche, J., and Navarro, J. R. (2004). Características edáficas y presencia de micorrizas en plantaciones de teca (*Tectona grandis*L.f.) en Costa Rica. *AgronomíaCostarricense*. 28, 89–100. Available online at: https://www.redalyc.org/articulo.oa?id=43628109

Andrade, G., and Cely, M.V.T. (2019). *Processo de Produção e Inoculação de Fungos Micorrízicos Arbusculares. BR Patent No 102019008109-0 A2.*

Awasthi, A., Bharti, N., Nair, P., Singh, R., Shukla, A., Gupta, M., et al. (2011). Synergistic effect of *Glomus mosseae* and nitrogen fixing *Bacillus subtilis* strain

Daz26 on artemisin content in *Artemisia annua* L. *Appl. Soil Ecol.* 49, 125–130. doi: 10.1016/j.apsoil.2011.06.005

Carneiro, M. F., Albuquerque, W. W., Souza, C. O., Câmara, A. P., Silva, J. G. M., and Galvão, E. K. S. (2018). Radial growth and artificial form factor of teak trees in Alta Floresta do Oeste, Rondônia. *Rev. Cienc. Agrar.* 61, 1–7. doi: 10.22491/rca.2018.2702

Cely, M. V. T., Siviero, M. A., Emiliano, J., Spago, F.R., Freitas, V. F., Barazetti, A. R., et al. (2016). Inoculation of *Schizolobiumparahyba* with mycorrhizal fungi and plant growth-promoting rhizobacteria increases wood yield under field conditions. *Front. Plant Sci.* 7, 1–13. doi: 10.3389/fpls.2016.01708

Chen, J.W, Zhanga, Q., Lia, X.S., and Cao, K.F. (2011). Steady and dynamic photosynthetic responses of seedlings from contrasting successional groups under low-light growth conditions. *Physiol. Plantarum.* 141, 84–95. doi: 10.1111/j.1399-3054.2010.01414.x

Duin, V. F. F., Liuti, G., Prado, N. V., Cely, M. V. T., Andreata, M. F. L., Santos, I. M. O., et al. (2019). Effect of the fertilization and growth promoting microrganisms on *Schizolobiumparahyba*. *Semina Ciênc. Agrár.* 40, 1747–1760. doi: 10.5433/1679-0359.2019v40n5p1747

FAO (1994). *Soil Map of the World. Revised Legend With Corrections*. Rome: FAO-UNESCOISRIC, 140.

Galaviz, C., Lopez, B. R., Bashan, L. E., Hirsch, A. M., Maymon, M., and Bashan, Y. (2018). Root growth improvement of mesquite seedlings and bacterial rhizosphere and soil community changes are induced by inoculation with plant growth-promoting bacteria and promote restoration of eroded desert soil. *Land Degrad. Dev.* 2018:2904. doi: 10.1002/ldr.2904

Giovannetti, M., and Mosse, B. (1980). An evaluation of techniques for measuring vesicular arbuscular mycorrhizal infection in roots. *New Phytol.* 84, 489–500. doi: 10.1111/j.1469-8137.1980.tb04556.x

Hashem, A., Abd_Allah, E. F., Alqarawi, A. A., Al-Huqail, A. A., and Shah, M. A. (2016). Induction of osmoregulation and modulation of salt stress in *Acacia gerrardii* Benth. by arbuscular mycorrhizal fungi and *Bacillus subtilis* (BERA 71). *Bio. Med. Res. Int.* 2016:6294098. doi: 10.1155/2016/6294098

IBÁ. (2019). *Relatório IBÁ 2019 – Desempenho do setor florestal em 2018*. Brasília.

Irianto, R.S.B., and Santoso, E. (2005). Effect of arbuscular mycorrhiza fungi inoculation on teak (*Tectona grandis* Linn. *F*) at cikampek, west Java. *J. For. Res.* 2, 69–73. doi: 10.20886/ijfr.2005.2.2.69-73

Jerez-Rico, M., and Coutinho, S. A. (2017). "Establishment and management of planted teak forests," in *The Global Teak Study. Analysis, Evaluation and Future Potential of Teak Resources IUFRO World Series*, Vol. 36, eds W. Kollert, and M. Kleine (Vienna), 49–65.

Köppen, W. (1936). "Das geographische System der Klimate," in *Handbuch der Klimatologie*, Vol. 1, eds W. Köppen, and R. Geiger (Berlin: Verlag von Gebrüder Borntraeger), 1–44.

Marulanda-Aguirre, A., Azcón, R., Ruiz-Lozano, J. M., and Aroca, R. (2007). Differential effects of a *Bacillusmegaterium* Strain on *Lactuca sativa* plant growth depending on the rigin of the arbuscular mycorrhizal fungus co-inoculated: physiologic and biochemical traits. *J. Plant. Growth Regul.* 27, 10–18. doi: 10.1007/s00344-007-9024-5

Matricardi, W. A. T. (1989). *Efeitos dos fatores do solo sobre o desenvolvimento da teca (Tectona grandis L. F.) cultivada na grande Cáceres - Mato Grosso* (Dissertação, Mestrado em Ciências Florestais), Universidade de São Paulo, Piracicaba, 135.

Medina, A., Probanza, A., Gutierrez Mañero, F. J., and Azcón, R. (2003). Interactions of arbuscular-mycorrhizal fungi and *Bacillus* strains and their effects on plant growth, microbial rhizosphere activity (thymidine and leucine incorporation) and fungal biomass (ergosterol and chitin). *App. Soil. Ecol.* 22, 15–28. doi: 10.1016/S0929-1393(02)00112-9

Meng, Q., Jiang, H., and Hao, J. J. (2016). Effects of *Bacillus velezensis* strain BAC03 in promoting plant growth. *Biol. Control.* 98, 18–26. doi: 10.1016/j.biocontrol.2016.03.010

Meza, A., Rodriguez, J., Gatti, K., and Espinoza, E. (2015). Propagación de arboles de teca *Tectona grandis* L. f. por miniestacas. *Temas Agrar.* 20, 43–48. doi: 10.21897/rta.v20i2.757

Pandey, D., and Brown, C. (2000). *Teak: A Global Overview* (Unasylva: FAO), 3–13.

Phillips, J. M., and Hayman, D. S. (1970). Improved procedures for clearing roots and staining parasitic and vesicular-arbuscular mycorrhizal for rapid assessment of infection. *Trans. Br. Mycol. Soc.* 55, 158–161. doi: 10.1016/S0007-1536(70)80110-3

Prayudyaningsih, R., and Sari, R. (2016). The application of arbuscular mycorrhizal fungi (AMF) and compost to Improve the growth of Teak seedlings (*Tectona grandis* Linn. f.) on limestone post-mining soil. *J. P. K. Wallacea* 5, 37–46. doi: 10.18330/jwallacea.2016.vol5iss1pp37-46

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/ (accessed December 3, 2020).

Radhakrishnan, R., Hashem, A., and Abd_Allah, E. F. (2017). *Bacillus*: a biological tool for crop improvement through bio-molecular changes in adverse environments. *Front. Physiol.* 8, 1–14. doi: 10.3389/fphys.2017.00667

Raghu, H. B., Ashwin, R., Ravi, J. E., and Bagyaraj, D. J. (2020). Microbial consortium improved growth and performance of Teak (*Tectona grandis* L.f.) in nursery and field trials. *Proc. Natl. Acad. Sci. India Sect. B Biol. Sci.* 90, 903–909. doi: 10.1007/s40011-019-01163-0

Rillig, M.C., and Mummey, D.L. (2006). Mycorrhizas and soil structure. *New Phytol.* 171, 41–53. doi: 10.1111/j.1469-8137.2006.01750.x

Rodrigues, L.A., Barroso, D.G., and Figueredo, F.A.M. (2018). Fungos micorrízicos arbusculares no crescimento e na nutrição mineral de mudas de *Tectona grandis* L. *F. Ciência Florest.* 28, 25–34. doi: 10.5902/1980509831572

Ryser, P., and Lambers, H. (1995). Root and leaf attributes accounting for the performance of fast- and slow-growing grasses at different nutrient supply. *Plant Soil.* 170, 251–265. doi: 10.1007/BF00010478

Schuhli, G.S., and Paludzyszyn Filho, E. (2010). O cenário nacional da silvicultura de teca (*Tectona grandis* L. f.) e perspectivas de melhoramento. *Pesqui. Florest. Bras.* 30, 217–230. doi: 10.4336/2010.pfb.30.63.217

Siviero, M. A., Marega, A., Lima Dos Santos, D., Rosselli, B. R., Huh, Y. S., Santinoni, I., et al. (2008). Interaction among N-fixing bacteria and AM fungi in Amazonian legume tree (*Schizolobiumamazonicum*) in field conditions. *Appl. Soil Ecol.* 39, 144–152. doi: 10.1016/j.apsoil.2007.12.004

Smith, S. E., and Read, D. J. (2008). "Growth and carbon economy of arbuscular mycorrhizal symbionts," in *Mycorrhizal Symbiosis, 3rd Edn*, eds S. E. Smith, and D. J. Read (London: Elseiver), 119–187. doi: 10.1016/B978-012370526-6.50006-4

Smith, S. E., and Smith, F. A. (2011). Roles of arbuscular mycorrhizas in plant nutrition and growth: new paradigms from cellular to ecosystem scales. *Ann. Rev. Plant Biol.* 62, 227–250. doi: 10.1146/annurev-arplant-042110-103846

Teixeira, D. A., Alfenas, A. C., Mafia, R. G., Ferreira, E. M., Siqueira, L., Maffia, L. A., et al. (2007). Rhizobacterial promotion of eucalypt rooting and growth. *Brazil. J. Microbiol.* 38, 118–123. doi: 10.1590/S1517-838220070001 00025

Zangaro, W., Nishidate, F.R., Vandresen, J., Andrade, G., and Nogueira, M.A. (2007). Root mycorrhizal colonization and plant responsiveness are related to root plasticity, soil fertility and successional status of native woody species in southern Brazil. *J. Trop. Ecol.* 23, 53–62. doi: 10.1017/S02664674060 03713

Zangaro, W., Torezan, M. D., Rostirola, L. V., Souza, P. B., and Nogueira, M. A. (2015). Influence of mycorrhizas, organic substrates and container volumes on the growth of *Heliocarpuspopayanensis* Kunth. *Cerne* 21, 395–403. doi: 10.1590/01047760201521031335

Conflict of Interest: JB, ES, and FT are employed by the Teak Resources Company (TRC).

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

frontiers in Plant Science

ORIGINAL RESEARCH
published: 15 July 2021
doi: 10.3389/fpls.2021.661842

Production of Organic Acids by Arbuscular Mycorrhizal Fungi and Their Contribution in the Mobilization of Phosphorus Bound to Iron Oxides

Alberto Andrino[1], Georg Guggenberger[1], Sarmite Kernchen[2], Robert Mikutta[3], Leopold Sauheitl[1] and Jens Boy[1]*

[1] Institute of Soil Science, Leibniz Universität Hannover, Hanover, Germany, [2] Bayreuth Center of Ecology and Environmental Research, University of Bayreuth, Bayreuth, Germany, [3] Soil Science and Soil Protection, Martin Luther University Halle-Wittenberg, Halle (Saale), Germany

Most plants living in tropical acid soils depend on the arbuscular mycorrhizal (AM) symbiosis for mobilizing low-accessible phosphorus (P), due to its strong bonding by iron (Fe) oxides. The roots release low-molecular-weight organic acids (LMWOAs) as a mechanism to increase soil P availability by ligand exchange or dissolution. However, little is known on the LMWOA production by AM fungi (AMF), since most studies conducted on AM plants do not discriminate on the LMWOA origin. This study aimed to determine whether AMF release significant amounts of LMWOAs to liberate P bound to Fe oxides, which is otherwise unavailable for the plant. *Solanum lycopersicum* L. plants mycorrhized with *Rhizophagus irregularis* were placed in a bicompartmental mesocosm, with P sources only accessible by AMF. Fingerprinting of LMWOAs in compartments containing free and goethite-bound orthophosphate (OP or GOE-OP) and phytic acid (PA or GOE-PA) was done. To assess P mobilization *via* AM symbiosis, P content, photosynthesis, and the degree of mycorrhization were determined in the plant; whereas, AM hyphae abundance was determined using lipid biomarkers. The results showing a higher shoot P content, along with a lower N:P ratio and a higher photosynthetic capacity, may be indicative of a higher photosynthetic P-use efficiency, when AM plants mobilized P from less-accessible sources. The presence of mono-, di-, and tricarboxylic LMWOAs in compartments containing OP or GOE-OP and phytic acid (PA or GOE-PA) points toward the occurrence of reductive dissolution and ligand exchange/dissolution reactions. Furthermore, hyphae grown in goethite loaded with OP and PA exhibited an increased content of unsaturated lipids, pointing to an increased membrane fluidity in order to maintain optimal hyphal functionality and facilitate the incorporation of P. Our results underpin the centrality of AM symbiosis in soil biogeochemical processes, by highlighting the ability of the AMF and accompanying microbiota in releasing significant amounts of LMWOAs to mobilize P bound to Fe oxides.

Keywords: arbuscular mycorrhiza, low-molecular-weight organic acid, iron oxides, membrane fluidity, organic P, inorganic P, ligand exchange, reductive dissolution

OPEN ACCESS

Edited by:
Sergio Saia,
University of Pisa, Italy

Reviewed by:
Philipp Franken,
Friedrich Schiller University Jena, Germany
Luisella Celi,
University of Turin, Italy

****Correspondence:***
Alberto Andrino
andrino@ifbk.uni-hannover.de

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

Received: *31 January 2021*
Accepted: *15 June 2021*
Published: *15 July 2021*

Citation:
Andrino A, Guggenberger G, Kernchen S, Mikutta R, Sauheitl L and Boy J (2021) Production of Organic Acids by Arbuscular Mycorrhizal Fungi and Their Contribution in the Mobilization of Phosphorus Bound to Iron Oxides. Front. Plant Sci. 12:661842. doi: 10.3389/fpls.2021.661842

INTRODUCTION

Phosphorus (P) is an essential plant macronutrient (Schachtman et al., 1998), and its deficiency limits the plant growth in both natural and agricultural systems (Oberson et al., 2001). Particularly, in acidic soils, the high affinity and strong specific adsorption of inorganic (Pi) and organic (Po) phosphorus forms to iron (Fe) oxides determine their accessibility to plants (He and Zhu, 1998). Rhizosphere acidification and the release of low-molecular-weight organic acids (LMWOAs) are the important plant response mechanisms to increase P availability in the soil solution (Wang et al., 2019). The LMWOAs may solubilize P from mineral surfaces either by ligand exchange or by ligand-promoted dissolution of Fe oxides (Owen et al., 2015). The ability of different LMWOAs to desorb P generally decreases with a decrease in the stability constants of Fe (III) acid complexes (Marschener, 1998; Deubel and Merbach, 2005). The adsorption of LMWOAs is driven by positively charged oxide surfaces and the negative charge of the carboxylate group and is influenced by the formation of metal complexes in solution, with adsorption generally increasing with their concentration in solution and the number of carboxylic groups (Oburger et al., 2011; Adeleke et al., 2017). Thus, tricarboxylic acids such as citrate have a higher efficiency to desorb P from Fe oxides than dicarboxylic or monocarboxylic ones (Geelhoed et al., 1999; Richardson, 2001).

The association of plants with symbiont organisms is one of the most widespread strategies employed to mobilize P in acidic tropical soils (Seguel et al., 2013). In particular, the association with arbuscular mycorrhizal fungi (AMF) is central to the P cycling, mobilization, and supply to plants adapted to acidic environments (Klugh and Cumming, 2007). The arbuscular mycorrhizal (AM) symbiosis promotes the formation of an extensive mycelium network that operates as functional extensions of the plant root system (Xu et al., 2007), exchanging the acquired P for fresh assimilated photosynthetic carbon (C) from the host plant (Zhang et al., 2016). Furthermore, AMF may act as hub translocating freshly assimilated C to soil microbes on the surfaces of mycorrhizal hyphae, spores, and the hyphosphere, the zone surrounding individual fungal hyphae (Zhang et al., 2014; Manchanda et al., 2017). The accompanying AMF microbiota may be functionally diverse and provide essential plant growth-promoting functions, such as phytate mineralization, siderophore production, Pi solubilization, and LMWOA production (Battini et al., 2016). In this way, the association of AMF with bacteria provides a beneficial partnership for accessing and mobilizing soil P pools, which otherwise would not be available to the plant (Wang et al., 2016; Drigo and Donn, 2017). Phosphorus mobilization by AMF may involve both Pi (Smith and Read, 2008) and Po forms (Andrino et al., 2020). There is also evidence that AMF can desorb OP from ferrihydrite (Gogala et al., 1995; Rakshit and Bhadoria, 2010), and recently, we confirmed the ability of *R. irregularis* to mobilize Po and Pi bound to goethite (GOE), one of the most abundant Fe (oxy)hydroxides in tropical soils, at differing host plant C cost (Andrino et al., 2019).

The release of P bound to pedogenic oxides requires the action of LMWOAs (Geelhoed et al., 1999), but the production of LMWOAs by AMF is still poorly documented (Bharadwaj et al., 2012). Sato et al. (2015) and Burghelea et al. (2018) pointed out that AMF exudates involved in P mobilization from Po and Pi sources may comprise phosphatases, phenolic compounds, protons, siderophores, and an increased root exudation of organic ligands; however, studies on the production of LMWOAs by AMF are scarce (Tawaraya et al., 2006; Toljander et al., 2007). Consequently, the present study seeks to understand the role of LMWOAs secreted by the AMF to the P mobilization from GOE-bound P sources. We hypothesize that the suite of LMWOAs produced when mobilizing P from GOE-bound orthophosphate (OP or GOE-OP) and phytic acid (PA or GOE-PA) sources differs from those in the presence of their free P forms, as a consequence of ligand dissolution processes. To this end, we used a bicompartmental mesocosm consisting of a plant compartment (PC) harboring one *Solanum lycopersicum* L. plant mycorrhized with *Rhizophagus irregularis*; however, the fungal compartment (FC) contained free or OP or GOE-OP and PA or GOE-PA only accessible by the AM fungus. To assess P mobilization via AM symbiosis, P and N contents, photosynthesis, and the degree of mycorrhization were determined in the plant; however, LMWOAs fingerprint and the AM hyphae abundance were determined using the FC.

MATERIALS AND METHODS

Phosphorus Sources

Four P sources were prepared to be added individually into the FCs as described in **Table 1**. OP was added as KH_2PO_4 (Sigma-Aldrich, Steinheim, Germany), whereas PA was added as sodium salt (Sigma-Aldrich, Steinheim, Germany). The adsorption

TABLE 1 | Description of the treatments tested during the time course experiment.

Codes	Treatment	Phosphorus content at the fungal compartment
M+	Control: treatment containing an arbuscular mycorrhizal (AM) plant and no phosphorus (P) source in the fungal compartment (FC)	Quartz sand (60 g) + MilliQ water (16 ml) containing no P
M-	Control: non-AM plant and no P source in the FC	Quartz sand (60 g) + MilliQ water (16 ml) containing no P
GOE	Control: AM plant and no P source in the FC	Bayferrox 920 Z goethite (24.3 g) + MilliQ water (28ml) containing no P
OP	AM plant and orthophosphate (KH_2PO_4) as P source in the FC	Quartz sand (60 g) + containing 30 mg P (16 ml)
PA	AM plant and phytic acid solution ($C_6H_{18}O_{24}P_6{\cdot}xNa^+{\cdot}yH_2O$) as P source in the FC	Quartz sand (60 g) + containing 30 mg P (16 ml)
GOE-PA	AM plant and phytic acid bound to goethite adsorption complex (1.79 g P/kg) as P source in the FC	GOE-PA (16.7 g) containing 30 mg P + Bayferrox 920 Z goethite (7.6 g) + MilliQ water (28 ml)
GOE-OP	AM plant and orthophosphate bound to goethite adsorption complex (1.24 g P/kg) as P source in the FC	GOE-OP (24.3 g) containing 30 mg P + MilliQ water (28 ml)

complexes were prepared by equilibrating P compounds with GOE (Bayferrox 920 Z. Lanxess, Cologne, Germany). The first step involved the equilibration of 50 g of GOE for 16 h in 250 ml ultrapure water adjusted to pH 4 by 0.5 M HCl. Second, 250 ml ultrapure water containing either 17 g KH_2PO_4 or 0.72 g $C_6H_{18}O_{24}P_6$ and adjusted to pH 4 by 0.5 M HCl was added to the GOE solution and equilibrated for 48 h on an overhead shaker. The GOE-P suspensions were centrifuged for 15 min (3,000 × *g*), and pellets were afterward rinsed with ultrapure water until the electric conductivity was <40 μS cm^{-1}. Finally, the resulting GOE-P associations were shock-frozen in liquid N_2 and freeze-dried. The loading of OP and PA onto the GOE was determined by hydrolyzing 5 mg of the GOE-P associations in concentrated HNO_3 (n = 3) and subsequent measurement of P contents by ICP-MS Agilent 7500C (Agilent Technologies, Santa Clara, CA, United States). The adsorption complexes contained 1.24 mg P g^{-1} for GOE-OP and 1.79 mg P g^{-1} for GOE-PA.

Plant Mycorrhization

Solanum lycopersicum L. var. Moneymaker seeds (Volmary GmbH) were surface-sterilized (5% H_2O_2, 10 min), soaked in distilled and autoclaved water, and pregerminated on petri dishes (72 h, 27°C). We selected *R. irregularis* DAOM 197198 as AMF due to its global distribution and well adaptation to intensive agricultural practices (Köhl et al., 2016). The inoculum consisted of 0.4 g containing AMF propagules (roots, spores, hyphae) of *Sorghum bicolor* inoculated with *R. irregularis* DAOM 197198 (Symplanta GmbH & Co. KG. Darmstadt, Germany) grown in a trap plant culture following the methodology of Brundrett et al. (1996). The combination of both organisms has been selected in several other research studies as a model of mutualistic association (Herrera-Medina et al., 2008; Fernández et al., 2014). Tomato pregerminated seeds were planted in QP96 cells (HerkuPlast Kubern GmbH, Ering, Germany) together with the inoculum of *R. irregularis* and 70 ml of autoclaved and acid-washed quartz sand. The quartz sand was used as a nutrient-free culture substrate suitable for the colonization of AMF (**Table 1**), where high-purity mycelium can develop (Johansen et al., 1996; Olsson and Johansen, 2000). For the non-mycorrhizal plants, we grew *S. bicolor* without including any AMF inoculum. Then, 0.4 g of non-inoculated inoculum was applied after checking that no endophyte was colonizing the roots. Mycorrhized and non-mycorrhized tomato plants were grown in a greenhouse (photoperiod, 16/8 h light/dark; temperature, 24/20°C light/dark; relative humidity, 50–60%; photon flux density, 175–230 μmol m^{-2} s^{-1}). *S. lycopersicum* seedlings were watered every day with 10 ml deionized water and on alternate days were fertilized with 5 ml low P (0.32 mM) modified Long Ashton nutrient solution pH 6.5 (Hewitt, 1966).

Time Course Experiment

The mesocosms were made of two compartments, a PC and a FC. In the latter, exclusively hyphae could enter and access the four P sources (**Figure 1**), as a polyamide mesh (20 μm pore diameter) (Franz Eckert GmbH, Waldkirch, Germany) separated

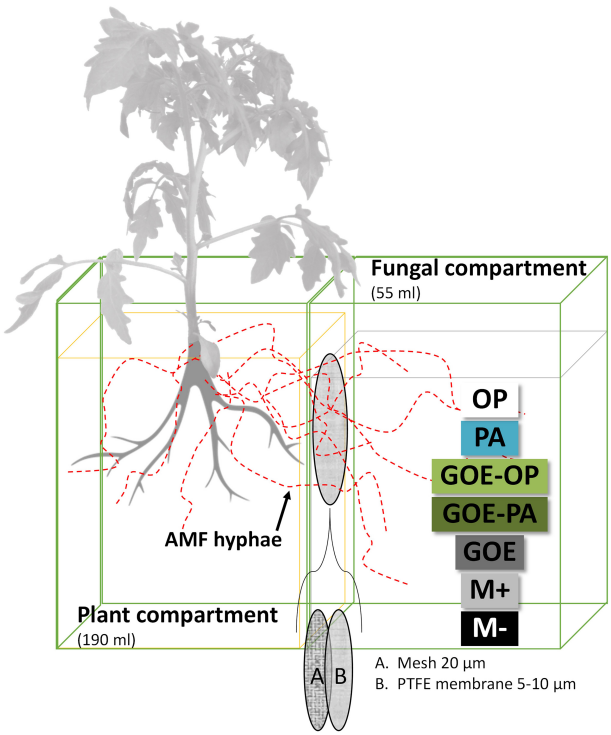

FIGURE 1 | Scheme of the bicompartmental mesocosms, comprising a plant and a fungal compartment. A polyamide mesh (20 μm pore diameter) separated mycorrhizal roots and mycelium, while the polytetrafluoroethylene (PTFE) membrane (5–10 μm pore diameter) allowed the AMF to cross and access the individual P sources but avoided the diffusion of ions into the plant compartment. OP, orthophosphate; PA, phytic acid; GOE-PA, phytic acid bound to goethite; GOE-OP, orthophosphate bound to goethite; GOE, mycorrhized plant, no P, and goethite; M+, mycorrhized plant, no P, quartz sand; M-, non-mycorrhized plant, no P, quartz sand.

mycorrhizal roots and mycelium (Watkins et al., 1996; Fitter et al., 1998). A second polytetrafluoroethylene membrane (5–10 μm pore diameter) (Pieper Filter GmbH, Bad Zwischenahn, Germany) allowed the AMF hyphae to cross but avoided the diffusion of ions into the PC (Mäder et al., 2000). The different P sources were placed into the FC as described in **Table 1**. Three types of controls were included, one without a mycorrhized plant and without a P source, to evaluate how the tomato plant responds to the absence of P (M-): two more controls both with mycorrhized plants and without P, one containing quartz sand (M+) and one with only GOE in the FC (GOE), to the evaluate possible effects related to the substrate where the fungus grows, but not to the P source (**Table 1**). Four-week-old mycorrhizal and control *S. lycopersicum* plants were planted into the PC. Mesocosms, comprising three biological replicates per P source and harvest point, were placed in a climatic controlled greenhouse (photoperiod, 16/8 h light/dark; temperature, 24/20°C light/dark; relative humidity, 50–60%; photon flux density, 175–230 μmol m^{-2} s^{-1}). They were watered two times a week with 10 ml ultrapure water. On alternate days, the pots were fertilized with 5 ml no-P Long Ashton

nutrient solution. Once a week, the mesocosms were rotated to achieve homogeneous growth conditions for all mesocosms. The first sampling point was at the day of transplanting (day 0) to determine the initial plant biomass, P and N contents, and photosynthetic activities (n = 5) followed by harvest points at days 7, 21, 35, 49, 77, and 91.

Plant Biomass, Phosphorus, and Nitrogen Contents

At each harvest point, shoots and roots were dried (70°C, 48 h), weighed, and ball-milled. Aliquots of shoot and root were incinerated at 480°C for 8 h, digested in 1 ml 30% HNO_3, and filtered to <0.45 μm (PVDF filters), and the solutions were analyzed for P content by ICP-MS Agilent 7500C (Agilent Technologies, Santa Clara, CA, United States). Total N content from the milled shoot material was measured by dry combustion using an Elementar vario MICRO cube C/N Analyzer (Elementar GmbH, Hanau, Germany). Shoot and root P contents in percentage of total plant dry weight (% dw) were calculated for each sampling point and treatment. Total P acquired into the plant tissues was calculated by subtracting the total P content (mg) in a subsample (n = 5) of the initial transplanted AM plants at day 0 from the total P content (mg) at each harvest point. The shoot N:P ratio, an indicator for P deficiency in the shoot tissues (Hayes et al., 2014; Ros et al., 2018), was calculated for plants accessing the different P sources and controls. Shoot N:P ratios are useful to investigate shifts from N to P limitation because they are easily determined and comparable across studies. Nitrogen limitation for terrestrial plants occurs at values below 10, while P limitation usually occurs above 20 and may cause the inhibition of photosynthesis (Güsewell, 2004).

Photosynthetic Capacity

At each harvest point, the photosynthetic capacity (μmol CO_2 m^{-2} s^{-1}) was measured on recently fully expanded third or fourth leaf from the top, in order to check the impact of the different P sources on the host carbohydrate metabolism. At each sampling point, CO_2 assimilation rate was measured with the LI-6400 (LiCor, Lincoln, NE, United States). Values were recorded at 22°C in the leaf cuvette, at approximately 50% relative humidity, airflow rate was set at 400 μmol/s, the external CO_2 concentration was 360 ppm, and the CO_2 mixer was set at 400 ppm. Irradiance was provided by a led source set to a photon flux density of 1,000 μmol m^{-2} s^{-1}.

Degree of Mycorrhization

Before planting the seedlings into the mesocosms and at each harvest point, a root subsample was digested in 10% KOH (35 min, 95°C) and stained using the ink and vinegar staining technique for AMF of Vierheilig et al. (1998). Then, the stained root fragments were arranged on microscope slides with fine tweezers, and the degree of mycorrhization was determined using the methodology of McGonigle et al. (1990). In brief, number of arbuscules, vesicles, and internal hyphae were counted using a compound microscope with an eye piece cross-hair, which is moved to randomly selected positions. Arbuscules, vesicles, and total mycorrhization were expressed as the percentage of the total counted intersections.

Low-Molecular-Weight Organic Acids

Concentration and composition of LMWOAs in the FC containing the different P sources were analyzed in order to determine their role in P acquisition from the different sources. For each treatment containing a P source, the mean content of each LMWOA was calculated using the dates of the harvesting points for the periods where no P incorporation was detected in the AM plant tissue, as well as for those where we detected P uptake in the AM plant. LMWOAs were determined by the method of Tani and Higashi (1999). In brief, 5 g moist sample of each FC was extracted with NH_4-phosphate buffer (0.1 M $NH_4H_2PO_4$–H_3PO_4, pH 2) at a sample (related to dry sample mass) to solution ratio of 1 (wt.):4 (vol), by shaking for 30 min on a horizontal shaker. Then, the crude extract was separated from the sample by centrifuging at 10,000 × g for 10 min, followed by further filtration through a 0.025-μm filter (Supor® PES membrane disk filters, Pall Life Sciences, Hampshire, United Kingdom). Filtered extracts were analyzed with an Agilent series 1100 liquid chromatograph (Agilent Technologies, Santa Clara, CA, United States) coupled to electrospray ionization (ESI) mass spectrometer (Agilent 6130 single quadrupole) to determine the different LMWOAs. Further details on the methods to analyze the LMWOAs can be found in the **Supplementary Material 1**.

Fatty Acid Analysis in the FC

The AMF *R. irregularis* DAOM 197198 has a fatty acid composition ranging from C16:0 to C22:2 (Wewer et al., 2014). The fungal phospholipid fatty acids (PLFAs) 16:1ω5, 18:1ω7c, 18:1ω9c, and 18:2ω6,9 were used as indicators for evaluating the amount of AMF extraradical mycelia, while neutral lipid fatty acids (NLFAs) 16:1ω5, 18:1ω7c, 18:1ω9c, and 18:2ω6,9 signatures were considered as indicators on energy storage by the fungus (Olsson and Wilhelmsson, 2000; Bååth, 2003; van Aarle and Olsson, 2003). Lipids were extracted from 8 g or 16 g fresh weight samples of the FCs containing goethite (GOE, GOE-OP, and GOE-PA) or the ones with quartz (M+, M-, OP, and PA), respectively. Then, extracts were fractionated into PLFA and NLFA by the solid-phase extraction with activated silica gel (Sigma Aldrich, pore size 60 Å, 70–230 mesh). Thereafter, the PLFA and NLFA fractions were saponified into fatty acids, and both types of lipids were esterified with methanol to free fatty acid methyl esters, as outlined in Frostegård et al. (1991) and with modifications by Bischoff et al. (2016). The fatty acid methyl esters were then separated by gas chromatography using an Agilent 7890A GC system (Agilent Technologies, Santa Clara, CA, United States) equipped with a Zebron capillary GC column (60 m, 0.25 mm diameter and 0.25 μm film thickness; Phenomenex, Torrance, California, United States) and quantified with a flame ionization detector, using He as carrier gas. Glyceryl tridodecanoate and non-adecanoic acid were used as internal standards during the extraction, and tridecanoic acid methyl ester was added to each sample and standard before the analysis as a recovery standard. At each time point, the relative abundance (%)

of fungal biomarkers (PLFA and NLFA) was calculated for each treatment containing a P source.

Data Analysis

Data for shoot P contents, root P contents, shoot N:P ratios, photosynthetic activities, and LMWOAs contents were tested for normality with Shapiro–Wilk's test and homogeneity of variances using the Levene's test, and the different variables were subjected to one-way ANOVA. The Duncan *post hoc* test was employed to check for differences of mean values ($p < 0.05$) between the different P sources offered in the FC at each sampling point. Moreover, two correlation tests ($p < 0.05$) were carried out: first, between the relative abundance of all detected fungal PLFA biomarkers in the FC and the amount of P acquired by the plant (mg), to assess the link between AMF presence and plant P allocation. In a second correlation, the degree of mycorrhization (arbuscules, vesicles, and mycorrhization), i.e., a proxy for AM activity (McCormack and Iversen, 2019), was related to the relative abundance of fungal biomarkers (PLFA and NLFA). This second correlation intends to determine whether P incorporation into the plant was linked to mycorrhizal root activity, and this was in turn linked to the development of the fungal symbiont inside the FC. The ANOVA and correlation tests were performed with SPSS v. 24 (IBM Corporation, 2016).

RESULTS

Phosphorus Contents in the Plant Tissues and Photosynthetic Capacity

All AM plants with access to a P source in the FC showed significantly larger P contents in the shoots compared to the roots (**Figure 2**). AM plants that accessed OP and PA or GOE-OP and GOE-PA showed a P dilution in their shoot and root tissues until day 35 or 49, then from day 49 or 77 onward, the P content in their plant tissues significantly increased, respectively, as compared to all controls (**Figure 2**).

Mycorrhizal plants with access to free OP and PA exhibited P deficiency from day 21 until day 35, as was reflected by their N:P ratios >20 (**Figure 3**). From day 49 to day 91, AM plants with access to OP were not P-deficient, as indicated by mean N:P values <20. In the case of AM plants accessing the FC containing PA, the N:P ratios were stable from day 49 until day 91, showing a slight P deficiency in the plant tissues. The AM plants with access to GOE-OP and GOE-PA exhibited P deficiency from day 35 until day 49. At day 77, AM plants accessing both P forms bound to GOE showed a slight P deficiency with N:P values close to 20. From day 21 until day 91, all controls exhibited a significantly higher P deficiency, i.e., higher N:P ratios, compared to all treatments that accessed a P source (**Figure 3**). All controls with no access to a P source showed a significantly lower photosynthetic capacity (μmol CO_2 m^{-2} s^{-1}) from day 21 until day 91, compared to all treatments that accessed P sources (**Figure 4**).

Low-Molecular-Weight Organic Acids in the FC

Acetic and gluconic acids dominated the group of monocarboxylic acids, while oxalic and citric acids dominated the group of dicarboxylic and tricarboxylic acids, respectively (**Figure 5**). Mesocosms containing free OP in the FC only showed significantly larger contents of gluconic acid before P was allocated to the plant tissues. The treatment containing PA showed a significantly larger content of acetic, butyric,

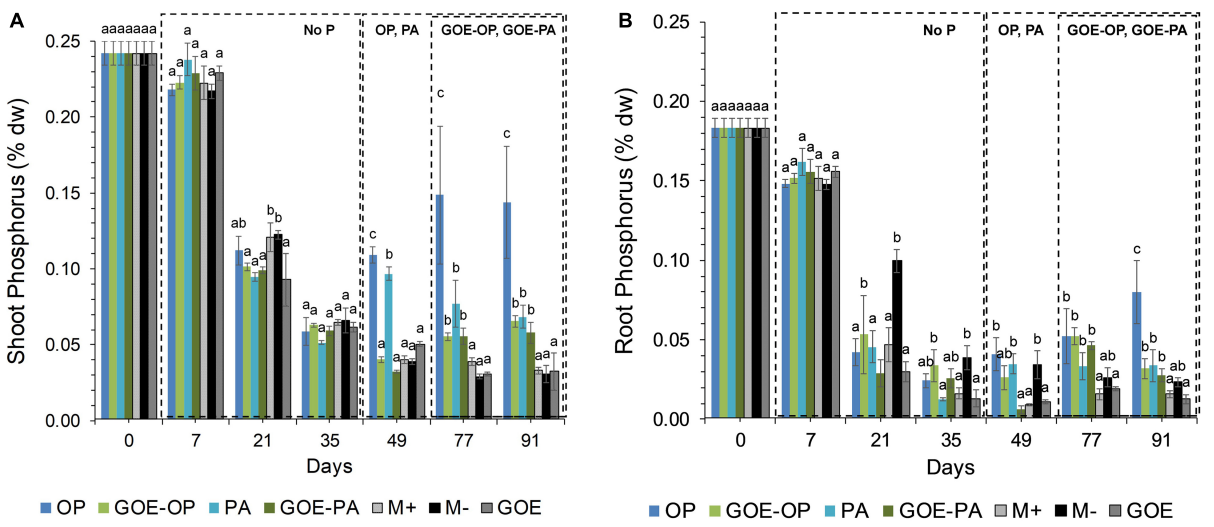

FIGURE 2 | Mean values and standard errors for shoot **(A)** and root **(B)** P content (% dry weight) of *Solanum lycopersicum* L. for the different available P sources and controls during the time course experiment. Three explicative boxes are included to differentiate the periods when we detected the AM plants acquired P from their respective sources. Within each P source and day, treatments with significant differences are labeled with different letters ($p < 0.05$) as result of a one-way ANOVA. OP, orthophosphate; PA, phytic acid; GOE-PA, phytic acid bound to goethite; GOE-OP, orthophosphate bound to goethite; GOE, mycorrhized plant, no P, and goethite; M+, mycorrhized plant, no P, quartz sand; M-, non-mycorrhized plant, no P, quartz sand.

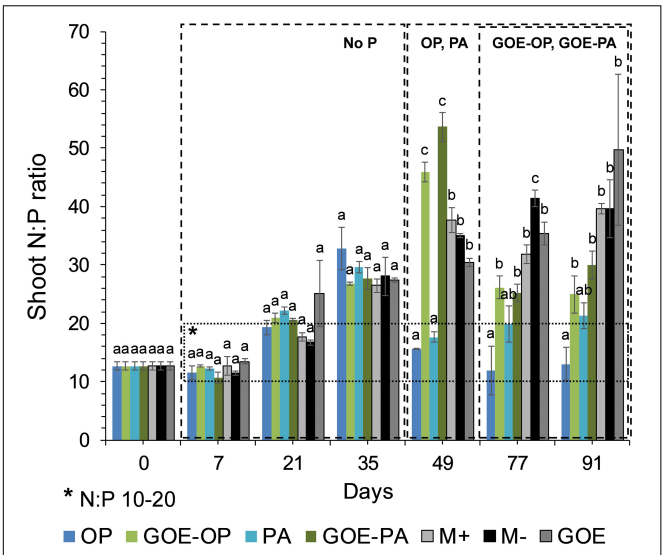

FIGURE 3 | Mean values and standard errors for shoot N:P ratio of *Solanum lycopersicum* L. for the different available P sources and controls during the time course experiment. A box has been drawn covering the values 10–20 of the N:P ratio, to represent the range of the ratio where no nitrogen (<10) or phosphorus (>20) deficiency would exist. Three explicative boxes are included to differentiate the periods when we detected the AM plants acquired P from their respective sources. Within each P source and day, treatments with significant differences are labeled with different letters ($p < 0.05$) as result of a one-way ANOVA. OP, orthophosphate; PA, phytic acid; GOE-PA, phytic acid bound to goethite; GOE-OP, orthophosphate bound to goethite; GOE, mycorrhized plant, no P, and goethite; M + , mycorrhized plant, no P, quartz sand; M-, non-mycorrhized plant, no P, quartz sand.

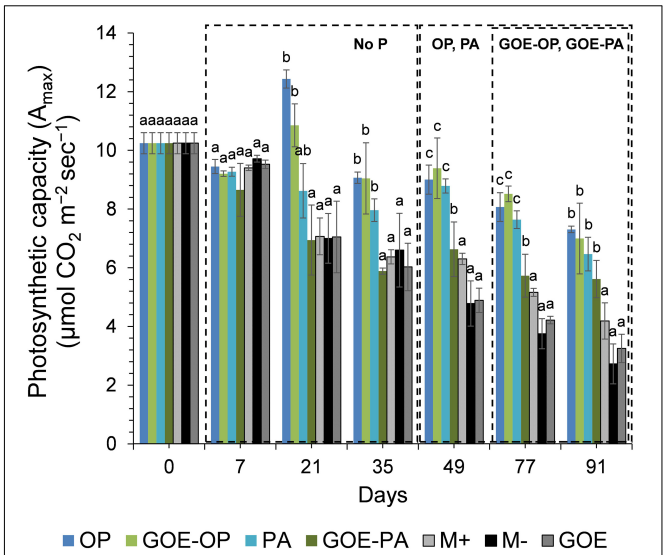

FIGURE 4 | Mean values and standard errors for photosynthetic capacity (A_{max}) of *Solanum lycopersicum* L. leaves for the different available P sources and controls during the time course experiment. Three explicative boxes are included to differentiate the periods when we detected the AM plants acquired P from their respective sources. Within each P source and day, treatments with significant differences are labeled with different letters ($p < 0.05$) as result of a one-way ANOVA. OP, orthophosphate; PA, phytic acid; GOE-PA, phytic acid bound to goethite; GOE-OP, orthophosphate bound to goethite; GOE, mycorrhized plant, no P, and goethite; M+, mycorrhized plant, no P, quartz sand; M-, non-mycorrhized plant, no P, quartz sand.

lactic, and citric acids before P uptake by the AM plant, while afterward only acetic and citric acids were present. The treatment containing GOE-OP exhibited a significantly larger content of all monocarboxylic acids, as well succinic, oxalic, and citric acids before P uptake by the AM plant. After the AM plants acquired P from GOE-OP, gluconic, lactic, malic, and oxalic acids were observed (**Figure 5**). Compared to the controls, the FC containing GOE-PA showed a significantly larger content of butyric, gluconic, and citric acids before the AM plant acquired P, while after P incorporation acetic, gluconic, lactic, malic, and oxalic acids were found in the FC.

Fungal Growth and Degree of Mycorrhization

Of the four AMF PLFA markers (16:1ω5c, 18:1ω7c, 18:2ω6,9, 18:1ω9c), only the three first ones exhibited a significant positive correlation ($p < 0.05$) with the P incorporation in the plant tissues (**Table 2**). The AMF PLFA biomarker 16:1ω5c always correlated positively with acquired P in the whole plant for the four P forms (**Table 2**). Additionally, treatments with access to GOE-OP showed a significant correlation between the AMF PLFA biomarker 18:1ω7c with P incorporation, while the GOE-PA treatment did it with the AMF PLFA biomarker 18:2ω6,9.

In all tomato roots mycorrhized with *R. irregularis*, we observed an arum-type AM association (Saito and Ezawa, 2016) (**Supplementary Table 1**). Correlation analysis of AMF-derived PLFA and NLFA biomarkers with the P acquired into the plant tissues, and mycorrhizal root traits revealed that there was a significant positive correlation between the abundance of arbuscules (%) and the acquired P over time for all offered P sources. A similar positive correlation was found for the 16:1ω5c PLFA and the abundance of arbuscules (%). All mycorrhizal root traits of AM plants accessing GOE-PA and GOE-OP correlated positively with the NLFA 16:1ω5c. Plants mobilizing P sources bound to GOE also showed a significant positive correlation between mycorrhizal root traits and the PLFA and NLFA 18:1ω7c, 18:2ω6,9, respectively (**Table 3**). The fatty acid 18:1ω9c did not show any significant correlation with the P incorporation from any of the P sources (**Table 3**); thus, it was not included in the correlation analysis between mycorrhizal root status parameters and fungal biomarkers within the FC (**Table 3**).

DISCUSSION

In the current study, we investigated the role of LMWOAs secreted by AMF and their accompanying microbiota in the mobilization of GOE-bound P sources. *R. irregularis* DAOM 197198 seemed not to be a specialist species in terms of mobilizing P bound to Fe oxides, since AM plants did not restore their initial P tissue contents during the time course experiment (**Figure 2**). It is not surprising, as it is a frequent dweller in agricultural contexts, thus not likely to be a functional specialist

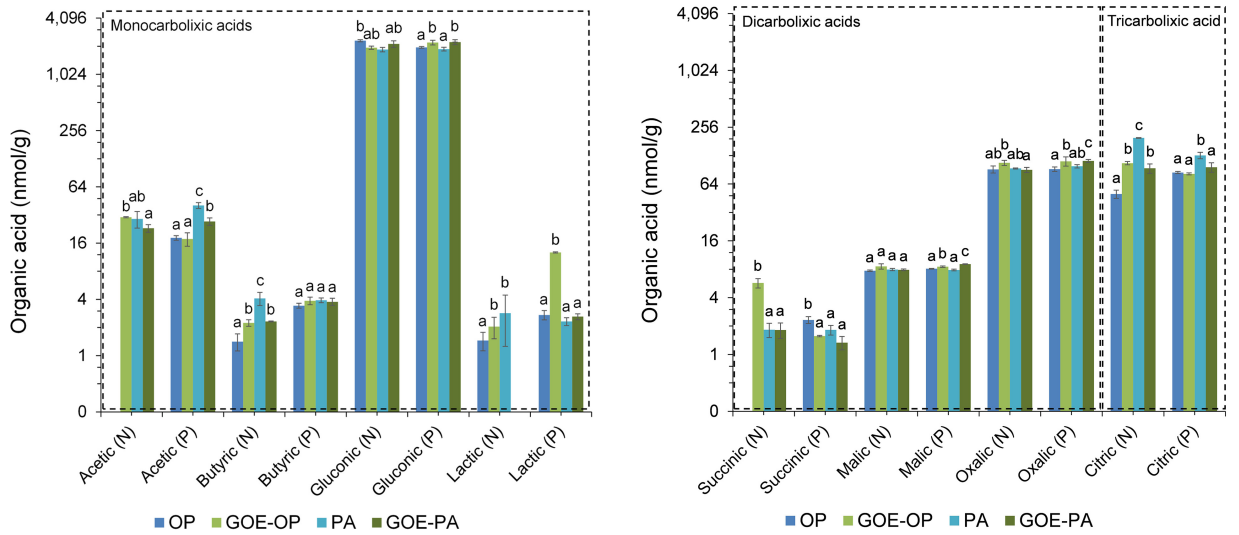

FIGURE 5 | Low-molecular-weight organic acids (LMWOAs) determined in the fungal compartment containing the different available P sources during the time course experiment. LMWOA are grouped depending on the number of carboxylic groups (–COOH) present in their molecular structure, differentiating between mono/di/tricarboxylic acids. Based on the results from **Figure 2**, each bar shows the mean value and standard errors for all those samples which belong either to a period of no P incorporation (N) or to a period when we detected P incorporation (P), for their respective P sources. Within each test, treatments with significant differences are labeled with different letters ($p < 0.05$) as result of a one-way ANOVA. OP, orthophosphate; PA, phytic acid; GOE-PA, phytic acid bound to goethite; GOE-OP, orthophosphate bound to goethite.

TABLE 2 | Result of the correlations between the fungal PLFA biomarkers and the acquired P (mg/plant).

Fungal biomarker	Acquired P (mg/plant)			
	OP	PA	GOE-OP	GOE-PA
16:1ω5c	**0.662****	**0.686****	**0.737****	**0.780****
18:2ω6,9	**−0.581***	−0.4002	−0.1088	**0.595***
18:1ω9c	0.103	−0.251	−0.482	−0.486
18:1ω7c	0.358	0.074	**0.798****	−0.232

*Correlation values in bold are significant (*p < 0.05, **p < 0.01). OP, orthophosphate; PA, phytic acid; GOE-PA, phytic acid bound to goethite; GOE-OP, orthophosphate bound to goethite.*

(Köhl et al., 2016). Our results show that before any P was acquired by the plant, there was a dilution in the P contents of shoots and roots for all treatments. Phosphorus was preferentially stored in the shoots, showing no P deficiency in case of AM plant mobilizing OP and PA, and only a slight P deficiency in case of those accessing GOE-OP and GOE-PA, as indicated by the N:P ratios (**Figure 3**). Furthermore, the AM plants with access to a P source exhibited significant higher photosynthetic activities until the end of the experiment, compared to the controls (**Figure 4**). Phosphorus is a key limiting nutrient and plays an important role in photosynthesis and the production of carbohydrates (Thuynsma et al., 2016). Plants may cycle P more efficiently at low soil P levels, by exhibiting a higher resorption efficiency (Dalling et al., 2016; Rychter et al., 2016). Hidaka and Kitayama (2013) proposed that in P-poor soils, plants tend to allocate P to the shoots, for keeping their productivity and growth and reducing the demand for P. Mycorrhizal benefit on the host plant is usually greater when plants are P limited (Hoeksema et al., 2010; Johnson et al., 2014) and this is particularly applicable to AM plants, which tend to store more P in the shoots, as compared to non-mycorrhizal plants (Yang et al., 2014; Holste et al., 2016). Furthermore, plants establishing AM symbiosis exhibit higher photosynthetic capacity, stomatal conductance, and transpiration rates, compared to non-mycorrhizal ones (Augé et al., 2016). The strength of the C sink in the mycorrhizal roots enhances plant photosynthetic capacity by wider opening of the stomata, allowing for more CO_2 to diffuse into the leaf, which in terms increase the level of sucrose and hexose in roots (Boldt et al., 2011). Furthermore, the C sink to the roots accelerates the utilization of triose phosphate for sucrose synthesis and the export toward the phloem. This increases plant P recycling rates by releasing P back to the chloroplast and activating the regeneration of ribulose 1,5-bisphosphate in the Calvin cycle. By this mechanism, more C is fixed per time and per unit of P, resulting in higher photosynthetic P-use efficiency (Tuomi et al., 2001; Valentine et al., 2001; Kaschuk et al., 2009). Our results on the reduction of the P dilution in the shoots, coupled with lower N:P ratios and higher photosynthetic capacities over time, in those treatments with access to the GOE-bound P sources, may be an indicator that AM symbiosis was conducive to more efficient use of P mobilized from less-accessible sources. A more efficient photosynthetic P use may also benefit the secretion of LMWOAs, as they entail a substantial C cost which is exclusively supported by the direct supply of photoassimilates (Plassard and Fransson, 2009).

Studies on the exudation of LMWOA by AMF are somewhat limited, and we found none where these mediated the mobilization of P bound to iron oxides. Conversely, D'Amico et al. (2020) recently demonstrated that in extremely

TABLE 3 | Result of the correlations between mycorrhization parameters and fungal biomarkers (%) at the fungal compartment.

		PLFA (%)			NLFA (%)			
		Inc. P (mg)	**16:1ω5c**	**18:2ω6,9**	**18:1ω7c**	**16:1ω5c**	**18:2ω6,9**	**18:1ω7c**
OP	A%	**0.711****	**0.565***	−0.326	**0.621***	0.342	**0.547***	0.469
	V%	0.444	0.116	−0.209	0.322	0.156	0.184	0.226
	M%	**0.664****	0.430	−0.316	**0.544***	0.279	0.424	0.412
PA	A%	**0.771****	**0.548***	−0.210	−0.072	0.414	0.121	0.380
	V%	0.429	0.396	0.052	−0.034	0.432	0.439	**0.530***
	M%	**0.533***	0.430	−0.016	0.027	0.441	0.357	**0.522***
GOE-OP	A%	**0.620****	**0.587***	0.133	**0.637***	**0.938*****	**0.767****	**0.932*****
	V%	0.008	0.267	0.258	0.206	**0.838*****	0.479	**0.779****
	M%	0.218	0.372	0.189	0.400	**0.910*****	0.599	**0.868*****
GOE-PA	A%	**0.560***	**0.774****	**0.597***	−0.005	**0.815****	0.483	**0.801****
	V%	**0.540***	**0.691****	**0.633***	−0.045	**0.809****	0.439	**0.820****
	M%	**0.654***	**0.743****	**0.628***	−0.031	**0.780****	0.478	**0.787****

*Correlation values in bold are significant (*p < 0.05, **p < 0.01, ***p < 0.001). PLFA, phospholipid fatty acid; NLFA, neutral lipid fatty acid; OP, orthophosphate; PA, phytic acid; GOE-PA, phytic acid bound to goethite; GOE-OP, orthophosphate bound to goethite; A, arbuscules; V, vesicles; M, mycorrhization; Inc. P, acquired P.*

P-poor environments, ectomycorrhizal fungi were able to release both Pi and Po from associations with goethite. We found two similar studies where AMF growth was isolated and organic acid production was measured: The one by Toljander et al. (2007) detected the presence of acetate and formiate, and other by Tawaraya et al. (2006) observed citrate and malate as part of the AMF hyphal exudates. We also detected three out of the four LMWOAs found in the two previous studies in similar concentration as for citric acid (100 nmol/g FC substrate). Furthermore, we found couple of studies investigating P desorption from goethite with non-mycorrhizal plants and incubation experiments. Parfitt (1979) concluded that one of the mechanisms by which GOE-OP could be solubilized and made available is through the action of the LMWOAs. He performed several extraction cycles on GOE-OP in combination with different LMWOAs, resulting in a higher OP desorption when it was incubated in the presence of citrate. In a more recent study, Martin et al. (2004) studied the effects of LMWOAs which may be released by non-mycorrhizal plant roots, such as citrate, on the desorption of PA and OP bound to GOE. They found a smaller amount of PA desorbed from GOE as compared to OP, which was attributed to the strength of chemical bonds and the high negative charge of the complexes. In our experimental setup, we detected the presence of significantly larger concentrations of LMWOAs in the FCs containing PA, GOE-OP, and GOE-PA before and after any P was allocated to the plant tissues, compared to those ones containing OP (**Figures 2**, **5**). The FCs where AM plants mobilized GOE-OP and GOE-PA contained the highest concentrations of malic and oxalic acid during the plant P incorporation phase. For citric acid, the trend was opposite to that of the dicarboxylic acids, having a significant larger concentration before any P was acquired in case of GOE-OP and GOE-PA treatments. The LMWOAs detected in the FCs of GOE-OP and GOE-PA treatments belong to the ones with higher chelation capacity, namely, malate, oxalate, and citrate, thus more effective mobilizing P from GOE or amorphous ferric hydroxides, as compared to the ones containing one carboxyl group (Muthukumar et al., 2014; Thorley et al., 2015). The release of mono/di/tri LMWOAs by the fungal partner of mycorrhizal plants refers to a possible mechanism involved in the acquisition of P from mineral-bound sources, where organic acids weaken and break the bonds between surface-coordinated P forms and structural metal ions before being mobilized by the AM plant. The presence of low contents of monocarboxylic acids (i.e., acetic, butyric, and lactic acids) before and after the AM plant acquired P from GOE-OP and GOE-PA, may be indicative of fermentation reactions occurring during transient periods of anaerobiosis, when reconstituting the water content to field capacity in the FCs. Although thermodynamically stable, goethite may undergo reductive dissolution in an anoxic environment when the redox potential drops (Torrent et al., 1987). This partial reductive dissolution may have taken place in FCs containing GOE-P compounds caused by the anaerobic respiration of microorganisms, transferring electrons from organic compounds to the Fe(III)-oxides (Peiffer and Wan, 2016). This reaction may have contributed, together with the action of di/tricarboxylic acids, in releasing adsorbed P from the goethite surfaces. However, the presence of the di/tri LMWOAs may suggest a mechanism used by AMF to desorb P from GOE surfaces. This desorption is done either by ligand exchange or by dissolution and subsequent desorption of P through the action of LMWOAs, such as oxalic, succinic, and citric acids. In the case of those plants accessing GOE-PA, the desorbed PA may be mineralized through the action of phosphatases secreted by the AMF (Tisserant et al., 2012).

We inoculated the tomato plants with *R. irregularis* DOAM 197198 grown under xenic conditions; thus, the inoculum carried the microorganisms naturally associated with its hyphae. In this sense, our results of LMWOAs production have to be examined under the possible joint influence of the AMF and its accompanying microbiota. In this regard, Battini et al. (2016) isolated microbiota from *Rhizophagus intraradices* and found plant growth-promoting activities such as phytate mineralization, siderophore production, Pi solubilization, and

LMWOA production in several representatives of Gram-positive (e.g., *Streptomyces* spp., *Arthrobacter* spp., *Nocardiodes* spp., and *Bacillus* spp.) and Gram-negative bacterial groups (e.g., *Sinorhizobium* spp.). Furthermore, Lecomte et al. (2011) and Selvakumar et al. (2016) reported bacteria closely associated with the mycelium of *R. irregularis* involved in the P mobilization from phytic acid. More recently, P transfer from phytate via AMF with the assistance of phytate-mineralizing bacteria was confirmed by Hara and Saito (2016). They isolated bacteria from the hyphosphere of the cosmopolitan AMF *R. irregularis* DAOM197198 and found that *Claroideoglomus etunicatum* can mineralize phytic acid. Taktek et al. (2015, 2017) and Wang et al. (2016) also isolated bacteria closely attached to the hyphosphere of *R. irregularis* DAOM197198. They showed that exudates from *R. irregularis* hyphae supported the growth and activity of bacteria with high potential for LMWOA production and Po mineralization. A possible mechanism used by the AMF and its accompanying microbiota to desorb P from the surface of GOE would involve the release of exudates containing LMWOAs. Following this, the desorbed OP could be taken up directly by the AM hyphae, while the desorbed PA still had to be hydrolyzed by phosphatases. As we observed in our results, it is likely that these previous steps delayed the incorporation of P mobilized from GOE-PA and GOE-OP into the plant tissues, compared to the other treatments (**Figure 2**). Additionally, several authors (Otani and Ae, 1999; Hayes et al., 2000; George et al., 2005) have pointed out that some LMWOAs (e.g., citric acid) have a synergistic effect on the secreted phosphatases (e.g., acid phosphatase), by changing the chemical structure or molecular size of the extracted Po and making it more accessible to enzymatic action. Summarizing, we found profiles of LMWOAs differing with the accessibilities of the offered P sources. The LMWOAs with two and three carboxylic groups were more abundant in case of P sources with lower P accessibility, before any P was acquired into the plant tissues. Hence, our results would point to a plant–fungus synchronous functioning that would adapt over time to respond to P accessibility in the soil. In this way, the mycorrhizal symbiosis would favor a more efficient P utilization, by maintaining an adequate photosynthetic capacity to ensure the soil volume exploration, together with the secretion of the LMWOAs.

Our results on the presence of AMF lipid biomarkers, together with the P acquisition from the different sources, highlight the central role played by *R. irregularis* in mobilizing P into the AM plant. This statement is founded on the fact that lipid biomarkers in the FC increased along with P in the plant tissues (**Table 2**); besides, both parameters positively correlated with the presence of arbuscules (**Table 3**). The arbuscules are short-lived structures with a turnover rate of 1–2 weeks (van Aarle and Olsson, 2003) and the interface between the plant and AMF (Wewer et al., 2014), where the P and photosynthates are exchanged in the periarbuscular space (Kobae et al., 2014; Saito and Ezawa, 2016). Thus, it would be consistent with the interpretation that P mobilization stimulated by the LMWOAs secretion was further supported by fungal growth and the exchange structures at the root level. The second conclusion stems from the correlation between the fungal PLFAs 18:1ω7c and 18:2ω6,9 with the acquired P and the arbuscules (%), for those AM plants mobilizing P from GOE-OP and GOE-PA, respectively. The PLFA are vital components of all biological membranes and play a key role in processes such a signal transduction, cytoskeletal rearrangement, membrane trafficking, etc., and remain at the place where they are synthesized (van Aarle and Olsson, 2003; Debiane et al., 2011; Dalpé et al., 2012). The AMF lack genes for a *de novo* biosynthesis of lipids and are enzymatically only able to elongate 16C lipid molecules; they mandatorily receive from their host plant (Bravo et al., 2017; Keymer et al., 2017). Since, AMF only elongates 16C lipid molecules, requiring the plant to produce them (Luginbuehl et al., 2017), the presence of the two unsaturated 18C PLFA fungal biomarkers in our experiment (18:1ω7c and 18:2ω6,9) might support the possibility of a modified composition in lipids constituting the hyphal membrane, which might be seen as an adaptation to the accessibility of the different P sources. Plasticity in fatty acid synthesis attributable to nutritional factors is common in filamentous fungi (Olsson et al., 2002). Based on the correlation data, our results point toward a change in the unsaturation level of AMF membrane lipids with the changing quality of the offered P sources. *R. irregularis*, therefore, might have modified its lipid composition in response to the different P sources. Consequently, the lipid membrane increased its fluidity to keep its integrity compatible with an optimal membrane functionality (Calonne et al., 2010). Membrane fluidity depends on its phospholipid composition of varying length and saturation with unsaturated lipid chains being more fluid than saturated ones. The unsaturated double bonds make it harder for the lipids to pack together by putting kinks into the otherwise straight hydrocarbon chain (Reichle, 1989). For successful adaptation to altered physicochemical environments, the active remodeling of membrane lipid composition is an essential feature and depends on both strain properties and cultivation conditions (Bentivenga and Morton, 1994; Čertík et al., 2005). Changes in membrane fluidity influence membrane processes such as transport, enzyme activities, and signal transduction (Benyagoub et al., 1996; Turk et al., 2007). In summary, it is plausible to consider that AMF modified its membrane lipid composition when mobilizing the GOE-bound P sources may have modulated the way in which the lipid membrane was organized for maintaining the growth state (Wang et al., 2017), by increasing unsaturated lipids in the case the AMF developed on a P source bound to GOE.

CONCLUSION

We found that free P sources were earlier acquired by the AM plant compared to their goethite-associated counterparts. Our results on the acquisition of P from GOE-bound sources suggest the AM symbiosis was conducive to greater P-use efficiency. Since we found evidence pointing to a synchronous response of the plant–fungus binomial, by mobilizing P desorbed from GOE to the photosynthetically active tissues and ensuring an adequate photosynthetic capacity for fueling the exploration of hyphae in the soil, as well as the costly production of LMWOAs. The LMWOAs with two and three carboxylic groups

(e.g., oxalic, succinic, and citric acids) were more abundant in those FCs where P was mobilized from sources with lower accessibility. This fact suggests that desorption of OP and PA from GOE was mediated either by ligand exchange or by ligand-controlled dissolution. Additionally, the presence of low contents of monocarboxylic acids characteristic of transient anaerobic conditions (i.e., acetic, butyric and lactic acids) before and after the AM plant acquired P from GOE-P associations may be indicative of reductive dissolution processes to release P from goethite surfaces. Finally, the fungal lipid analysis may indicate the AMF modified its membrane lipid composition by increasing the amount of unsaturated lipids when mobilizing the GOE-bound P sources, for maintaining the growth state and functionality. The AM symbiosis with *R. irregularis* and accompanying microbiota played a central role mobilizing P from GOE-bound sources to the host plant, highlighting the potentially pervasive influence of AMF on key ecosystem processes as the cycling of essential plant nutrients.

DATA AVAILABILITY STATEMENT

Raw and derived data supporting the findings of this study are available from the corresponding author AA on request.

AUTHOR CONTRIBUTIONS

AA, JB, GG, RM, and LS designed the experiment. AA prepared the plant and fungal material, conducted the experiment, analyzed the data, and wrote the manuscript with contributions from JB, GG, RM, LS, and SK. SK analyzed the LMWOAs on the FC samples and described the material and methods. JB, GG, LS, and RM supervised the research. All authors contributed to the article and approved the submitted version.

FUNDING

We want to thank the German Federal Ministry of Education and Research for the funding of this project in the framework of the DFG-RTG 1798 Signaling at the Plant-Soil Interface, as well as the DFG priority program SPP 1685 Forest Strategies for limited Phosphorus Resources. The publication of this article was funded by the Open Access fund of the Leibniz Universität Hannover.

ACKNOWLEDGMENTS

We are thankful for the great help and guidance received at the laboratory by Silke Bokeloh, Elke Eichmann-Prusch, Ulrike Pieper, Anne Katrin Herwig, Heike Steffen, Michael Klatt, and Martin Schrapel. We also want to thank the support received by Marcus Horn for the LMWOAs sample preparation and analysis.

SUPPLEMENTARY MATERIAL

The Supplementary Material for this article can be found online at: https://www.frontiersin.org/articles/10.3389/fpls.2021.661842/full#supplementary-material

REFERENCES

Adeleke, R., Nwangburuka, C., and Oboirien, B. (2017). Origins, roles and fate of organic acids in soils: a review. *S. Afric. J. Bot.* 108, 393–406. doi: 10.1016/j.sajb.2016.09.002

Andrino, A., Boy, J., Mikutta, R., Sauheitl, L., and Guggenberger, G. (2019). Carbon investment required for the mobilization of inorganic and organic phosphorus bound to goethite by an arbuscular mycorrhiza (*Solanum lycopersicum* x *Rhizophagus irregularis*). *Front. Environ. Sci.* 7:26. doi: 10.3389/fenvs.2019.00026

Andrino, A., Guggenberger, G., Sauheitl, L., Burkart, S., and Boy, J. (2020). Carbon investment into mobilization of mineral and organic phosphorus by arbuscular mycorrhiza. *Biol. Fertil. Soils* 57, 47–64. doi: 10.1007/s00374-020-01505-5

Augé, R. M., Toler, H. D., and Saxton, A. M. (2016). Mycorrhizal stimulation of leaf gas exchange in relation to root colonization, shoot size, leaf phosphorus and nitrogen: a quantitative analysis of the literature using meta-regression. *Front. Plant Sci.* 7:1084. doi: 10.3389/fpls.2016.01084

Bååth, E. (2003). The use of neutral lipid fatty acids to indicate the physiological conditions of soil fungi. *Microb. Ecol.* 45, 373–383. doi: 10.1007/s00248-003-2002-y

Battini, F., Cristani, C., Giovannetti, M., and Agnolucci, M. (2016). Multifunctionality and diversity of culturable bacterial communities strictly associated with spores of the plant beneficial symbiont *Rhizophagus intraradices*. *Microbiol. Res.* 183, 68–79. doi: 10.1016/j.micres.2015.11.012

Bentivenga, S. P., and Morton, J. B. (1994). Stability and heritability of fatty-acid methyl-ester profiles of Glomalean Endomycorrhizal fungi. *Mycol. Res.* 98, 1419–1426. doi: 10.1016/S0953-7562(09)81073-3

Benyagoub, M., Willemot, C., and Bélanger, R. R. (1996). Influence of a subinhibitory dose of antifungal atty acids from *Sporothrix flocculosa* on cellular lipid composition in fungi. *Lipids* 31, 1077–1082. doi: 10.1007/BF02522465

Bharadwaj, D. P., Alström, S., and Lundquist, P.-O. O. (2012). Interactions among *Glomus irregulare*, arbuscular mycorrhizal spore-associated bacteria, and plant pathogens under in vitro conditions. *Mycorrhiza* 22, 437–447. doi: 10.1007/s00572-011-0418-7

Bischoff, N., Mikutta, R., Shibistova, O., Puzanov, A., Reichert, E., Silanteva, M., et al. (2016). Land-use change under different climatic conditions: consequences for organic matter and microbial communities in Siberian steppe soils. *Agric. Ecosyst. Environ.* 235, 253–264. doi: 10.1016/j.agee.2016.10.022

Boldt, K., Pörs, Y., Haupt, B., Bitterlich, M., Kühn, C., Grimm, B., et al. (2011). Photochemical processes, carbon assimilation and RNA accumulation of sucrose transporter genes in tomato arbuscular mycorrhiza. *J. Plant Physiol.* 168, 1256–1263. doi: 10.1016/j.jplph.2011.01.026

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

Brundrett, M., Bougher, N., Dell, B., Grove, T., and Malajczuk, N. (1996). *Working with Mycorrhizas in Forestry and Agriculture*. Canberra: Australian Centre for International Agricultural Research.

Burghelea, C. I., Dontsova, K., Zaharescu, D. G., Maier, R. M., Huxman, T., Amistadi, M. K., et al. (2018). Trace element mobilization during incipient bioweathering of four rock types. *Geochim. Cosmochim. Acta* 234, 98–114. doi: 10.1016/j.gca.2018.05.011

Calonne, M., Fontaine, J. J., Debiane, D., Laruelle, F. F., Grandmougin-ferjani, A., Sahraoui, A. L., et al. (2010). "Propiconazole toxicity on the non-target organism, the arbuscular mycorrhizal fungus, glomus irregulare," in *Fungicides*, ed. O. Carisse (London: InTech Open), 325–346.

Čertík, M., Breierová, E., and Juršíková, P. (2005). Effect of cadmium on lipid composition of *Aureobasidium pullulans* grown with added extracellular

polysaccharides. *Int. Biodeterior. Biodegrad.* 55, 195–202. doi: 10.1016/j.ibiod.2004.11.005

Dalling, J., Heineman, K., Lopez, O., Wright, S., and Turner, B. (2016). "Nutrient availability in tropical rain forests: the paradigm of phosphorus limitation," in *Tropical Tree Physiology*, eds G. Goldstein and L. S. Santiago (Cham: Springer International Publishing), 261–273. doi: 10.1007/978-3-319-27422-5_12

Dalpé, Y., Trépanier, M., Sahraoui, A. L.-H., Fontaine, J., and Sancholle, M. (2012). Lipids of Mycorrhizas. *Fungal Assoc.* 9, 137–169. doi: 10.1007/978-3-642-30826-0_8

D'Amico, M., Almeida, J. P., Barbieri, S., Castelli, F., Sgura, E., Sineo, G., et al. (2020). Ectomycorrhizal utilization of different phosphorus sources in a glacier forefront in the Italian Alps. *Plant Soil* 446, 81–95. doi: 10.1007/s11104-019-04342-0

Debiane, D., Calonne, M., Fontaine, J., Laruelle, F., Grandmougin-Ferjani, A., and Lounes-Hadj Sahraoui, A. (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

Deubel, A., and Merbach, W. (2005). "Influence of microorganisms on phosphorus bioavailability in soils," in *Microorganisms in Soils: Roles in Genesis and Functions*, eds A. Varma and F. Buscot (Berlin: Springer-Verlag), 177–191. doi: 10.1007/3-540-26609-7_9

Drigo, B., and Donn, S. (2017). "Trading carbon between arbuscular mycorrhizal fungi and their hyphae-associated microbes," in *Mycorrhizal Mediation of Soil: Fertility, Structure, and Carbon Storage*, eds N. C. Johnson, C. A. Gehring, and J. Jansa (Amsterdam: Elsevier), 395–412. doi: 10.1016/B978-0-12-804312-7.00022-X

Fernández, I., Merlos, M., López-Ráez, J. A., Martínez-Medina, A., Ferrol, N., Azcón, C., et al. (2014). Defense related phytohormones regulation in arbuscular mycorrhizal symbioses depends on the partner genotypes. *J. Chem. Ecol.* 40, 791–803. doi: 10.1007/s10886-014-0473-6

Fitter, A. H., Graves, J. D., Watkins, N. K., Robinson, D., and Scrimgeour, C. (1998). Carbon transfer between plants and its control in networks of arbuscular mycorrhizas. *Funct. Ecol.* 12, 406–412. doi: 10.1046/j.1365-2435.1998.00206.x

Frostegård, Å, Tunlid, A., and Bååth, E. (1991). Microbial biomass measured as total lipid phosphate in soils of different organic content. *J. Microbiol. Methods* 14, 151–163. doi: 10.1016/0167-7012(91)90018-L

Geelhoed, J. S., Van Riemsdijk, W. H., and Findenegg, G. R. (1999). Simulation of the effect of citrate exudation from roots on the plant availability of phosphate adsorbed on goethite. *Eur. J. Soil Sci.* 50, 379–390. doi: 10.1046/j.1365-2389.1999.00251.x

George, T. S., Simpson, R. J., Hadobas, P. A., and Richardson, A. E. (2005). Expression of a fungal phytase gene in *Nicotiana tabacum* improves phosphorus nutrition of plants grown in amended soils. *Plant Biotechnol. J.* 3, 129–140. doi: 10.1111/j.1467-7652.2004.00116.x

Gogala, N., Virant-Klun, I., and Gogala, N. (1995). Impact of vam on phosphorus nutrition of maize with low soluble phosphate fertilization. *J. Plant Nutr.* 18, 1815–1823. doi: 10.1080/01904169509365025

Güsewell, S. (2004). N: P ratios in terrestrial plants: variation and functional significance. *New Phytol.* 164, 243–266.

Hara, S., and Saito, M. (2016). Isolation of inositol hexaphosphate (IHP)-degrading bacteria from arbuscular mycorrhizal fungal hyphal compartments using a modified baiting method involving alginate beads containing IHP. *Microbes Environ.* 31, 234–243. doi: 10.1264/jsme2.ME15206

Hayes, J. E. E., Richardson, A. E. E., and Simpson, R. J. J. (2000). Components of organic phosphorus in soil extracts that are hydrolysed by phytase and acid phosphatase. *Biol. Fertil. Soils* 32, 279–286. doi: 10.1007/s003740000249

Hayes, P., Turner, B. L., Lambers, H., and Laliberté, E. (2014). Foliar nutrient concentrations and resorption efficiency in plants of contrasting nutrient-acquisition strategies along a 2-million-year dune chronosequence. *J. Ecol.* 102, 396–410. doi: 10.1111/1365-2745.12196

He, Z., and Zhu, J. U. N. (1998). Microbial utilization and transformation of phosphate adsorbed by variable charge minerals. *Soil Biol. Biochem.* 30, 917–923. doi: 10.1016/S0038-0717(97)00188-0

Herrera-Medina, M. J., Tamayo, M. I., Vierheilig, H., Ocampo, J. A., and García-Garrido, J. M. (2008). The jasmonic acid signalling pathway restricts the development of the arbuscular mycorrhizal association in tomato. *J. Plant Growth Regul.* 27, 221–230. doi: 10.1007/s00344-008-9049-4

Hewitt, E. J. (1966). Sand and water culture methods used in the study of plant nutrition. *Soil Sci. Soc. Am.* 17, 301–301.

Hidaka, A., and Kitayama, K. (2013). Relationship between photosynthetic phosphorus-use efficiency and foliar phosphorus fractions in tropical tree species. *Ecol. Evol.* 3, 4872–4880. doi: 10.1002/ece3.861

Hoeksema, J. D., Chaudhary, V. B., Gehring, C. A., Johnson, N. C., Karst, J., Koide, R. T., et al. (2010). A meta-analysis of context-dependency in plant response to inoculation with mycorrhizal fungi. *Ecol. Lett.* 13, 394–407. doi: 10.1111/j.1461-0248.2009.01430.x

Holste, E. K., Kobe, R. K., and Gehring, C. A. (2016). Plant species differ in early seedling growth and tissue nutrient responses to arbuscular and ectomycorrhizal fungi. *Mycorrhiza* 27, 211–223. doi: 10.1007/s00572-016-0744-x

IBM Corporation (2016). *IBM SPSS Statistics for Windows, Version 24.0, 2016*. Armonk, NJ: IBM Corporation.

Johansen, A., Finlay, R. D., and Olsson, P. A. (1996). Nitrogen metabolism of external hyphae of the arbuscular mycorrhizal fungus *Glomus intraradices*. *New Phytol.* 133, 705–712. doi: 10.1111/j.1469-8137.1996.tb01939.x

Johnson, N. C., Wilson, G. W. T., Wilson, J. A., Miller, R. M., and Bowker, M. A. (2014). Mycorrhizal phenotypes and the law of the minimum. *New Phytol.* 205, 1473–1484. doi: 10.1111/nph.13172

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

Keymer, A., Pimprikar, P., Wewer, V., Huber, C., Brands, M., Bucerius, S. L., et al. (2017). Lipid transfer from plants to arbuscular mycorrhiza fungi. *eLife* 6:e29107. doi: 10.7554/eLife.29107

Klugh, K. R., and Cumming, J. R. (2007). Variations in organic acid exudation and aluminum resistance among arbuscular *Mycorrhizal* species colonizing *Liriodendron tulipifera*. *Tree Physiol.* 27, 1103–1112. doi: 10.1093/treephys/27.8.1103

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

Köhl, L., Lukasiewicz, C. E., and Van der Heijden, M. G. A. (2016). Establishment and effectiveness of inoculated arbuscular mycorrhizal fungi in agricultural soils. *Plant Cell Environ.* 39, 136–146. doi: 10.1111/pce.12600

Lecomte, J., St-Arnaud, M., and Hijri, M. (2011). Isolation and identification of soil bacteria growing at the expense of arbuscular mycorrhizal fungi. *FEMS Microbiol. Lett.* 317, 43–51. doi: 10.1111/j.1574-6968.2011.02209.x

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

Mäder, P., Vierheilig, H., Streitwolf-Engel, R., Boller, T., Frey, B., Christie, P., et al. (2000). Transport of 15N from a soil compartment separated by a polytetrafluoroethylene membrane to plant roots via the hyphae of arbuscular mycorrhizal fungi. *New Phytol.* 146, 155–161. doi: 10.1046/j.1469-8137.2000.00615.x

Manchanda, G., Singh, R. P., Li, Z. F., and Zhang, J. J. (2017). "Mycorrhiza: creating good spaces for interactions," in *Mycorrhiza - Function, Diversity, State of the Art*, eds A. Varma, R. Prasad, and N. Tuteja (Cham: Springer International Publishing), 39–60. doi: 10.1007/978-3-319-53064-2_4

Marschener, H. (1998). Role of root growth, arbuscular mycorrhiza, and root exudates for the efficiency in nutrient acquisition. *Field Crop. Res.* 56, 203–207. doi: 10.1016/S0378-4290(97)00131-7

Martin, M., Celi, L., and Barberis, E. (2004). Desorption and plant availability of myo-inositol hexaphosphate adsorbed on goethite. *Soil Sci.* 169, 115–124. doi: 10.1097/01.ss.0000117787.98510.9d

McCormack, M. L., and Iversen, C. M. (2019). Physical and functional constraints on viable belowground acquisition strategies. *Front. Plant Sci.* 10:1215. doi: 10.3389/fpls.2019.01215

McGonigle, T. P., Miller, M. H., Evans, D. G., Fairchild, G. L., and Swan, J. A. (1990). A new method which gives an objective measure of colonization of roots by vesicular—arbuscular mycorrhizal fungi. *New Phytol.* 115, 495–501. doi: 10.1111/j.1469-8137.1990.tb00476.x

Muthukumar, T., Priyadharsini, P., Uma, E., Jaison, S., and Pandey, R. R. (2014). "Role of arbuscular mycorrhizal fungi in alleviation of acidity stress on plant growth," in *Use of Microbes for the Alleviation of Soil Stresses*, Vol. 1, ed. M. Miransari (New York, NY: Springer), 43–71. doi: 10.1007/978-1-4614-9466-9_3

Oberson, A., Friesen, D. K., Rao, I. M. M., Bühler, S., Frossard, E., Buhler, S., et al. (2001). "Phosphorus transformations in an oxisol under contrasting land-use systems: the role of the soil microbial biomass," in *Plant and Soil*, (Amsterdam: Kluwer Academic Publishers), 197–210. doi: 10.1023/A:1013301716913

Oburger, E., Jones, D. L., and Wenzel, W. W. (2011). Phosphorus saturation and pH differentially regulate the efficiency of organic acid anion-mediated P solubilization mechanisms in soil. *Plant Soil* 341, 363–382. doi: 10.1007/s11104-010-0650-5

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

Olsson, P. A., van Aarle, I. M., Allaway, W. G., Ashford, A. E. A. E., and Rouhier, H. (2002). Phosphorus effects on metabolic processes in Monoxenic Arbuscular Mycorrhiza cultures. *Plant Physiol.* 130, 1162–1171. doi: 10.1104/pp.009639

Olsson, P. A., and Wilhelmsson, P. (2000). The growth of external AM fungal mycelium in sand dunes and in experimental systems. *Plant Soil* 226, 161–169. doi: 10.1023/A:1026565314345

Otani, T., and Ae, N. (1999). Extraction of organic phosphorus in Andosols by various methods. *Soil Sci. Plant Nutr.* 45, 151–161. doi: 10.1080/00380768.1999.10409331

Owen, D., Williams, A. P., Griffith, G. W., and Withers, P. J. A. (2015). Use of commercial bio-inoculants to increase agricultural production through improved phosphrous acquisition. *Appl. Soil Ecol.* 86, 41–54. doi: 10.1016/j.apsoil.2014.09.012

Parfitt, R. L. (1979). The availability of P from phosphate-goethite bridging complexes. Desorption and uptake by ryegrass. *Plant Soil* 53, 55–65. doi: 10.1007/BF02181879

Peiffer, S., and Wan, M. (2016). "Reductive dissolution and reactivity of Ferric (Hydr)Oxides: new insights and implications for environmental Redox processes," in *Iron Oxides: From Nature to Applications*, ed. D. Faivrein (Hoboken, NJ: Wiley), 31–52. doi: 10.1002/9783527691395.ch3

Plassard, C., and Fransson, P. (2009). Regulation of low-molecular weight organic acid production in fungi. *Fungal Biol. Rev.* 23, 30–39. doi: 10.1016/j.fbr.2009.08.002

Rakshit, A., and Bhadoria, P. S. (2010). Role of VAM on growth and phosphorus nutrition of maize with low soluble phosphate fertilization. *Acta Agronóm.* 59, 119–123.

Reichle, D. E. (1989). "Biomembranes: molecular structure and function," in *Springer Advanced Texts in Chemistry*, (Berkeley, CA: University of California Press), 533. doi: 10.1007/978-1-4757-2065-5

Richardson, A. E. (2001). Prospects for using soil microorganisms to improve the acquisition of phosphorus by plants. *Aust. J. Plant Physiol.* 28, 897–906. doi: 10.1071/PP01093

Ros, M. B. H., De Deyn, G. B., Koopmans, G. F., Oenema, O., and van Groenigen, J. W. (2018). What root traits determine grass resistance to phosphorus deficiency in production grassland? *J. Plant Nutr. Soil Sci.* 181, 323–335. doi: 10.1002/jpln.201700093

Rychter, A., Rao, I., and Cardoso, J. (2016). "Role of phosphorus in photosynthetic carbon assimilation and partitioning," in *Handbook of Photosynthesis*, ed. M. Pessarakli (Boca Raton, FL: CRC Press), 603–625. doi: 10.1201/b19498-45

Saito, K., and Ezawa, T. (2016). "Phosphorus metabolism and transport in arbuscular mycorrhizal symbiosis," in *Molecular Mycorrhizal Symbiosis*, (Hoboken, NJ: John Wiley & Sons, Inc.), 197–216. doi: 10.1002/9781118951446.ch12

Sato, T., Ezawa, T., Cheng, W., and Tawaraya, K. (2015). Release of acid phosphatase from extraradical hyphae of arbuscular mycorrhizal fungus *Rhizophagus clarus*. *Soil Sci. Plant Nutr.* 61, 269–274. doi: 10.1080/00380768.2014.993298

Schachtman, D. P., Reid, R. J., and Ayling, S. M. (1998). Update on phosphorus uptake phosphorus uptake by plants: from soil to cell. *Plant Physiology* 116, 447–453.

Seguel, A., Cumming, J. R., Klugh-Stewart, K., Cornejo, P., and Borie, F. (2013). The role of arbuscular mycorrhizas in decreasing aluminium phytotoxicity in acidic soils: a review. *Mycorrhiza* 23, 167–183. doi: 10.1007/s00572-013-0479-x

Selvakumar, G., Krishnamoorthy, R., Kim, K., and Sa, T. M. (2016). Genetic diversity and association characters of bacteria isolated from arbuscular mycorrhizal fungal spore walls. *PLoS One* 11:e0160356. doi: 10.1371/journal.pone.0160356

Smith, S., and Read, D. (2008). *Mycorrhizal Symbiosis*. New York, NY: Academic Press. doi: 10.1016/B978-0-12-370526-6.X5001-6

Taktek, S., St-Arnaud, M., Piché, Y., Fortin, J. A., and Antoun, H. (2017). Igneous phosphate rock solubilization by biofilm-forming mycorrhizobacteria and hyphobacteria associated with *Rhizoglomus irregulare* DAOM 197198. *Mycorrhiza* 27, 13–22. doi: 10.1007/s00572-016-0726-z

Taktek, S., Trépanier, M., Servin, P. M., St-Arnaud, M., Piché, Y., Fortin, J.-A. A., et al. (2015). Trapping of phosphate solubilizing bacteria on hyphae of the arbuscular mycorrhizal fungus *Rhizophagus irregularis* DAOM 197198. *Soil Biol. Biochem.* 90, 1–9. doi: 10.1016/j.soilbio.2015.07.016

Tani, M., and Higashi, T. (1999). Vertical distribution of low molecular weight aliphatic carboxylic acids in some forest soils of Japan. *Eur. J. Soil Sci.* 50, 217–226. doi: 10.1046/j.1365-2389.1999.00228.x

Tawaraya, K., Naito, M., and Wagatsuma, T. (2006). Solubilization of insoluble inorganic phosphate by hyphal exudates of arbuscular mycorrhizal fungi. *J. Plant Nutr.* 29, 657–665. doi: 10.1080/01904160600564428

Thorley, R. M. S., Taylor, L. L., Banwart, S. A., Leake, J. R., and Beerling, D. J. (2015). The role of forest trees and their mycorrhizal fungi in carbonate rock weathering and its significance for global carbon cycling. *Plant. Cell Environ.* 38, 1947–1961. doi: 10.1111/pce.12444

Thuynsma, R., Kleinert, A., Kossmann, J., Valentine, A. J., and Hills, P. N. (2016). The effects of limiting phosphate on photosynthesis and growth of *Lotus japonicus*. *S. Afric. J. Bot.* 104, 244–248. doi: 10.1016/j.sajb.2016.03.001

Tisserant, E., Kohler, A., Dozolme-Seddas, P., Balestrini, R., Benabdellah, K., Colard, A., et al. (2012). The transcriptome of the arbuscular mycorrhizal fungus *Glomus intraradices* (DAOM 197198) reveals functional tradeoffs in an obligate symbiont. *New Phytol.* 193, 755–769. doi: 10.1111/j.1469-8137.2011.03948.x

Toljander, J. F., Lindahl, B. D., Paul, L. R., Elfstrand, M., and Finlay, R. D. (2007). Influence of arbuscular mycorrhizal mycelial exudates on soil bacterial growth and community structure. *FEMS Microbiol. Ecol.* 61, 295–304. doi: 10.1111/j.1574-6941.2007.00337.x

Torrent, J., Schwertmann, U., and Barron, V. (1987). The reductive dissolution of synthetic goethite and hematite in dithionite. *Clay Miner.* 22, 329–337. doi: 10.1180/claymin.1987.022.3.07

Tuomi, J., Kytoviita, M.-M. M., and Hardling, R. (2001). Cost efficiency of nutrient acquisition and the advantage of mycorrhizal symbiosis for the host plant. *Oikos* 92, 62–70. doi: 10.1034/j.1600-0706.2001.920108.x

Turk, M., Abramović, Z., Plemenitaš, A., and Gunde-Cimerman, N. (2007). Salt stress and plasma-membrane fluidity in selected extremophilic yeasts and yeast-like fungi. *FEMS Yeast Res.* 7, 550–557. doi: 10.1111/j.1567-1364.2007.00209.x

Valentine, A. J., Osborne, B. A., and Mitchell, D. T. (2001). Interactions between phosphorus supply and total nutrient availability on mycorrhizal colonization, growth and photosynthesis of cucumber. *Sci. Hortic.* 88, 177–189. doi: 10.1016/S0304-4238(00)00205-3

van Aarle, I. M., and Olsson, P. A. (2003). Fungal lipid accumulation and development of mycelial structures by two arbuscular mycorrhizal. *Appl. Environ. Microbiol.* 69, 6762–6767. doi: 10.1128/AEM.69.11.6762

Vierheilig, H., Coughlan, A. P., Wyss, U., and Piché, Y. (1998). Ink and vinegar, a simple staining technique for arbuscular-mycorrhizal fungi. *Appl. Environ. Microbiol.* 64, 5004–5007.

Wang, F., Shi, N., Jiang, R., Zhang, F., and Feng, G. (2016). In situ stable isotope probing of phosphate-solubilizing bacteria in the hyphosphere. *J. Exp. Bot.* 67, 1689–1701. doi: 10.1093/jxb/erv561

Wang, J., Chen, W., Nian, H., Ji, X., Lin, L., Wei, Y., et al. (2017). Inhibition of polyunsaturated fatty acids synthesis decreases growth rate and membrane fluidity of *Rhodosporidium kratochvilovae* at low temperature. *Lipids* 52, 729–735. doi: 10.1007/s11745-017-4273-y

Wang, P., Wang, T. Y., Wu, S. H., Wen, M. X., Lu, L. M., Ke, F. Z., et al. (2019). Effect of arbuscular mycorrhizal fungi on rhizosphere organic acid content and microbial activity of trifoliate orange under different low P conditions. *Arch. Agron. Soil Sci.* 65, 2029–2042. doi: 10.1080/03650340.2019.1590555

Watkins, N. K., Fitter, A. H., Graves, J. D., and Robinson, D. (1996). Carbon transfer between C3 and C4 plants linked by a common mycorrhizal network, quantified using stable carbon isotopes. *Soil Biol. Biochem.* 28, 471–477. doi: 10.1016/0038-0717(95)00189-1

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/tpj.12566

Xu, G. H., Chague, V., Melamed-Bessudo, C., Kapulnik, Y., Jain, A., Raghothama, K. G., et al. (2007). Functional characterization of LePT4: a phosphate transporter in tomato with mycorrhiza-enhanced expression. *J. Exp. Bot.* 58, 2491–2501. doi: 10.1093/jxb/erm096

Yang, Y., Tang, M., Sulpice, R., Chen, H., Tian, S., and Ban, Y. (2014). Arbuscular Mycorrhizal fungi alter fractal dimension characteristics of *Robinia pseudoacacia* L. seedlings through regulating plant growth, leaf water status, photosynthesis, and nutrient concentration under drought stress. *J. Plant Growth Regul.* 33, 612–625. doi: 10.1007/s00344-013-9410-0

Zhang, L., Fan, J., Ding, X., He, X., Zhang, F., and Feng, G. (2014). Hyphosphere interactions between an arbuscular mycorrhizal fungus and a phosphate solubilizing bacterium promote phytate mineralization in soil. *Soil Biol. Biochem.* 74, 177–183. doi: 10.1016/j.soilbio.2014.03.004

Zhang, L., Xu, M., Liu, Y., Zhang, F., Hodge, A., and Feng, G. (2016). Carbon and phosphorus exchange may enable cooperation between an arbuscular mycorrhizal fungus and a phosphate-solubilizing bacterium. *New Phytol.* 210, 1022–1032. doi: 10.1111/nph.13838

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

frontiers in Plant Science

SYSTEMATIC REVIEW
published: 06 August 2021
doi: 10.3389/fpls.2021.711720

Nitrogen Fertilisation Increases Specific Root Respiration in Ectomycorrhizal but Not in Arbuscular Mycorrhizal Plants: A Meta-Analysis

Bahareh Bicharanloo[1]*, *Timothy R. Cavagnaro*[2], *Claudia Keitel*[1] and *Feike A. Dijkstra*[1]

[1] School of Life and Environmental Sciences, Sydney Institute of Agriculture, The University of Sydney, Camden, NSW, Australia, [2] School of Agriculture, Food and Wine, The University of Adelaide, Adelaide, SA, Australia

Plants spend a high proportion of their photosynthetically fixed carbon (C) belowground to support mycorrhizal associations in return for nutrients, but this C expenditure may decrease with increased soil nutrient availability. In this study, we assessed how the effects of nitrogen (N) fertiliser on specific root respiration (SRR) varied among mycorrhizal type (Myco type). We conducted a multi-level meta-analysis across 1,600 observations from 32 publications. SRR increased in ectomycorrhizal (ECM) plants with more than 100 kg N ha^{-1} applied, did not change in arbuscular mycorrhizal (AM) and non-mycorrhizal (NM) plants, but increased in plants with a dual mycorrhizal association in response to N fertilisation. Our results suggest that high N availability (>100 kg N ha^{-1}) could disadvantage the growth of ECM plants because of increased C costs associated with maintaining higher root N concentrations, while the insensitivity in SRR by AM plants to N fertilisation may be because AM fungi are more important for phosphorus (P) uptake.

Keywords: association, carbon cost, expenditure, meta-regression, multi-level, multi-model inference, symbiosis, uptake

OPEN ACCESS

Edited by:
Sergio Saia, University of Pisa, Italy

Reviewed by:
Néstor Fernández Del-Saz, University of Concepcion, Chile
Qiang-Sheng Wu, Yangtze University, China

***Correspondence:**
Bahareh Bicharanloo
bahareh.bicharanloo@sydney.edu.au

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

Received: *19 May 2021*
Accepted: *12 July 2021*
Published: *06 August 2021*

Citation:
Bicharanloo B, Cavagnaro TR, Keitel C and Dijkstra FA (2021) Nitrogen Fertilisation Increases Specific Root Respiration in Ectomycorrhizal but Not in Arbuscular Mycorrhizal Plants: A Meta-Analysis. Front. Plant Sci. 12:711720. doi: 10.3389/fpls.2021.711720

INTRODUCTION

Plants allocate up to 50% of photosynthetically fixed carbon (C) to root biomass and rhizodeposition (Pausch and Kuzyakov, 2018). In addition, plants spend a significant portion of fixed C to support mycorrhizal fungi in exchange for soil-derived nutrients (Smith et al., 2009); most of this C expenditure is associated with root and hyphal respiration (Hughes et al., 2008). Up to 86% of all flowering plants can form symbiotic associations with mycorrhizal fungi (Brundrett, 2009; van der Heijden et al., 2015), where associations with arbuscular mycorrhizal (AM) and ectomycorrhizal (ECM) fungi are the most widespread type of mycorrhiza (Brundrett, 2009). The C cost associated with forming and maintaining different types of mycorrhizas remains highly uncertain, with estimates as low as 4% and as high as 25% of the net primary production of a plant allocated to the fungi (Hobbie, 2006; Johnson and Gehring, 2007; Stuart and Plett, 2020). Plants associated with AM and ECM fungi differ in their nutrient economies and related biogeochemical transformations of C and nutrients (Phillips et al., 2013). Thus, it can, therefore, be expected that variation in nutrient availability will also affect root respiratory C costs of AM and ECM plants, with consequences for their productivity and abundance in terrestrial ecosystems.

In general, the mycorrhizal contribution to plant nitrogen (N) acquisition is higher for ECM than for AM fungi, while AM fungi tend to have a more important role in phosphorus (P) uptake than ECM fungi (van der Heijden et al., 2015). Whereas, ECM plants also tend to dominate in organic soils with relatively high C:N ratios (Averill et al., 2014; Jo et al., 2019; Soudzilovskaia et al., 2019), AM plants tend to dominate in mineral soils with lower C:N ratios and higher net N mineralisation rates (Johnson, 2010; Lin et al., 2017; Jo et al., 2019). ECM plants have an advantage in organic soils, because ECM fungi are able to decompose soil organic matter and acquire organic N *via* the production of specialised enzymes (Hobbie et al., 2013). However, because the production of these C enzymes is expensive (Soudzilovskaia et al., 2015), ECM symbiosis of C may also be costly to the plant under these conditions. Unlike ECM, AM fungi have no specialised enzymes for organic N acquisition (Terrer et al., 2016; Jansa et al., 2019) and are not able to decompose soil organic matter (SOM) directly, but they can accelerate the decomposition indirectly by translocating plant-derived C to soil microorganisms to stimulate their activity (Hodge and Fitter, 2010; Taylor et al., 2016).

Nitrogen availability can affect the plant-fungi relationship by changing the balance between costs and benefits of forming mycorrhizal associations (Phillips et al., 2013). Reliance of plants on mycorrhizal symbiosis reduces when N availability increases and is more easily accessible to plants; therefore, plants may reduce their C investment to mycorrhizas with increased N fertilisation (Högberg et al., 2010; Thirkell et al., 2019). N fertilisation further can induce P limitation, which could shift C expenditure from acquiring N to acquiring P (Johnson, 2010; Treseder et al., 2018). The effects of N availability on mycorrhizas have mostly been assessed through changes in colonisation percentage per length of root (for AM) or root tips (for ECM), showing an overall decrease of 15% with N fertilisation across different studies, in a meta-analysis (Treseder, 2004), but there is large variation among studies. Indeed, AM colonisation could increase with N fertilisation because AM fungi also have a substantial N demand, thereby competing with plants for N (Puschel et al., 2016; Wang et al., 2018), or because of a shift towards increased plant P demand (Corkidi et al., 2002).

Given that N fertilisation affects mycorrhizal colonisation, this should result in changes in C costs for plants to maintain mycorrhizal associations. Indeed, specific root respiration (SRR) rate, which is defined as the root respiration rate per unit of root biomass (Pregitzer et al., 2007), tends to be greater for roots in association with mycorrhizal fungi, because mycorrhizal respiration also contributes to SRR (Hughes et al., 2008). SRR can be directly measured on excised roots (Makita et al., 2012; Wang and Liu, 2014), which in most cases severs external hyphae, and, therefore, most likely only includes the contribution of intraradical hyphae (for AM) and other mycorrhizal structures in or on the surface of roots (for AM and ECM). When root respiration is indirectly measured as the difference in soil respiration rates between soils with and without live roots present (Ding et al., 2010; Tu et al., 2013), it not only includes respiration of external hyphae but also respiration from recent rhizodeposits.

In theory, increased N availability with N fertilisation reduces plant reliance on mycorrhizal symbiosis, which may result in a decline of mycorrhizal colonisation and, thence, a lower SRR rate. However, evidence for this is sparse; most N fertilisation studies have concentrated on examining changes in root respiration and have not accounted for concurrent changes in root biomass. For instance, root respiration in an ECM boreal forest increased at a low level of N fertilisation (20 kg N ha^{-1}) but decreased at a high level of N (100 kg N ha^{-1}) (Hasselquist et al., 2012), while chronic N fertilisation had no effect on root respiration in northern hardwood forests dominated by AM trees (Burton et al., 2004). It is difficult to pinpoint the cause of the variability in responses in root respiration, which could be due to the level of N fertiliser applied and due to the variable N fertilisation effects on root biomass (so that fertilisation effects on root respiration may not be the same as SRR), on mycorrhizal colonisation rates of ECM and AM, and/or on root N concentrations. Indeed, increased root N concentrations are linked to higher SRR rates (Reich et al., 2008), but increased N in roots can be a result of either direct root uptake of N or mycorrhizal support.

While mycorrhizas represent a significant sink for plant photoassimilates, and the amount of C invested is likely to differ with Myco type and N supply, the impacts of Myco type and N fertilisation on SRR are less clear. Therefore, our meta-analysis included an investigation of the effects of N fertilisation on SRR to assess N fertiliser effects on C loss per unit of root biomass for plants associated with different types of mycorrhizas. The effects of N fertilisation on soil C fluxes and their components (heterotrophic and autotrophic respiration) have been reviewed (Zhou et al., 2014), but to our knowledge, this is the first study synthesising N fertilisation effects on SRR by plants varying in mycorrhizal symbiosis type through a meta-analysis. We focused on SRR (i.e., respiration per unit of root biomass) rather than total root respiration as a measure of plant C costs because it accounts for potential confounding effects of N fertilisation on root biomass. Environmental conditions such as soil moisture (Soil moist) and temperature can also affect SRR mediated by N fertilisation (Zhang et al., 2014). We, therefore, used multi-model inference to identify the most important predictors on SRR rates in response to N fertilisation; predictors include Soil moist and soil temperature (Soil temp), mycorrhizal symbiosis types [AM, ECM, AM+ECM, and non-mycorrhizal (NM)], root respiration measurement method, and N fertiliser amount. To examine potential confounding effects of plant growth forms from that of mycorrhizal association, we compared N fertilisation effects on the variation of SRR between woody and herbaceous plants. We further assessed the effect of N fertilisation on root N concentration for the same studies that also had information on SRR. We hypothesised that N fertilisation would reduce SRR in ECM plants because of reduced reliance on the symbiotic relationship with ECM fungi but that N fertilisation would not affect SRR in plants associated with AM because these plants still rely on the symbiotic relationship with AM fungi for P uptake. Furthermore, given the lack of symbiosis in NM plants, we hypothesise that N fertilisation would have no effect on SRR in NM plants.

METHODS

Literature Survey and Inclusion Criteria

We collected data related to SRR from published literature studies by searching in the Scopus and ScienceDirect databases in June 2021. We used the advanced search engine and searched for research articles published using the following keywords: (1) "specific root respiration," (2) "root respiration" AND "root biomass," (3) "autotrophic respiration" AND "root biomass" OR "fertilisation," (4) "rhizosphere respiration" AND "root biomass" OR "fertilisation," and (5) "below-ground respiration" AND "root biomass" OR "fertilisation." After excluding conference study papers, we found a total of 1,051 study papers. We applied the following criteria to be included in our meta-analysis: studies should have an N fertilisation treatment (without or in combination with other treatments) and should either report SRR directly or report root biomass and root respiration (or autotrophic respiration) allowing us to calculate SRR. We considered three different methods for calculating SRR: (1) based on the measurements of root respiration on excised roots ("root excavation"), (2) based on the difference in respiration between trenched and non-trenched plots ("trenching"), and (3) based on the difference in respiration between planted and unplanted or root-free/bare soil treatments ("unplanted"). For the trenching and unplanted methods, root biomass measurements in non-trenched and planted treatments also needed to be reported to calculate SRR. We excluded publications that were conducted in (1) hydroponic systems, (2) pots filled with sand only, (3) experiments that used oxygen (O_2) consumption to estimate respiration, and (4) C isotope pulse labelling experiments that were unable to quantify root respiration. Multiple respiration measurements with time were included as separate observations. We found a total of 32 study papers that fit these criteria (**Supplementary Table 1**) and retrieved a total of 1,600 observations from these study papers (1,559 observations from field experiments and 41 observations from pot experiments, **Supplementary Information**).

Data Collection

We used Plot Digitizer software Ver. 2.6.8 to extract the data from figures. We also extracted Soil moist, temperature, and N concentrations in roots when reported. Soil moist units were reported differently in different publications including volumetric, gravimetric, and water-filled pore space (WFPS). We converted all Soil moist data to gravimetric moisture using the bulk density when reported (including five study papers), or otherwise, we used a bulk density of 1.2 g cm^{-3} (another five study papers) (Bache et al., 2008). For each plant species, we collected information on mycorrhizal association type, including AM association, ECM association, AM+ECM (dual association), and non-symbiotic (NM) association based on plant species known to form an association with different types of mycorrhizas (Brundrett, 2009; Cosme et al., 2018; Teste et al., 2020). We considered AM+ECM association for those observations from mixed forests containing species associated with both AM and ECM and where respiration measurements were obtained by the trenching method. We further recorded whether plants were herbaceous or woody. Fertiliser application amount, experimental type (field vs. pot experiment), and method used for respiration measurement were also recorded. The fertiliser application rate was grouped into low ($\leq$ 100 kg N ha^{-1}) and high fertilisation rates ($>$ 100 kg N ha^{-1}).

Calculation and Statistical Analysis

Data were back-transformed when they were reported in a natural log. For those study papers that reported SE, we calculated the SD (σ) by multiplying the SE with the root square of the sample size (N). In cases where root respiration was calculated based on the difference between the mean total (non-trenched, planted) and heterotrophic (trenched, unplanted) respiration, the SD for root respiration was calculated using the pooled Equation (1) (Kaltenbach, 2012):

$$\sigma_{tr-hr} = \sqrt{\frac{\sigma_{tr}^2}{n} + \frac{\sigma_{hr}^2}{m}} \quad (1)$$

where σ_{tr} and n are the SD and sample size for total respiration, and σ_{hr} and m are the SD and sample size for heterotrophic respiration, respectively.

The Taylor expansion Equation (2) was used to calculate the SD for SRR when it was derived from the mean of root respiration divided by the mean of root biomass (Kendall, 1987):

$$\sigma_{rr/rb} = \sqrt{\frac{\mu_{rr}^2}{\mu_{rb}^2} \times \left[\frac{\sigma_{rr}^2}{\mu_{rr}^2} - 2\frac{Cov\left(rr,\ rb\right)}{\mu_{rr}\mu_{rb}} + \frac{\sigma_{rb}^2}{\mu_{rb}^2}\right]} \quad (2)$$

where $\sigma_{rr/rb}$ is the SD of SRR, μ_{rr} and σ_{rr} are the mean and SD for root respiration, and μ_{rb} and σ_{rb} are the mean and SD for root biomass, respectively.

Finally, for those means with no SD reported, the SD based on the coefficient of variance (CV) averaged across all other observations where SDs were reported was calculated.

The effect size of N fertilisation (LnRR) on SRR and root N concentration and their variance of the effect size Var(LnRR) (Borenstein et al., 2009; Lajeunesse, 2011) were calculated using Equations (3) and (4), respectively:

$$LnRR = \ln\left(\frac{\mu_t}{\mu_c}\right) \quad (3)$$

$$Var\left(LnRR\right) = \frac{(\sigma_c)^2}{N_c\mu_c^2} + \frac{(\sigma_t)^2}{N_t\mu_t^2} \quad (4)$$

where μ_t and μ_c are the mean of SRR or root N concentration under fertilisation and control treatments, and σ and N are the SDs and sample size of μ_t and μ_c, respectively. An inverse variance method including two variance components (see below) was used to calculate the weight of individual studies (Veroniki et al., 2016). The restricted maximum likelihood (REML) method was used to estimate the between-study variance τ^2 (residual heterogeneity), and a Q-profile method was applied to calculate the 95% CIs (Veroniki et al., 2016).

Mixed effect models were used for meta-estimates and meta-regressions to examine the association of categorical and continuous moderators (or predictor as explanatory variables) with the magnitude of the effect size (Terrer et al., 2019; Rubio-Aparicio et al., 2020). We examined how effect sizes differed among categorical factors of Myco type (AM, ECM, AM+ECM, and NM, both at fertilisation levels $\leq$ 100 and $>$ 100 kg N ha^{-1}), experimental type (field and pot experiments), method (root excavation, trenching, and unplanted), and plant growth form (herbaceous and woody), and continuous factors of Soil moist, Soil temp, and N fertilisation level. Effect sizes and their corresponding variances were calculated in R using the function "*escalc*" from the package *metafor* (Viechtbauer, 2010). Because the corresponding effect size estimates for studies with multiple observations (multiple measurements over time) are likely to be correlated (Gleser and Olkin, 2009; Lajeunesse, 2011; Nakagawa and Santos, 2012), we generated a variance-covariance (VCV) matrix and conducted a multi-level mixed effect model that accounts for the non-independency among effect sizes (Noble et al., 2017; Midolo et al., 2019). We used the function "*rma.mv*" from the package *metafor* where observation ID was nested within the study as a random effect (second variance σ^2 component), and a VCV matrix was used for weighing the effect sizes (Noble et al., 2017; Midolo et al., 2019; Terrer et al., 2021).

A multi-model inference was further conducted by modelling all possible predictor (moderator) combinations to examine which predictor provided the best fit with N fertilisation effect sizes on SRR, based on the sum of Akaike weights (AICc) and $p > 0.05$ (Burnham, 2002; Gurka, 2006). We further conducted a meta-regression relating LnRR of SRR to N fertilisation with root N concentration in fertilised treatments.

All analyses were done in R, version 4.0.0 (R Development Core Team, 2020). We used the package *metafor* to conduct the multi-level meta-analysis and meta-regressions (Viechtbauer, 2010) and *glmulti* for model selection and multi-model inference (Calcagno and de Mazancourt, 2010).

RESULTS

Across 1,600 observations from 32 publications, SRR was not affected by N fertilisation in field or pot experiments (**Table 1**). Because the majority of observations came from field experiments (1,559 observations), the remainder of our analysis is focused on these studies. Given the large variation in effect sizes among observations, we conducted a multi-model inference to find out the most important moderators on effect sizes of SRR. We included Myco type, Soil moist, Soil temp, N fertiliser level ($\leq$ 100 kg N ha^{-1} and $>$ 100 kg N ha^{-1}), and measurement method. Results of our model selection showed that Myco type and N fertiliser level were the two most important moderators explaining variation in N fertilisation effect sizes, with both moderators showing a sum of AICc larger than the cut-off value 0.8 (**Figure 1**).

We found no significant variation for SRR to N fertilisation among different respiration measurement methods (**Table 2**) and not between woody and herbaceous plants (**Table 3**).

TABLE 1 | Effect sizes (LnRR) of specific root respiration (SRR) and root N concentration to N fertilisation for all observations and separated by field and pot experiments.

	n	Estimate	Lower CI	Upper CI	*p*	Model p
Specific root respiration	1,600	0.059	−0.049	0.181	0.3	
Field experiments	1,559	0.041	−0.079	0.162	0.5	0.3
Pot experiments	41	0.240	−0.087	0.567	0.2	
Root N concentration	258	0.200	−0.006	0.407	0.06	
Field experiments	234	0.247	0.012	0.482	0.03	0.1
Pot experiments	24	−0.012	−0.549	0.512	0.9	

Estimates for LnRR are associated with lower and upper 95% CIs. Significant p-values ($p < 0.05$) are shown in bold (n is the number of observations).

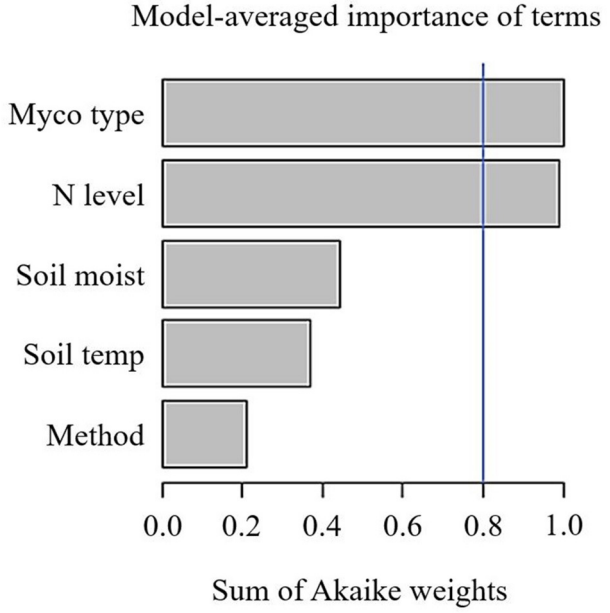

FIGURE 1 | Sum of Akaike weights (AICc) from the multi-model inference for predictors including mycorrhizal type (Myco type), N fertilisation levels (N level), soil temperature (Soil temp), gravimetric soil moisture (Soil moist), Specific root respiration measurement method on N fertilisation effect sizes of specific root respiration. Cut-off value (blue line) is set at 0.8 differentiating between important and less important predictors. N.B. only observations from field experiments are included.

Among the Myco types, SRR increased in plants with dual association (AM+ECM) but was not affected in plants associated with AM only, ECM only, and NM plants only (NM), although NM plants showed a higher variation in the effect size compared to the other Myco types (**Figure 2**).

We then tested whether the effect size of SRR in plants associated with different types of mycorrhizas varied between low ($\leq$ 100 kg N ha^{-1}) and high N ($>$ 100 kg N ha^{-1}) fertilisation levels (**Figure 3**). SRR significantly increased in

TABLE 2 | Effect sizes (LnRR) of SRR to N fertilisation for different respiration measurement methods.

Measurement method	*n*	Estimate	Lower CI	Upper CI	*p*-value	Model p
Root excavation	26	0.073	−0.387	0.533	0.7	0.5
Trenching	1,268	0.026	−0.119	0.171	0.7	
Unplanted	306	0.173	−0.065	0.412	0.1	

Estimates for LnRR are associated with lower and upper 95% CIs. Significant p-values ($p < 0.05$) are shown in bold (n is the number of observations).

TABLE 3 | Effect sizes (LnRR) of SRR to N fertilisation for different plant growth forms.

Plant growth form	*n*	Estimate	Lower CI	Upper CI	*p*-value	Model p
Herbaceous plants	874	0.052	−0.150	0.254	0.6	0.8
Woody plants	685	0.035	−0.136	0.205	0.7	

Estimates for LnRR are associated with lower and upper 95% CIs. Significant p-values ($p < 0.05$) are shown in bold (n is the number of observations, and only observations from field experiments are included).

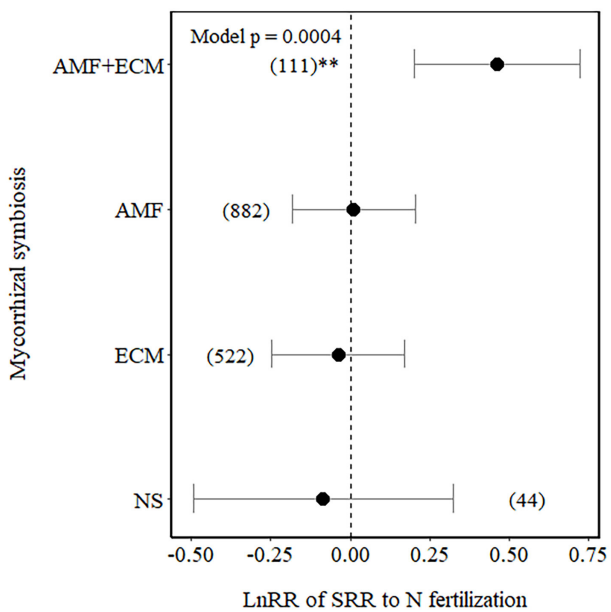

FIGURE 2 | Effect sizes (LnRR) of specific root respiration (SRR) to N fertilisation in plants with no mycorrhizal associations (NM), with ectomycorrhizal associations (ECM) only, arbuscular mycorrhizal associations (AM) only, or AM+ECM associations. Numbers in brackets represent the number of observations, and error bars represent the 95% CIs. **indicates significance at $p < 0.01$. Only observations from field experiments are included.

plants with AM+ECM under low N fertilisation ($p = 0.01$, **Figure 3A**). Under high N fertilisation, SRR increased in ECM plants (**Figure 3B**), while it showed a non-significant

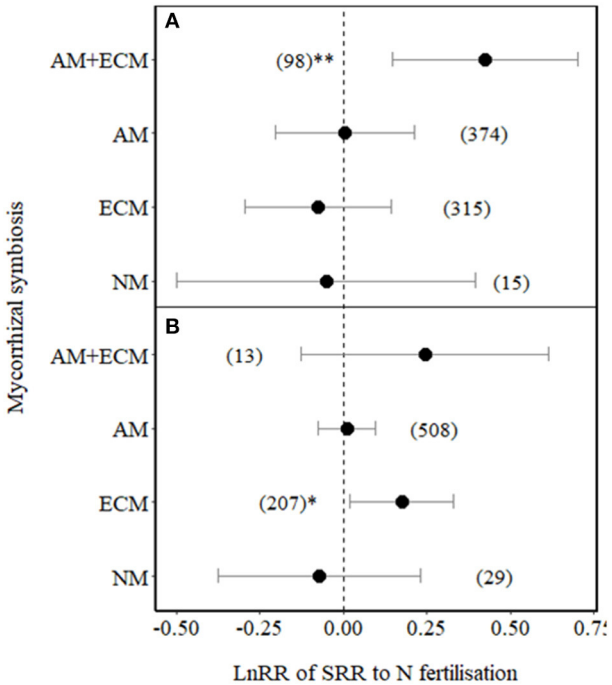

FIGURE 3 | Effect sizes (LnRR) of SRR in plants with no mycorrhizal associations (NM), with ECM associations only, AM associations only, or AM+ECM associations, for **(A)** low N fertilisation levels ($N \leq 100$ kg ha^{-1}) and **(B)** high N fertilisation levels ($N > 100$ kg ha^{-1}). Numbers in brackets represent the number of observations and error bars represent the 95% CIs. ** and * indicate significance at $p < 0.01$ and $p < 0.05$, respectively. Only observations from field experiments are included.

increase in plants associated with AM+ECM (but note the low number of observations for AM+ECM association with high variation in effect sizes in response to high N fertilisation). SRRs in AM and NM plants were not affected by either low or high N fertilisation (also note the relative low number of observations and high variation in effect sizes in response to both low and high N fertilisation for NM plants, **Figure 3**).

We examined whether differences among Myco type in SRR effect sizes to N fertilisation could be explained by their differences in root N concentration. As expected, root N concentration increased with N fertilisation across all Myco types in field experiments ($p = 0.03$) but not in pot experiments (**Table 1**). Plants associated with ECM showed the largest increase in root N concentration with N fertilisation and with smaller increases in root N concentration of AM and NM plants (**Figure 4A**), while there were no observations for AM+ECM plants. Due to the low number of observations, we were unable to test whether there were differences in the effect sizes of root N concentration between low and high N fertilisation levels. A meta-regression relating SRR affected by N fertilisation to root N concentration in fertilised treatments revealed a positive relationship explaining 44% of the variation ($p < 0.001$, $r^2 = 0.44$, **Figure 4B**).

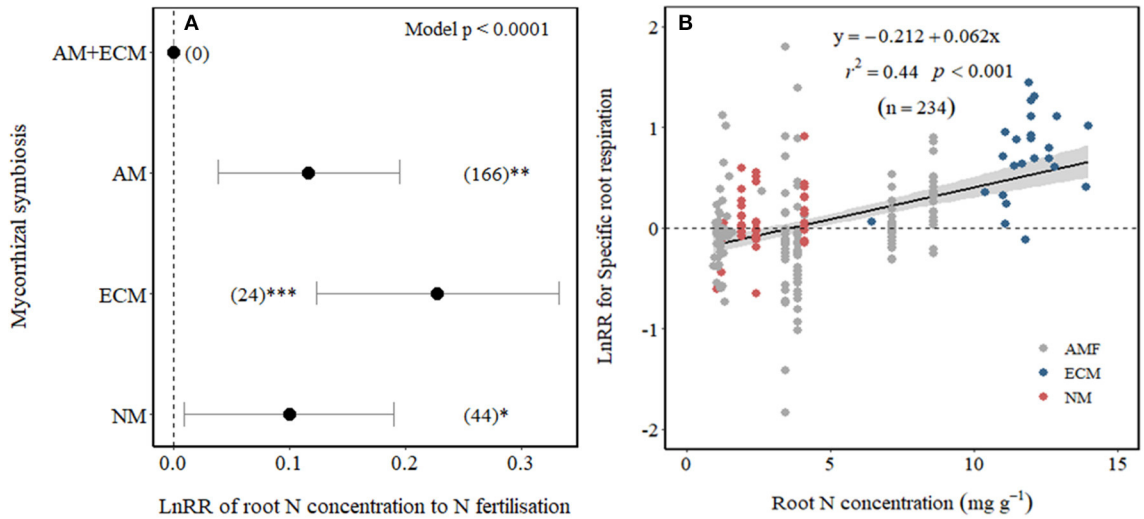

FIGURE 4 | Effect sizes (LnRR) of root N concentration to N fertilisation **(A)** in association with different mycorrhizal symbiosis and **(B)** meta-regression relating LnRR of SRR to N fertilisation with root N concentration in fertilised treatments (NM, non-mycorrhizal—red symbols; ECM, ectomycorrhizal—blue symbols; and AM, arbuscular mycorrhizal—grey symbols). ***, **, and *indicate significance at $p < 0.0001$, $p < 0.01$, and $p < 0.05$, respectively. Shade band around the regression fit represents 95% CIs. Only observations from field experiments are included.

DISCUSSION

The mycorrhizal type was the most important predictor of N fertilisation effect sizes on SRR, with no changes for AM, ECM, and NM plants and no increases for AM+ECM plants. N fertilisation level was the second most important moderator of SRR effect sizes. In particular, when both factors were examined together, SRR increased in ECM plants at high N fertilisation rates (>100 kg N ha^{-1}) and for AM+ECM plants at low N fertilisation rates (≤100 kg N ha^{-1}). While N fertilisation effects on SRR can be due to several factors, such as fertiliser-induced changes in root architecture and morphology (López-Bucio et al., 2003), root N concentrations (Reich et al., 2008), and mycorrhizal colonisation (Nilsson and Wallander, 2003; Treseder, 2004; Emmanuel et al., 2012), in this study, we focus on explaining these effects from a C cost and N benefit perspective of mycorrhizal symbiosis (Phillips et al., 2013).

Measurement method did not play an important role on SRR (**Table 2**), despite the fact that respiration measured by "trenching" and "unplanted" methods would also include external mycelial respiration (and possibly decomposition of rhizodeposition), while the "root excavation" method would only include internal mycelial respiration in SRR. This suggests that external mycelial respiration and decomposition of rhizodeposition were little affected by N fertilisation (Nilsson and Wallander, 2003; Hobbie, 2008; Wang et al., 2021). Further, we found no variation in SRR to N fertilisation between woody and herbaceous plants (**Table 3**), indicating that our results were not confounded by plant growth form. Interestingly, Soil temp and moist did not contribute significantly to explaining the variance in SRR effect sizes. Although Soil temp and moisture have been shown as effective factors controlling SRR (Atkin et al., 2000; Zhou et al., 2016), our results highlight that N fertilisation plays a more important role than Soil temp and moist on root respiration (Chen et al., 2019).

From the C cost and N benefit perspective, increased N availability (i.e., with increased N fertilisation) can decrease plant C cost as a result of direct N uptake so that mycorrhizal symbiosis may become too C expensive. A decrease in C cost may particularly occur in ECM plants. For instance, the excretion of extracellular enzymes by ECM fungi to decompose organic matter and release N for plant use (Pritsch and Garbaye, 2011; Nicolás et al., 2019) is C expensive, and it may be more C cost-effective for those plants to take up N directly from the soil when the availability of N increases. Furthermore, the extraradical mycelial networks that ECM fungi build to forage for nutrients in nutrient-poor soils (Chen et al., 2019) require a substantial amount of plant C (Soudzilovskaia et al., 2015) and may not be a C-efficient way for plants to acquire nutrients. Consequently, a reduction in SRR, due to reduced formation of mycorrhizal associations with increased N availability (Nilsson and Wallander, 2003; Treseder, 2004; Emmanuel et al., 2012), is expected as a plant C cost management strategy for ECM plants. To our surprise, we did not observe this but, instead, observed an increase in SRR in ECM plants at high levels of N fertilisation.

There are several possibilities that could explain our results. For instance, high rates of N fertilisation may not have decreased mycorrhizal colonisation and hyphal growth of ECM fungi and associated C cost. Although this is not supported by some studies in ECM trees where N fertilisation caused a decrease in mycelial production (Nilsson and Wallander, 2003; Ekblad et al., 2016), in a meta-analysis Treseder (2004) observed no significant effect of N fertilisation on ECM colonisation. It is also possible that N fertilisation may have changed the mycorrhizal communities (Avis et al., 2003; Parrent et al., 2006) towards species that require more C from their plant host, thereby increasing SRR. It is

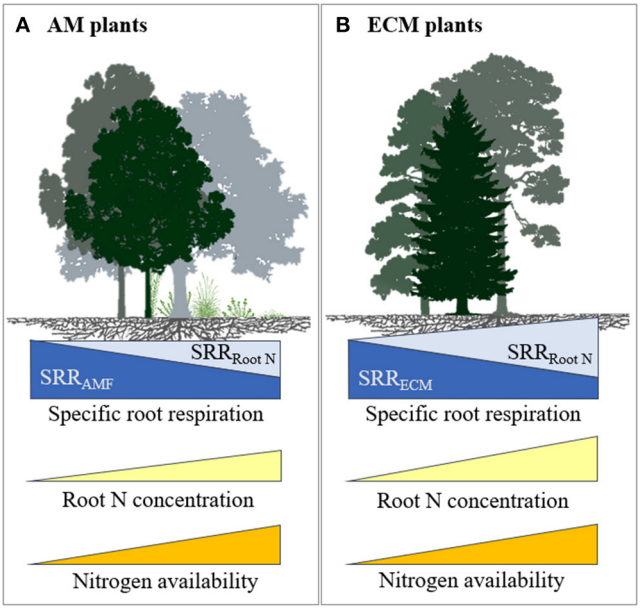

FIGURE 5 | Conceptual model illustrating the influence of soil N availability on SRR and root N concentration for plants in association with **(A)** AM and **(B)** ECM fungi. With increased soil N availability, both ECM and AM plants spend less C to support mycorrhiza resulting in reduced SRR associated with mycorrhizal symbiosis (a dark blue component of SRR) but respire more C due to an increase in root N concentration (a light blue component of SRR). However, the increase in plants C costs associated with higher root N concentration outweighs the reduction in C to support ECM fungi, while it counterbalances the reduction in C to support AM fungi. Furthermore, in AM plants, C costs for mycorrhizal symbiosis may remain relatively high at high soil N availability, because AM symbiosis is more important for P uptake. We refrained from drawing models for NM and AM+ECM plants due to limited data.

further possible that N fertilisation increased SRR in ECM plants because of an associated increase in root N concentration. Our results showed that when plants respired more C per unit of root biomass, they also took up more N with increased N fertilisation. The positive relationship between root N concentration and SRR suggests that higher N uptake results in greater C cost, possibly as a result of increased metabolic activities related to nutrient uptake, assimilation, and transportation (Reich et al., 2008; Burton et al., 2012). Therefore, any reductions in C costs due to lower mycorrhizal colonisation may have been outweighed by the increased C costs associated with the higher root N concentrations through increased direct uptake, which resulted in increased SRR at high N fertilisation rates (>100 kg N ha^{-1}) (see conceptual diagram in **Figure 5**).

Unlike ECM, our results showed that N fertilisation did not affect SRR in AM plants, under low or high N (**Figure 3**). One possible explanation is that AM colonisation is less affected by N availability and is more important for P supply so that the fungal contribution to SRR may not be affected by N availability (Hughes et al., 2008). However, AM colonisation on average decreased with N fertilisation by 15% in a meta-analysis (Treseder, 2004), although there was large variation among studies, possibly due to large variation in available N in control treatments. An alternative explanation may be that a reduction in mycorrhizal colonisation with N fertilisation may have been counterbalanced by an increase in root N concentration on SRR (**Figure 5**). Therefore, in the case of AM plants, reduced C costs *via* reduced AM colonisation may have been offset by the increased C costs associated with greater plant N uptake (Reich et al., 2008), resulting in no net change in SRR in response to N fertilisation.

It might be expected that SRR declines with N fertilisation in plants with the dual mycorrhizal association (AM+ECM) because supporting both types of mycorrhizal association could be more C expensive for plants than supporting one type. However, our results showed that SRR increased with N fertilisation (significant at N$\leq$100 kg ha^{-1}) in plants with the dual association (**Figure 3A**). Considering the different capabilities of AM and ECM fungi to acquire nutrients from organic and inorganic sources, they may play a complementary role in N and P uptake (Teste et al., 2020). Increased inorganic N availability with N fertilisation could shift the dominance of mycorrhizal association from ECM acquiring organic forms of N to AM fungi acquiring inorganic N (Phillips et al., 2013), without causing a reduction in mycorrhizal colonisation, but where fertilisation-induced enhancement in root N concentrations would increase respiratory C cost. N fertilisation can also induce a shift from N to P limitation in plants, which could increase plant C cost, given that soil P availability is usually much lower and less mobile compared to N.

Specific root respiration in NM plants was not affected by N fertilisation. If the effects of N fertilisation on SRR are controlled by mycorrhizal associations, then this would be expected. Conversely, if N fertilisation also increased root N concentration, then this would increase SRR. However, the large variation in the effect size of SRR in NM plants among studies precludes making any generalisations. NM plants can be categorised into early colonisers that have no morphological root adaptations and thrive in disturbed or relatively fertile soils, and plants with strong morphological root adaptations, such as cluster or dauciform roots that help plants with P acquisition in severely P-impoverished soils (Lambers and Teste, 2013). These two groups grow in soils that contrast in nutrient availability, and this could explain the large variation in N responses for NM plants, although we did not have enough observations to examine this for low and high N fertilisation levels.

We found that SRR increased in ECM plants supplied with more than 100 kg N ha^{-1} but not in AM and NM plants, while root N concentrations also increased the most in ECM plants in response to N fertilisation. These results provide insights into why ECM plants tend to dominate in organic or high C:N ratio soils and AM plants in mineral or low C:N ratio soils (Taylor et al., 2016; Jo et al., 2019; Soudzilovskaia et al., 2019). N acquisition through ECM association seems less C expensive for plants when soil N availability is low. This frequently occurs in high C:N ratio soils where most of the soil N is in organic form. Under these conditions, ECM plants may have an advantage over AM plants in acquiring N from organic sources *via* excretion of extracellular enzymes (Pritsch and Garbaye, 2011; Sulman et al., 2017). With increased inorganic N availability caused by N fertilisation, root N concentrations increase, and at some point this may become too C expensive and no longer economical

for plants to support ECM associations. In contrast, supporting AM associations when soil N availability is high may still be beneficial to plants. Although C costs could increase because of an increase in root N concentration, other nutrients like P may become more limiting to plant growth and AM symbiosis may be advantageous (Jo et al., 2019), given that AM fungi play an important role in P acquisition (van der Heijden et al., 2015) and are believed to be less C costly for plants than the ECM symbiosis (Viertelhauzen, 2013). SRR may, therefore, be a key variable in explaining responses of plant growth and their dependence on mycorrhizal associations with N fertilisation and increased atmospheric N deposition. Our meta-analysis further highlights the need for future research studies addressing mechanisms (e.g., biochemical and metabolic pathways) causing variation in SRR and plant N uptake associated with different Myco types in response to N fertilisation.

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

FD conceived the research idea and participated in the evaluation of the results. BB collected the data, conducted the meta-analysis, performed the model-selection and multi-model inference, and drafted the manuscript. All authors contributed to editing the manuscript.

FUNDING

This research was supported with a Postgraduate Research Scholarship funded by the Australian Research Council Discovery (Grant No. DP190102262) and the Thomas Lawrance Pawlett Postgraduate Research Scholarship from the Sydney Institute of Agriculture.

SUPPLEMENTARY MATERIAL

The Supplementary Material for this article can be found online at: https://www.frontiersin.org/articles/10.3389/fpls.2021.711720/full#supplementary-material

REFERENCES

Atkin, O. K., Edwards, E. J., and Loveys, B. R. (2000). Response of root respiration to changes in temperature and its relevance to global warming. *New Phytol*. 147, 141–154. doi: 10.1046/j.1469-8137.2000.00683.x

Averill, C., Turner, B. L., and Finzi, A. C. (2014). Mycorrhiza-mediated competition between plants and decomposers drives soil carbon storage. *Nature* 505, 543–545. doi: 10.1038/nature12901

Avis, P. G., McLaughlin, D. J., Dentinger, B. C., and Reich, P. B. (2003). Long-term increase in nitrogen supply alters above- and below-ground ectomycorrhizal communities and increases the dominance of Russula spp. in a temperate oak savanna. *New Phytol*. 160, 239–253. doi: 10.1046/j.1469-8137.2003.00865.x

Bache, B. W., Chesworth, W., Chesworth, W., Gessa, C., and Lewis, D. T. (2008). "Bulk density," in *Encyclopedia of Soil Science*, ed W. Chesworth (Dordrecht: Springer Netherlands). doi: 10.1007/978-1-4020-3995-9_80

Borenstein, M., Hedges, L. V., Higgins, J. P. T., and Rothstein, H. R. (2009). Introduction to meta-analysis. *John Wiley & Sons, Ltd* doi: 10.1002/9780470743386

Brundrett, M. C. (2009). Mycorrhizal associations and other means of nutrition of vascular plants: understanding the global diversity of host plants by resolving conflicting information and developing reliable means of diagnosis. *Plant Soil*. 320, 37–77. doi: 10.1007/s11104-008-9877-9

Burnham, K. P. (2002). *Model Selection and Multimodel Inference: A Practical Information-Theoretic Approach*. 2nd edn. New York, NY: Springer Netherlands.

Burton, A. J., Jarvey, J. C., Jarvi, M. P., Zak, D. R., and Pregitzer, K. S. (2012). Chronic N deposition alters root respiration-tissue N relationship in northern hardwood forests. *Glob. Chang. Biol*. 18, 258–266. doi: 10.1111/j.1365-2486.2011.02527.x

Burton, A. J., Pregitzer, K. S., Crawford, J. N., Zogg, G. P., and Zak, D. R. (2004). Simulated chronic NO3– deposition reduces soil respiration in northern hardwood forests. *Glob. Chang. Biol*. 10, 1080–1091. doi: 10.1111/j.1365-2486.2004.00737.x

Calcagno, V., and de Mazancourt, C. (2010). glmulti: An R package for easy automated model selection with (generalized) linear models. *J. Stat. Softw*. 1:12. doi: 10.18637/jss.v034.i12

Chen, F., Yan, G., Xing, Y., Zhang, J., Wang, Q., Wang, H., et al. (2019). Effects of N addition and precipitation reduction on soil respiration and its components in a temperate forest. *Agric For Meteorol*. 271, 336–345. doi: 10.1016/j.agrformet.2019.03.021

Corkidi, L., Rowland, D. L., Johnson, N. C., and Allen, E. B. (2002). Nitrogen fertilization alters the functioning of arbuscular mycorrhizas at two semiarid grasslands. *Plant Soil*. 240, 299–310. doi: 10.1023/A:1015792204633

Cosme, M., Fernández, I., Van der Heijden, M. G. A., and Pieterse, C. M. J. (2018). Non-mycorrhizal plants: The exceptions that prove the rule. *Trends Plant Sci*. 23, 577–587. doi: 10.1016/j.tplants.2018.04.004

Ding, W., Yu, H., Cai, Z., Han, F., and Xu, Z. (2010). Responses of soil respiration to N fertilization in a loamy soil under maize cultivation. *Geoderma*. 155, 381–389. doi: 10.1016/j.geoderma.2009.12.023

Ekblad, A., Mikusinska, A., Ågren, G. I., Menichetti, L., Wallander, H., Vilgalys, R. et al. (2016). Production and turnover of ectomycorrhizal extramatrical mycelial biomass and necromass under elevated CO2 and nitrogen fertilization. *New Phytol*. 211, 874–885. doi: 10.1111/nph.13961

Emmanuel, B., Fagbola, O., and Osonubi, O. (2012). Influence of fertiliser application on the occurrence and colonisation of arbuscular mycorrhizal fungi (AMF) under maize/Centrosema and sole maize systems. *Soil Res*. 50, 76–81. doi: 10.1071/SR11254

Gleser, L. J., and Olkin, I. (2009). "Stochastically dependent effect sizes," in *The Handbook of Research Synthesis and Meta-Analysis. 2nd ed*, eds H. Cooper, L. V. Hedges, J. C. Valentine (New York, NY: Russell Sage Foundation).

Gurka, M. J. (2006). Selecting the best linear mixed model under REML. *Am. Stat*. 60, 19–26. doi: 10.1198/000313006X90396

Hasselquist, N. J., Metcalfe, D. B., and Högberg, P. (2012). Contrasting effects of low and high nitrogen additions on soil CO2 flux components and ectomycorrhizal fungal sporocarp production in a boreal forest. *Glob. Chang. Biol*. 18, 3596–3605. doi: 10.1111/gcb.12001

Hobbie, E. A. (2006). Carbon allocation to ectomycorrhizal fungi correlates with belowground allocation in culture studies. *Ecology*. 87, 563–569. doi: 10.1890/05-0755

Hobbie, E. A., Ouimette, A. P., Schuur, E. A. G., Kierstead, D., Trappe, J. M., Bendiksen, K., et al. (2013). Radiocarbon evidence for the mining of organic nitrogen from soil by mycorrhizal fungi. *Biogeochemistry* 114, 381–389. doi: 10.1007/s10533-012-9779-z

Hobbie, S. E. (2008). Nitrogen effects on decomposition: a five-year experiment in eight temperate sites. *Ecology* 89, 2633–2644. doi: 10.1890/07-1119.1

Hodge, A., and Fitter, A. H. (2010). Substantial nitrogen acquisition by arbuscular mycorrhizal fungi from organic material has implications for N cycling. *PNAS* 107, 13754–13759. doi: 10.1073/pnas.1005874107

Högberg, M. N., Briones, M. J., Keel, S. G., Metcalfe, D. B., Campbell, C., Midwood, A. J., et al. (2010). Quantification of effects of season and nitrogen supply on tree below-ground carbon transfer to ectomycorrhizal fungi and other soil organisms in a boreal pine forest. *New Phytol.* 187, 485–493. doi: 10.1111/j.1469-8137.2010.03274.x

Hughes, J. K., Hodge, A., Fitter, A. H., and Atkin, O. K. (2008). Mycorrhizal respiration: implications for global scaling relationships. *Trends Plant Sci.* 13, 583–588. doi: 10.1016/j.tplants.2008.08.010

Jansa, J., Forczek, S. T., Rozmoš, M., Püschel, D., Bukovská, P., and Hršelová, H. (2019). Arbuscular mycorrhiza and soil organic nitrogen: network of players and interactions. *Chem. Biol. Technol. Agric.* 6:10. doi: 10.1186/s40538-019-0147-2

Jo, I., Fei, S., Oswalt, C., Domke, G., and Phillips, R. (2019). Shifts in dominant tree mycorrhizal associations in response to anthropogenic impacts. *Sci. Adv.* 5, 1–8. doi: 10.1126/sciadv.aav6358

Johnson, N. C. (2010). Resource stoichiometry elucidates the structure and function of arbuscular mycorrhizas across scales. *New Phytol.* 185, 631–647. doi: 10.1111/j.1469-8137.2009.03110.x

Johnson, N. C., and Gehring, C. A. (2007). "Mycorrhizas: symbiotic mediators of rhizosphere and ecosystem processes," in *The Rhizosphere*, eds Z. G. Cardon and J. L. Whitbeck (Burlington: Academic Press). doi: 10.1016/B978-012088775-0/50006-9

Kaltenbach, H.-M. (2012). *A Concise Guide to Statistics. SpringerBriefs in Statistics.* Heidelberg: Springer. doi: 10.1007/978-3-642-23502-3

Kendall, M. G. (1987). *Kendall's Advanced Theory of Statistics*. 5th edn. London: C. Griffin.

Lajeunesse, M. J. (2011). On the meta-analysis of response ratios for studies with correlated and multi-group designs. *Ecology* 92, 2049–2055. doi: 10.1890/11-0423.1

Lambers, H., and Teste, F. P. (2013). Interactions between arbuscular mycorrhizal and non-mycorrhizal plants: do non-mycorrhizal species at both extremes of nutrient availability play the same game? *Plant Cell Environ.* 36, 1911–1915. doi: 10.1111/pce.12117

Lin, G., McCormack, M. L., Ma, C., and Guo, D. (2017). Similar below-ground carbon cycling dynamics but contrasting modes of nitrogen cycling between arbuscular mycorrhizal and ectomycorrhizal forests. *New Phytol.* 213, 1440–1451. doi: 10.1111/nph.14206

López-Bucio, J., Cruz-Ramirez, A. and, Herrera-Estrella, L. (2003). The role of nutrient availability in regulating root architecture. *Curr. Opin. Plant Biol.* 6, 280–287. doi: 10.1016/S1369-5266(03)00035-9

Makita, N., Kosugi, Y., Dannoura, M., Takanashi, S., Niiyama, K., Kassim, A. R., et al. (2012). Patterns of root respiration rates and morphological traits in 13 tree species in a tropical forest. *Tree Physiol.* 32, 303–312. doi: 10.1093/treephys/tps008

Midolo, G., De Frenne, P., Hölzel, N., and Wellstein, C. (2019). Global patterns of intraspecific leaf trait responses to elevation. *Glob. Chang. Biol.* 25, 2485–2498. doi: 10.1111/gcb.14646

Nakagawa, S., and Santos, E. S. A. (2012). Methodological issues and advances in biological meta-analysis. *Evol. Ecol.* 26, 1253–1274. doi: 10.1007/s10682-012-9555-5

Nicolás, C., Martin-Bertelsen, T., Floudas, D., Bentzer, J., Smits, M., Johansson, T., et al. (2019). The soil organic matter decomposition mechanisms in ectomycorrhizal fungi are tuned for liberating soil organic nitrogen. *ISME J.* 13, 977–988. doi: 10.1038/s41396-018-0331-6

Nilsson, L. O., and Wallander, H. (2003). Production of external mycelium by ectomycorrhizal fungi in a norway spruce forest was reduced in response to nitrogen fertilization. *New Phytol.* 158, 409–416. doi: 10.1046/j.1469-8137.2003.00728.x

Noble, D. W. A., Lagisz, M., O'Dea, R. E., and Nakagawa, S. (2017). Nonindependence and sensitivity analyses in ecological and evolutionary meta-analyses. *Mol. Ecol.* 26, 2410–2425. doi: 10.1111/mec.14031

Parrent, J. L., Morris, W. F., and Vilgalys, R. (2006). CO2-enrichment and nutrient availability alter ectomycorrhizal fungal communities. *Ecology* 87, 2278–2287. doi: 10.1890/0012-9658(2006)87[2278:CANAAE]2.0.CO;2

Pausch, J., and Kuzyakov, Y. (2018). Carbon input by roots into the soil: Quantification of rhizodeposition from root to ecosystem scale. *Glob. Chang. Biol.* 24, 1–12. doi: 10.1111/gcb.13850

Phillips, R. P., Brzostek, E., and Midgley, M. G. (2013). The mycorrhizal-associated nutrient economy: a new framework for predicting carbon-nutrient couplings in temperate forests. *New Phytol.* 199, 41–51. doi: 10.1111/nph.12221

Pregitzer, K. S., Zak, D. R., Loya, W. M., Karberg, N. J., King, J. S., and Burton, A. J. (2007). "The contribution of root - rhizosphere interactions to biogeochemical cycles in a changing world," in *The Rhizosphere*, eds Z. G. Cardon and J. L. Whitbeck (Burlington: Academic Press). doi: 10.1016/B978-012088775-0/50009-4

Pritsch, K., and Garbaye, J. (2011). Enzyme secretion by ECM fungi and exploitation of mineral nutrients from soil organic matter. *Ann. For. Sci.* 68, 25–32. doi: 10.1007/s13595-010-0004-8

Puschel, D., Janouskova, M., Hujslova, M., Slavikova, R., Gryndlerova, H., and Jansa, J. (2016). Plant-fungus competition for nitrogen erases mycorrhizal growth benefits of Andropogon gerardii under limited nitrogen supply. *Ecol. Evol.* 6, 4332–4346. doi: 10.1002/ece3.2207

R Development Core Team (2020). *R: A Language and Environment for Statistical Computing*. Vienna: The R Foundation for Statistical Computing.

Reich, P. B., Tjoelker, M. G., Pregitzer, K. S., Wright, I. J., Oleksyn, J., and Machado, J.-L. (2008). Scaling of respiration to nitrogen in leaves, stems and roots of higher land plants. *Ecol. Lett.* 11, 793–801. doi: 10.1111/j.1461-0248.2008.01185.x

Rubio-Aparicio, M., López-López, J. A., Viechtbauer, W., Marín-Martínez, F., Botella, J., and Sánchez-Meca, J. (2020). Testing categorical moderators in mixed-effects meta-analysis in the presence of heteroscedasticity. *J. Exp. Educ.* 88, 288–310. doi: 10.1080/00220973.2018.1561404

Smith, F. A., Grace, E. J., and Smith, S. E. (2009). More than a carbon economy: nutrient trade and ecological sustainability in facultative arbuscular mycorrhizal symbioses. *New Phytol.* 182, 347–358. doi: 10.1111/j.1469-8137.2008.02753.x

Soudzilovskaia, N. A., van Bodegom, P. M., Terrer, C., Zelfde, M., McCallum, I. (2019). Global mycorrhizal plant distribution linked to terrestrial carbon stocks. *Nat. Commun.* 10:5077. doi: 10.1038/s41467-019-13019-2

Soudzilovskaia, N. A., van der Heijden, M. G. A., Cornelissen, J. H. C., Makarov, M. I., Onipchenko, V. G., Maslov, M. N., et al. (2015). Quantitative assessment of the differential impacts of arbuscular and ectomycorrhiza on soil carbon cycling. *New Phytol.* 208, 280–293. doi: 10.1111/nph.13447

Stuart, E. K., and Plett, K. L. (2020). Digging deeper: In search of the mechanisms of carbon and nitrogen exchange in ectomycorrhizal symbioses. *Front. Plant Sci.* 10:1658. doi: 10.3389/fpls.2019.01658

Sulman, B. N., Brzostek, E. R., Medici, C., Shevliakova, E., Menge, D. N. L., and Phillips, R. P. (2017). Feedbacks between plant N demand and rhizosphere priming depend on type of mycorrhizal association. *Ecol. Lett.* 20, 1043–1053. doi: 10.1111/ele.12802

Taylor, M. K., Lankau, R. A., and Wurzburger, N. (2016). Mycorrhizal associations of trees have different indirect effects on organic matter decomposition. *J. Ecol.* 104, 1576–1584. doi: 10.1111/1365-2745.12629

Terrer, C., Jackson, R. B., Prentice, I. C., Keenan, T. F., Kaiser, C., Vicca, S., et al. (2019). Nitrogen and phosphorus constrain the CO2 fertilization of global plant biomass. *Nat. Clim. Change.* 9, 684–689. doi: 10.1038/s41558-019-0545-2

Terrer, C., Phillips, R. P., Hungate, B. A., Rosende, J., Pett-Ridge, J., Craig, M. E., et al. (2021). A trade-off between plant and soil carbon storage under elevated CO2. *Nature* 591, 599–603. doi: 10.1038/s41586-021-03306-8

Terrer, C., Vicca, S., Hungate, B. A., Phillips, R. P., and Prentice, I. C. (2016). Mycorrhizal association as a primary control of the CO_2 fertilization effect. *Science* 353, 72–74. doi: 10.1126/science.aaf4610

Teste, F. P., Jones, M. D., and Dickie, I. A. (2020). Dual-mycorrhizal plants: their ecology and relevance. *New Phytol.* 225, 1835–1851. doi: 10.1111/nph.16190

Thirkell, T., Cameron, D., and Hodge, A. (2019). Contrasting nitrogen fertilisation rates alter mycorrhizal contribution to barley nutrition in a field trial. *Front. Plant Sci.* 10:1312. doi: 10.3389/fpls.2019.01312

Treseder, K. K. (2004). A meta-analysis of mycorrhizal responses to nitrogen, phosphorus, and atmospheric CO2 in field studies. *New Phytol.* 164, 347–355. doi: 10.1111/j.1469-8137.2004.01159.x

Treseder, K. K., Allen, E. B., Egerton-Warburton, L. M., Hart, M. M., Klironomos, J. N., Maherali, H., et al. (2018). Arbuscular mycorrhizal fungi as mediators

of ecosystem responses to nitrogen deposition: A trait-based predictive framework. *J. Ecol*. 106, 480–489. doi: 10.1111/1365-2745.12919

Tu, L., Hu, T., Zhang, J., and Li, X. (2013). Nitrogen addition stimulates different components of soil respiration in a subtropical bamboo ecosystem. *Soil Biol. Biochem*. 58, 255–264. doi: 10.1016/j.soilbio.2012.12.005

van der Heijden, M. G. A., Martin, F. M., Selosse, M.-A., and Sanders, I. R. (2015). Mycorrhizal ecology and evolution: the past, the present, and the future. *New Phytol*. 205, 1406–1423. doi: 10.1111/nph.13288

Veroniki, A. A., Jackson, D., Viechtbauer, W., Bender, R., Bowden, J., Knapp, G., et al. (2016). Methods to estimate the between-study variance and its uncertainty in meta-analysis. *Res. Synth. Methods* 7, 55–79. doi: 10.1002/jrsm.1164

Viechtbauer, W. (2010). Conducting meta-analyses in R with the metafor package. *J. Stat. Softw*. 36, 1–48. doi: 10.18637/jss.v036.i03

Viertelhauzen, L. (2013). *Arbuscular vs Ectomycorrhizal fungi: Limiting or Stimulating Tree Growth With N Deposition?* MSc thesis, Utrecht University, Utrecht.

Wang, G., and Liu, F. (2014). Carbon allocation of Chinese pine seedlings along a nitrogen addition gradient. *For Ecol Manag*. 334, 114–121. doi: 10.1016/j.foreco.2014.09.004

Wang, R., Bicharanloo, B., Shirvan, M. B., Cavagnaro, T. R., Jiang, Y., Keitel, C., et al. (2021). A novel 13C pulse-labelling method to quantify the contribution of rhizodeposits to soil respiration in a grassland exposed to drought and nitrogen addition. *New Phytol*. 230, 857–866. doi: 10.1111/nph.17118

Wang, X.-X., Wang, X., Sun, Y., Cheng, Y., Liu, S., Chen, X., et al. (2018). Arbuscular mycorrhizal fungi negatively affect nitrogen acquisition and grain yield of maize in a N deficient soil. *Front. Microbiol*. 9:418. doi: 10.3389/fmicb.2018.00418

Zhang, C., Niu, D., Hall, S. J., Wen, H., Li, X., Fu, H., et al. (2014). Effects of simulated nitrogen deposition on soil respiration components and their temperature sensitivities in a semiarid grassland. *Soil Biol. Biochem*. 75, 113–123. doi: 10.1016/j.soilbio.2014.04.013

Zhou, L., Zhou, X., Shao, J., Nie, Y., He, Y., Jiang, L., et al. (2016). Interactive effects of global change factors on soil respiration and its components: a meta-analysis. *Glob. Chang. Biol*. 22, 3157–3169. doi: 10.1111/gcb.13253

Zhou, L., Zhou, X., Zhang, B., Lu, M., Luo, Y., Liu, L., et al. (2014). Different responses of soil respiration and its components to nitrogen addition among biomes: a meta-analysis. *Glob. Chang. Biol*. 20, 2332–2343. doi: 10.1111/gcb.12490

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.

Publisher's Note: All claims expressed in this article are solely those of the authors and do not necessarily represent those of their affiliated organizations, or those of the publisher, the editors and the reviewers. Any product that may be evaluated in this article, or claim that may be made by its manufacturer, is not guaranteed or endorsed by the publisher.

frontiers in Plant Science

ORIGINAL RESEARCH
published: 07 September 2021
doi: 10.3389/fpls.2021.687347

Stoichiometry of Carbon, Nitrogen and Phosphorus in Shrub Organs Linked Closely With Mycorrhizal Strategy in Northern China

Shuang Yang[1,2,3], Zhaoyong Shi[1,2,3], Menghan Zhang[1,2,3], Yang Li[1,2,3], Jiakai Gao[1,2,3], Xugang Wang[1,2,3] and Dehong Liu[1,2,3]*

[1] College of Agriculture, Henan University of Science and Technology, Luoyang, China, [2] Luoyang Key Laboratory of Symbiotic Microorganism and Green Development, Luoyang, China, [3] Henan Engineering Research Center of Human Settlements, Luoyang, China

Mycorrhizal strategies include mycorrhizal statuses and mycorrhizal types, which are important reflections of the functional characteristics of ecosystems. The stoichiometry of carbon, nitrogen, and phosphorus in plant organs is an important part of ecosystem functions, which has an important impact on the nutrient cycle of the ecosystem. The concentration of carbon, nitrogen, and phosphorus played a crucial role in ecosystem functioning and dynamics. The purpose of this study is to provide theoretical basis and data support for improving the properties of global terrestrial ecosystems by exploring the impact of mycorrhizal strategies on the stoichiometry of C, N, and P in different shrub organs. In this study, stoichiometric patterns of carbon (C), nitrogen (N) and phosphorus (P) in different shrub organs under different mycorrhizal status or types were analyzed at 725 samples across Northern China. Results showed that in different mycorrhizal status, the highest carbon concentration in shrub organs appeared in the facultatively mycorrhizal (FM) mycorrhizal status, and the highest nitrogen concentration appeared in the Non-mycorrhizal (NM) mycorrhizal status. Under different mycorrhizal types, the nitrogen concentration in the shrub organs under the arbuscular mycorrhiza (AM) mycorrhizal type was the highest, and the phosphorus concentration under the ecto-mycorrhiza (ECM) mycorrhizal type was the highest. In the OM or FM mycorrhizal status, the concentrations of C, N, and P in the stems and leaves increase with the increase of the concentrations of C, N, and P in the roots. In the NM mycorrhizal status, the N concentration in the stems and leaves increases with the increase of the N concentration in the roots. Under AM, AM+ECM, and ECM mycorrhizal type, the concentrations of C, N, and P are closely related in roots, stems and leaves. The content of plant nutrients in different organs is closely related. It turned out that mycorrhizal statuses or types are able to alter the allocation of C, N, and P in different organs, and the relationships of C, N, and P among different organs are able to present different trend with the varying of mycorrhizal statuses or types.

Keywords: stoichiometry, mycorrhizal status, mycorrhizal types, shrub organs, Northern China

OPEN ACCESS

Edited by:
Sergio Saia,
University of Pisa, Italy

Reviewed by:
Néstor Fernández Del-Saz,
University of Concepcion, Chile
Paula Aguilera,
Scientific and Technological Bioresource Nucleus (BIOREN), Chile

***Correspondence:**
Zhaoyong Shi
shizy1116@126.com

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

Received: *29 March 2021*
Accepted: *18 August 2021*
Published: *07 September 2021*

Citation:
Yang S, Shi Z, Zhang M, Li Y, Gao J, Wang X and Liu D (2021) Stoichiometry of Carbon, Nitrogen and Phosphorus in Shrub Organs Linked Closely With Mycorrhizal Strategy in Northern China. Front. Plant Sci. 12:687347. doi: 10.3389/fpls.2021.687347

INTRODUCTION

The stoichiometry in plant leaves, stems, and roots is able to characterize the nutrient restriction status of nutrient elements (Wassen et al., 2005). Carbon (C), nitrogen (N) and phosphorus (P) are considered to be important stoichiometry because these elements are the basic elements that form the structure and function of all living things, which coupled strongly in their biochemical processes (Vrede et al., 2004). Carbon, which accounts for about 38% of plant dry matter, is an indispensable element in the growth and development of plants (Vrede et al., 2004). Leaf nitrogen concentration plays a vital role in photosynthesis, plant production, and litter decomposition (LeBauer and Treseder, 2008). Phosphorus is an important part of energy storage and cell structure, and it is critical for energy conversion, respiration, and photosynthesis (Sterner and Elser, 2002; Vrede et al., 2004). Moreover, stoichiometric characteristics of C, N, and P affect ecosystem functions and nutrient cycling (Lü et al., 2013). Stoichiometry provides a framework for understanding the role of ecosystems and the balance of elements. Previous studies have shown that evolutionary history, environmental stress and plant functional groups are possible factors affecting the stoichiometry of C, N, and P in different organs (Craine et al., 2005; Kerkhoff et al., 2006; Li et al., 2010; Liu et al., 2010). Moreover, some studies have suggested that C, N, and P stoichiometry of plants are affected by many factors, including climate and soil (Yang et al., 2016; Zhang et al., 2019), legumes and non-legumes (Yang et al., 2014), arbuscular mycorrhiza (Chen et al., 2010), productivity (Tang et al., 2018), nitrogen deposition (Jing et al., 2017), latitude (Fang et al., 2019), and environmental conditions (Gong et al., 2018). However, it is still unknown that the effect of the complete mycorrhizal strategy functional group on the stoichiometry of C, N, and P. This study considers that different mycorrhizal statuses or types will also affect the stoichiometry in different plant organs.

Mycorrhiza is a combination of certain fungi and plant roots. Mycorrhizal fungi are important members of the plant microbiota and can form a symbiotic relationship with the root of most plants on the earth (Averill et al., 2019). More than 92% of plant species have mycorrhiza (Sizonenko et al., 2020). Because mycorrhizal fungi are ubiquitous in terrestrial ecosystems, they are an important part of the soil biota and affect where and how plants grow (Johnson et al., 2010). There is evidence that plant-mycorrhizal symbiosis is essential for maintaining biogeochemical cycles and ecosystem functions (van der Heijden et al., 2015). Fungi can't only protect plants from pathogens, but also regulate the relationship between plants and water (Veresoglou and Rillig, 2012). According to reports, plants have different growth responses to different fungi species (Treseder et al., 2018). Mycorrhizal strategies are divided into mycorrhizal status and mycorrhizal types. The status of mycorrhiza is closely related to the leaf economic spectrum (Shi et al., 2020). The mycorrhizal status reflects the consistency of the colonization of fungi within a species (Menzel et al., 2016). A recent hypothesis suggests that the mycorrhizal types are able to be used as a predictive framework for carbon and nutrient cycles within and throughout ecosystems (Wurzburger and Brookshire, 2017). Different mycorrhizal types of plants have different nutrient acquisition strategies (van der Heijden et al., 2015). In recent years, the status and type of mycorrhiza have been the focus of attention in the interaction between plants and environmental factors. The relationship between the mycorrhizal statuses or types of plant species and other plant traits is important to the understand plant's ecology and distribution. There have been many reports on the importance of mycorrhiza to forest ecosystems (Kubisch et al., 2016; Wu et al., 2019; Tarquin et al., 2021) and grassland ecosystems (Kriszta and Matthias, 2015; Maarten et al., 2018). A vast number of hypotheses explain the observed relationships between mycorrhizal association and plant ecology are available. However, it is unknown that the importance of mycorrhizal strategy to the shrub ecosystem. Therefore, this research will focus on whether the status or types of mycorrhiza have an effect on the stoichiometry of shrub organs and how they affect it.

Shrubs cover over 1.23 million square kilometers (equal to12.5% of national territory area) in China (Geological Publishing House, 2007). As the main plant type in Northern China, shrubs are the top-level vegetation adapted to the arid climate in Northern China (Yang et al., 2016), which are able to conserve water, regulate runoff, and improve soil fertility (Zhang et al., 2013). The shrubs are generally smaller than trees and are relatively more uniform in size among species, which weakens the "dilution effect" of carbon, nitrogen and phosphorus allocated to the structural components of woody plants (Kerkhoff et al., 2006). Exploring the stoichiometry in different organs of shrubs is essential to improve the properties of global terrestrial ecosystems. There have been previous studies on shrub stoichiometry (Yang et al., 2014; He et al., 2016; Zhang Q. et al., 2018), but the importance of mycorrhizal strategy to shrub stoichiometry is still unclear. Therefore, this study explores the stoichiometric characteristics based on shrubs, which is conducive to a deeper assessment in a larger ecological background. In this study, we explore the effects of mycorrhizal statuses or types on the content of C, N, and P among leaves, stems and roots and their relationships based on shrubs in Northern China. The purpose of this study is to explore the effects of mycorrhizal types or statuses on the distribution and relationship of carbon, nitrogen, and phosphorus among different shrub organs. The research aims to enhance the understanding of the stoichiometry of mycorrhiza in shrub organs by analyzing plant traits related to mycorrhiza development. Therefore, our hypotheses are: (1) Mycorrhizal statuses or types will alter the allocation of C, N, and P in different organs, (2) the relationship of C, N, and P among different organs is closely related to the mycorrhizal statuses or types.

MATERIALS AND METHODS

Study Site and Investigation

This study collected 725 samples in Northern China between July and September (mostly July and August). A species that is always found to form mycorrhizas is considered to be obligately mycorrhizal (OM), while a species that is found to form mycorrhizas in one habitat but not in another habitat is considered to be facultatively mycorrhizal (FM). And a species

is considered to be Non-mycorrhizal (NM) if it always not found to form mycorrhizas (Smith and Read, 2008). We classified all the plants with typical arbuscular mycorrhiza (AM) structures as AM type and others unable to form AMs as non-AM type, according to the method employed by Koele et al. (2012) in studying ectomycorrhizal effect on global foliar traits and Shi et al. (2020) in exploring the relationship between the worldwide leaf economic spectrum traits and mycorrhizal traits. The typical arbuscular mycorrhizal structure including arbuscule or vesicle was considered when AM type was identified. Ecto-mycorrhiza (ECM) and non-ECM are classified in the same way. The species forming both AM and ECM are classified as AM+ECM type. The mycorrhizal types of some plant species were determined this study, while others were ascertained according to the published literatures based on the method employed by Averill et al. (2019), Wang and Qiu (2006) and Shi et al. (2020). The references included mainly Wang and Qiu (2006); Akhmetzhanova et al. (2012), Li et al. (2011), and Shi et al. (2008). Based on these references the plant species were assigned a mycorrhizal status (OM, FM, or NM), and type (AM, ECM, and AM+ECM) (**Supplementary Data Sheet 1**). In total, out of 725 plant samples in this study, we obtained mycorrhizal information for 582 samples (80.28%) and analyzed a total of 543 samples (74.90%). This was due to the species with a small sample size (39 samples) were omitted from the analysis in order to ensure the rigor of mycorrhizal categories and the accuracy of the results. Out of these 473 (87.11%) were OM, 33 (6.08%) FM and 37 (6.81%) NM; 316 (66.81%) formed AM, 64 (13.53%) AM+ECM, 93 (19.66%) ECM.

We selected a 20 m × 20 m plot with relatively uniform species composition and community structure as the sample area to represent the shrub community at the site. Three 5 m × 5 m quadrats were arranged in the center and any two diagonal corners of the sample area to collect soil samples. We identified all individuals to the species level and determined the mycorrhizal type according to the species. In total, we analyzed 543 individuals of 49 shrub species from 37 genera and 17 families from 155 sites. The main species include *Corylus heterophylla, Ostryopsis davidiana, Prunus sibirica, Spiraea pubescens, Tamarix chinensis, Vitex negundo,* etc. Please refer to the **Supplementary Data Sheet 1** for detailed species information. We determined the level of shrubs based on the base stem, plant height, growth environment and growth years of the shrub. We generally divided it into three levels: large, medium, and small, and took three individuals at each level. We had at most 17 levels and at least one level. We collected all the adult leaves capable of normal photosynthesis, all the stems except the new stems of the current year and the whole roots of each individual outside and adjacent to the quadrate. The collected plant samples were dried and ground in the laboratory to the next analysis and determination.

The soil nutrient status of the sampling site was characterized by soil nitrogen and soil phosphorus. We analyzed the soil N content and soil P content of 49 species in 155 locations. Among them, the variation range of soil nitrogen concentration was 0.13–18.03 mg/g, the average value was 1.75 mg/g, and the variation range of soil phosphorus concentration was 0.08–2.01 mg/g, the average value was 0.55 mg/g. The specific soil nutrient values were recorded in the **Supplementary Data Sheet 1**. The soil samples were collected in three one-meter-deep pits on the diagonal of each 5 m × 5 m quadrat. And then the nine soil samples from each sample area were mixed well. The soil samples were air-dried and the roots were removed and then were ground through a 100-mesh sieve in order to facilitate the next measurement. Please refer to Yang et al. (2014) for more detailed information on data collections and locations of the sampling sites.

Determination of Carbon, Nitrogen, and Phosphorus Concentrations

The carbon and nitrogen concentrations in different shrub organs and total nitrogen concentrations of soil (STN) were analyzed using an elemental analyzer (2400 II CHNS; Perkin-Elmer, Boston, MA, United States) under 950°C for combustion then reduced to 640°C. The phosphorus concentrations in plant samples and total phosphorus concentrations of soil (STP) were measured using the molybdate/ascorbic acid method after H_2SO_4-H_2O_2 digestion.

Data Analysis

Because the element concentrations of leaves, stems, and roots at each sampling point were average at the species level, the data was statistically summarized, and the average value of three individual plants from each level was calculated to represent the element concentration at the same point. We used the average value of all data to represent the element concentration under this mycorrhizal status or type and make a significant comparison of different element concentrations under different mycorrhizal types and statuses. At the level of $P < 0.05$, the concentration of C, N, and P in shrub organs was analyzed by one-way analysis of variance (ANOVA), and Tukey multiple comparison method was used to test the significance of the difference. All analysis of significance was performed using SPSS 23.0. The element concentration and stoichiometric ratio in different shrub organs were converted to logarithm 10, and then linear regression analysis was performed. All the figures were generated using EXCEL 2016 (microsoft office) or SPSS 23.0. Under AM, AM+ECM, and ECM mycorrhizal types, the effects of site, species, and their interactions on nine categories (leafC, leafN, leafP, stemC, stemN, stemP, rootC, rootN, and rootP) were examined by linear mixed effect models. Each category was treated as a response variable. Site, species and their interaction were fixed effects. Mycorrhizal type was considered as random effects. The analysis of linear mixed effect models were conduct in R 4.0.5[1].

The relationships between soil nutrient concentration and shrub organs P concentration were analyzed used a bivariate correlation analysis. Pearson correlation coefficient and bilateral significance test were selected for these statistical analyses in SPSS 23.0.

In order to show that the distributions of C, N, and P contents varied with the mycorrhizal category, density histogram was performed using SPSS 23.0.

[1]http://R-project.org/

RESULTS

Carbon, Nitrogen and Phosphorus Concentration in Shrub Organs Under Different Mycorrhizal Status

The concentration of carbon, nitrogen, and phosphorus in shrub organs (leaves, stems, and roots) under different mycorrhizal status were presented in **Figure 1**. The carbon concentration in the leaves was 455.41, 379.02, and 440.73 mg/g in the different mycorrhizal status of FM, NM, and OM, respectively (**Figure 1A**). The carbon concentration in the stems was 464.88, 437.42, and 457.52 mg/g in the FM, NM, and OM mycorrhizal status, respectively (**Figure 1B**). The carbon concentration of leaves and stems in different mycorrhizal status of FM, NM, and OM presented the similar trend with the significant lower carbon concentration in the NM species than in OM and FM plants (**Figures 1A,B**). However, no remarkable difference was observed between FM and OM on carbon concentration either leaves or stems. In the roots, only a significantly higher carbon concentration was observed in FM shrubs than in NM shrubs. The carbon concentration in roots did not change markedly in FM and OM, and the same was true in NM and OM (**Figure 1C**).

Leaf nitrogen concentrations were 17.01, 20.07, and 20.35 mg/g (**Figure 1D**) in FM, NM, and OM species, respectively. And the nitrogen concentrations in the stem and root were 4.96, 11.85, and 6.68 mg/g (**Figure 1E**), 5.17, 14.60, and 8.01 mg/g (**Figure 1F**), respectively. The highest nitrogen concentration in the leaves was observed in OM species (OM plants have significantly higher leaf nitrogen concentrations than FM plants) (**Figure 1D**), however, this was not true for stems and roots, for which the highest nitrogen concentration was observed in NM species (**Figures 1E,F**). The nitrogen concentration in the stems of NM species was significantly higher than that of FM and OM species (**Figure 1E**). In the roots, the nitrogen concentration was highest in the NM, followed by the OM, and finally the FM, and the nitrogen concentration of roots was significantly different in the three mycorrhizal statuses (**Figure 1F**).

The phosphorus concentration in the stems was 0.50, 0.60, and 0.48 mg/g in FM, NM, and OM, respectively (**Figure 1H**). We found that NM species had markedly higher phosphorus concentration than FM and OM plants, while the FM did not exhibit significant variation of phosphorus concentration than OM (**Figure 1H**). In the leaves and roots of shrubs, there was no significant difference in the phosphorus concentration of the three mycorrhizal statuses (**Figures 1G,I**). The **Figure 1** showed that different mycorrhizal status had an impact on the concentration of carbon, nitrogen, and phosphorus in the shrubs, but the impact was different.

Carbon, Nitrogen and Phosphorus Concentration in Shrub Organs Under Different Mycorrhizal Types

Figure 2 showed the concentration of carbon, nitrogen, and phosphorus in shrub organs under different mycorrhizal types. Regarding mycorrhizal types, carbon concentration of leaves was significantly higher in AM+ECM plants (455.10 mg/g) than in AM species (436.14 mg/g) and the same pattern was observed in the stems, in which the carbon concentration in AM, AM+ECM and ECM was 456.50, 462.68, and 457.38 mg/g, respectively (**Figures 2A,B**). But in the leaves and stems, the carbon concentration did not exhibit significant variation among AM and ECM, AM+ECM, and ECM. In the roots, the carbon concentration was greater in AM compared to ECM, while both the carbon concentration in AM and ECM showed no significant difference from the carbon concentration in AM+ECM (**Figure 2C**).

There was no significant difference in nitrogen concentration between the three mycorrhizal types in shrub leaves (**Figure 2D**). The stem nitrogen concentration under AM, AM+ECM, and ECM was 7.28, 5.34, and 5.58 mg/g, respectively, and the nitrogen concentration in stems under AM was significantly higher than that under AM+ECM and ECM (**Figure 2E**). There was a similar rule in roots, for which the nitrogen concentration was 8.98, 5.55, and 6.46 mg/g, respectively (**Figure 2F**). However, we did not observed a significant difference of nitrogen concentration between AM+ECM and ECM in either stems or roots.

The leaf phosphorus concentration was 1.23, 1.39, and 1.58 mg/g (**Figure 2G**), and the stem phosphorus concentration was 0.45, 0.46, and 0.58 mg/g, respectively (**Figure 2H**), the root phosphorus concentration was 0.56, 0.53, and 0.93 mg/g, respectively (**Figure 2I**), in AM, AM+ECM, and ECM species. The leaf phosphorus concentration was significantly higher in ECM plants than in AM+ECM plants, and significantly higher in AM+ECM plants than in AM plants (**Figure 2G**). In the stems and roots of shrubs, there was a significant difference in the phosphorus concentration between ECM and AM, and there was also a significant difference in phosphorus concentration between ECM and AM+ECM (**Figures 2H,I**). The **Figure 2** showed that the mycorrhizal types affected the concentration of carbon, nitrogen, and phosphorus in the shrub.

Linear mixed effect model analyses confirmed that, the concentration of carbon, nitrogen, and phosphorus in shrub organs varied with site and leafC, leafN, leafP, stemN, and rootN also varied with species. And the significant interactions were observed between the two variables (except stemC) (**Table 1**).

Effect of Ecto-Mycorrhizas and Arbuscular Mycorrhizas on Carbon, Nitrogen and Phosphorus Concentration in Shrub Organs

The carbon concentration in leaves, stems, and roots was 445.65, 457.38, and 444.03 mg/g, respectively, in shrubs where ecto-mycorrhizas were dominant; however, the carbon concentration in leaves, stems, and roots was severally 434.70, 455.45, and 450.25 mg/g, in shrubs with no ecto-mycorrhiza dominant (**Figures 3A–C**). The carbon concentration in the stems and leaves of shrubs with ecto-mycorrhiza was higher than that without ecto-mycorrhiza (**Figures 3A,B**), further the leaf carbon concentration with ecto-mycorrhiza was significantly higher than that without ecto-mycorrhiza (**Figure 3A**). The shrub roots showed the opposite trend, which was, the carbon concentration

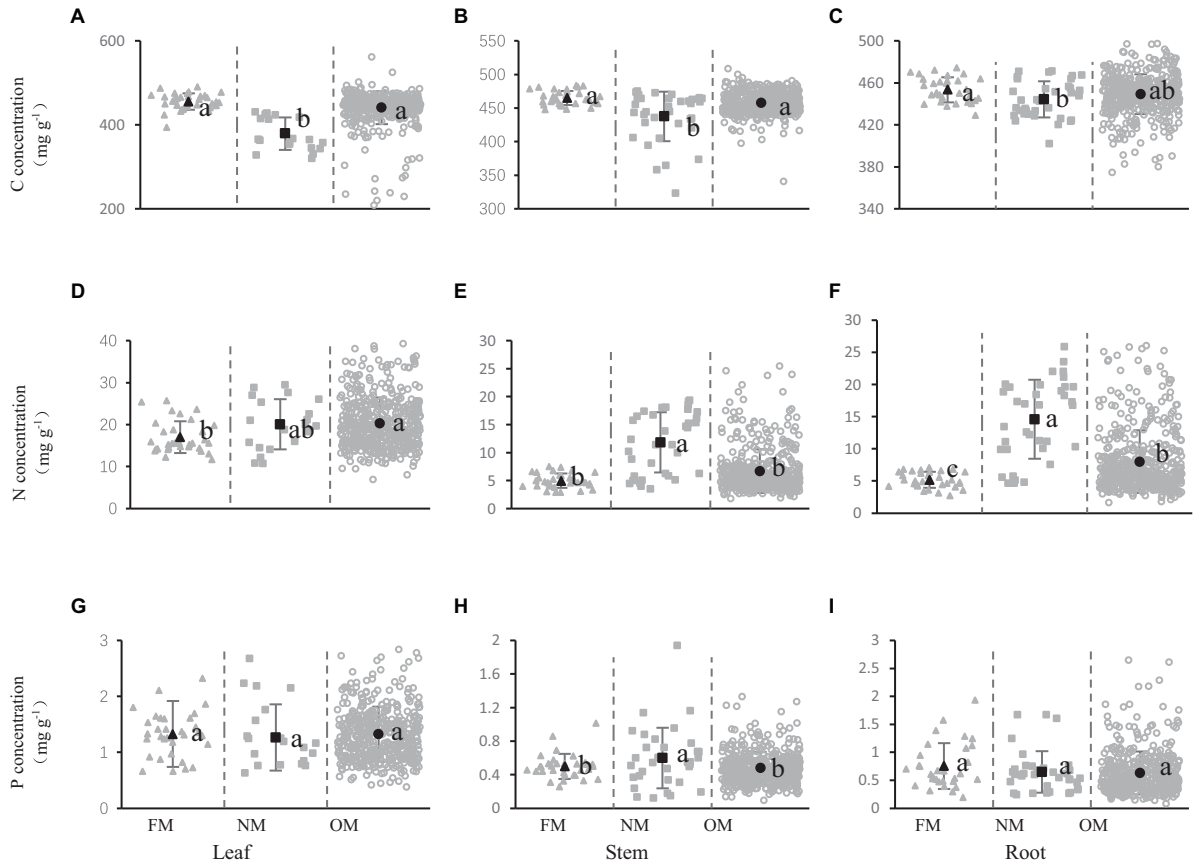

FIGURE 1 | Carbon **(A–C)**, nitrogen **(D–F),** and phosphorus **(G–I)** concentration in shrub organs [leaf **(A,D,G)**, stem **(B,E,H)**, and root **(C,F,I)**] under different mycorrhizal status. The gray solid triangles represent the concentration of the elements in the FM mycorrhizal status, the gray solid squares represent the concentration of the elements in the NM, the gray open cycles represent the C, N, and P concentration of OM. The black dots represent the average value of C, N, and P concentration in this mycorrhizal status. Line bars show 95% confidence intervals. Letters above the line bars show the results of Tukey tests. Slopes with the same letters are not significantly different ($P \geq 0.05$), while those with different letters are significantly different ($P < 0.05$).

without ecto-mycorrhiza was significantly higher than that with ecto-mycorrhiza (**Figure 3C**).

The nitrogen concentration of leaves, stems and roots was 20.29, 7.50, and 9.21 mg/g without ecto-mycorrhiza, while the nitrogen concentration of leaves, stems and roots was 20.00, 5.58, and 6.46 mg/g when there was ecto-mycorrhiza (**Figures 3D–F**). In stems and roots, the nitrogen concentration without ecto-mycorrhizas was significantly higher than that with ecto-mycorrhiza (**Figures 3E,F**). But this significant difference did not appear in the leaves (**Figure 3D**).

Phosphorus concentration in leaves, stems and roots was 1.25, 0.47, and 0.59 mg/g without ecto-mycorrhiza. In contrast, the phosphorus concentrations of leaves, stems and roots was 1.58, 0.58, and 0.93 mg/g when there were ecto-mycorrhizas (**Figures 3G–I**). In the all shrub organs (leaves, stems and roots), the phosphorus concentration in when there was ecto-mycorrhiza was very significantly higher than when there was no ecto-mycorrhiza (**Figures 3G–I**).

Except for ecto-mycorrhizas, arbuscular mycorrhizas also affected the concentration of carbon, nitrogen and phosphorus in different shrub organs. The carbon concentration in leaves, stems and roots under non-AM or AM was 433.86, 451.92, and 444.07 mg/g (non-AM), 436.14, 456.50, and 450.65 mg/g (AM), respectively (**Figures 4A–C**). Carbon concentration of all organs (leaves, stems, and roots) was higher in AM than in non-AM. Further, we found that AM had markedly higher carbon concentration than non-AM in stems (**Figure 3B**), and the carbon concentration exhibited vary significant variation among AM and non-AM in roots (**Figure 3C**).

The nitrogen concentration in leaves, stems and roots was 20.00, 7.31, and 8.80 mg/g in non-AM, respectively. On the contrary, when AM is present, the nitrogen concentration of leaves, stems and roots was 20.67, 7.28, and 8.98 mg/g, respectively (**Figures 3D–F**). The leaves and roots of shrubs showed a tendency of higher nitrogen concentration when there was arbuscular mycorrhiza than when there was no arbuscular mycorrhiza. On the contrary, the stems of shrubs showed a tendency to have a higher nitrogen concentration when there was no arbuscular mycorrhiza than when there was arbuscular mycorrhiza. However, no significant difference was observed in nitrogen concentration in shrub organs (leaf, stem and root) (**Figures 3D–F**).

Phosphorus concentrations in leaves, stems and roots were 1.53, 0.59, and 0.85 mg/g without arbuscular mycorrhiza, and

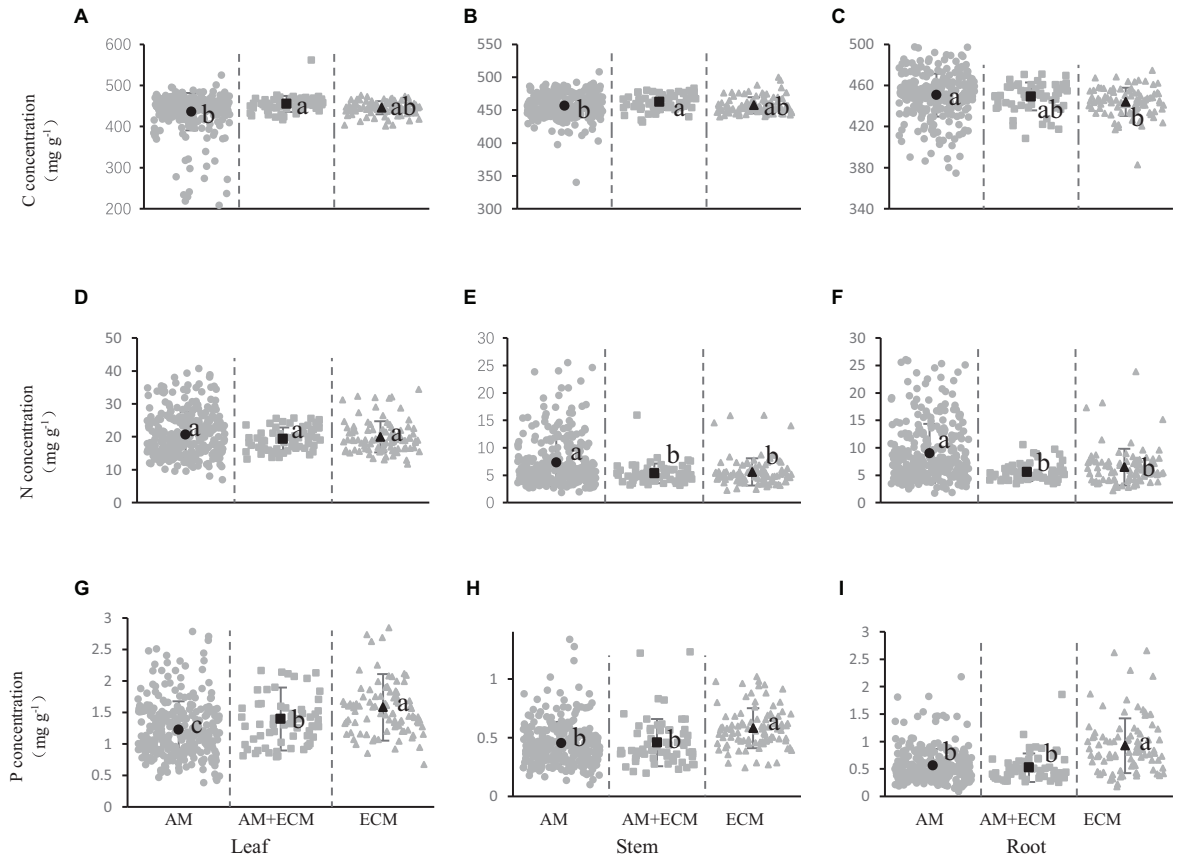

FIGURE 2 | Carbon **(A–C)**, nitrogen **(D–F),** and phosphorus **(G–I)** concentration in shrub organs [leaf **(A,D,G),** stem **(B,E,H)**, and root **(C,F,I)**] under different mycorrhizal types. The gray solid cycles represent the C, N, and P concentration of AM species, the gray solid squares represent the element concentration of AM+ECM plants, and the gray solid triangles represent the element concentration of ECM mycorrhiza. The black dots represent the average value of C, N, and P concentration in this mycorrhizal types. Line bars show 95% confidence intervals. Letters above the line bars show the results of Tukey tests. Slopes with the same letters are not significantly different ($P \geq 0.05$), while those with different letters are significantly different ($P < 0.05$).

TABLE 1 | The effect of site, species, and their interaction on the element concentration in shrub organs based on linear mixed effect models.

Concentration	Site		Species		Site × Species	
	F	*P*	*F*	*P*	*F*	*P*
leafC	7.19	<0.001	40.05	<0.001	10.96	<0.001
leafN	7.36	<0.001	58.94	<0.001	3.63	<0.001
leafP	6.93	<0.001	20.13	<0.001	2.30	<0.001
stemC	4.39	<0.001	0.93	0.33	1.21	0.20
stemN	4.78	<0.001	16.01	<0.001	2.45	<0.001
stemP	2.16	0.003	2.15	0.15	2.56	<0.001
rootC	5.08	<0.001	0.39	0.54	1.48	0.049
rootN	5.42	<0.001	37.02	<0.001	4.22	<0.001
rootP	2.10	0.007	0.76	0.38	1.76	0.008

Site, species and their interaction are fixed effects.
Mycorrhizal type is random effects.
$P < 0.01$ means extremely significant, $P < 0.05$ means significant.

1.23, 0.45, 0.56 mg/g when there was arbuscular mycorrhiza, respectively (**Figure 3G–I**). The leaves, stems and roots of shrubs all showed a tendency of higher phosphorus concentration when there was no arbuscular mycorrhiza than when there was arbuscular mycorrhiza. For the phosphorus concentration, extremely significant difference was observed among the AM and non-AM of all shrub organs (leaves, stems, and roots) (**Figures 3G–I**).

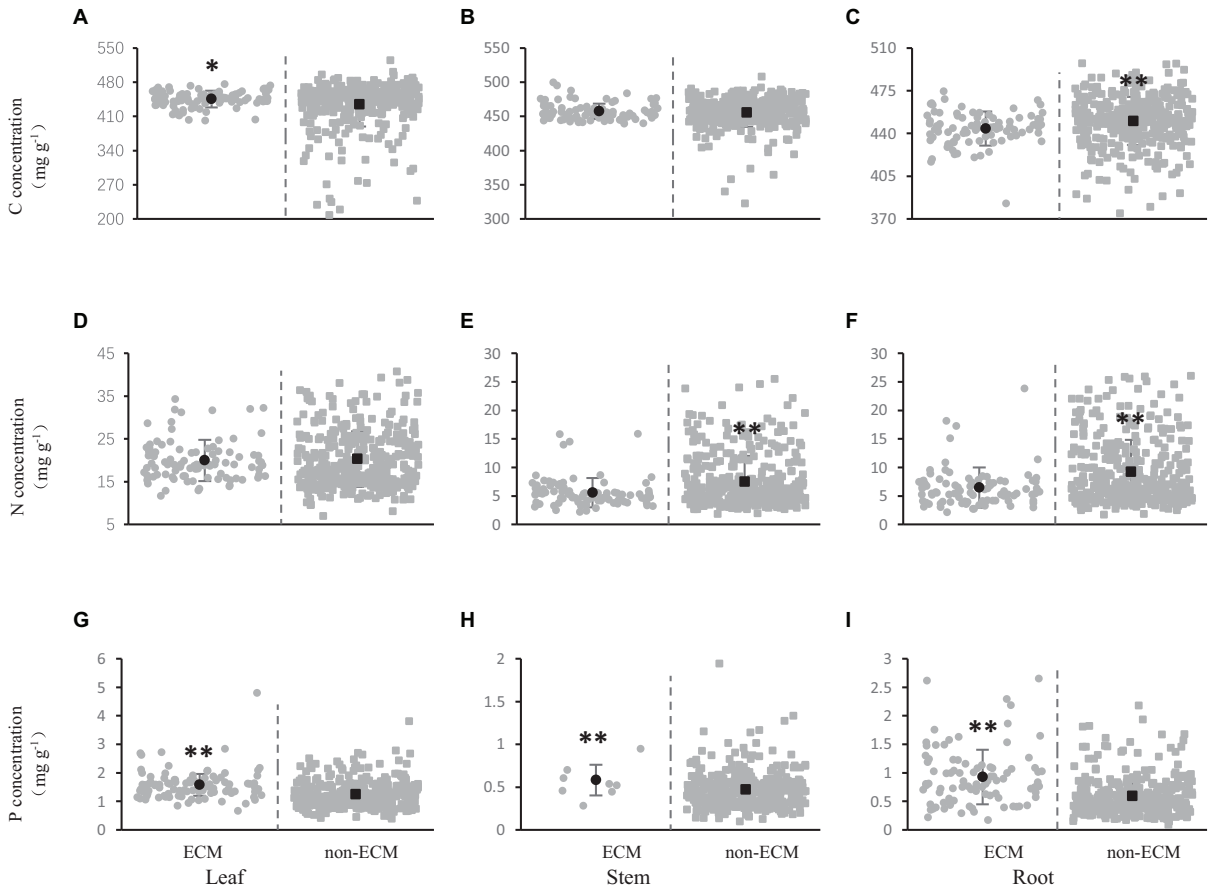

FIGURE 3 | Effect of ecto-mycorrhizas on carbon **(A–C)**, nitrogen **(D–F),** and phosphorus **(G–I)** concentration in shrub organs [leaf **(A,D,G)**, stem **(B,E,H),** and root **(C,F,I)**]. The gray solid circles represent the C, N, and P concentrations of ECM species, and the gray solid squares represent the C, N, and P element concentrations of non-ECM species. The black dots represent the average value of C, N, and P concentration in this mycorrhizal types. The asterisks above the line bars show the results of Tukey tests at the level of $P < 0.05$. Two asterisks indicate extremely significant differences, and one asterisk indicates significant differences.

Figures 1–4 collectively showed that there were higher C, N, and P concentrations in leaves than in stems and roots. Mycorrhiza can promote the accumulation of carbon in shrub organs, and AM has a better promoting effect than ECM (**Figures 1–4**). On the contrary, mycorrhiza was not conducive to the absorption of nitrogen by shrub organs. Even so, plants dominated by AM can absorb more nitrogen than ECM-dominated plants. In addition, the nitrogen concentration of leaves, stems and roots under the AM+ECM treatment was the lowest, which indicated that the co-existence of AM and ECM may have a certain interaction and weaken the absorption of nitrogen by plants (**Figures 1–4**). ECM was significantly better than AM in promoting phosphorus uptake by shrubs (**Figures 2–4**). The contents of C, N, and P in different organs of shrubs were closely related. Generally speaking, plants with high nutrient contents in roots and stems also had higher nutrient concentrations in leaves (**Figures 5**, **6**).

The C, N concentration in shrub organs varied greatly for both AM and non-AM plants, but the ranges were much larger for AM plants (except stem C) (**Supplementary Figures 2–4**). For example, leafC ranged from 208.89 to 525.34 mg/g for AM plants, while from 320.17 to 476.46 mg/g for non-AM plants; leafN ranged from 7.02 to 40.80 mg/g and from 10.84 to 34.35 mg/g for AM and non-AM plants, respectively; stemN from 1.86 to 25.53 mg/g and from 2.19 to 19.40 mg/g, respectively. And the C, N concentration in shrub organs varied greatly for both ECM and non-ECM plants, but the ranges were much larger for non-ECM plants (**Supplementary Figures 5–7**). For example, stemC ranged from 323.24 to 508.60 mg/g for non-ECM plants, while from 439.84 to 500.10 mg/g for ECM plants; stemN ranged from 1.86 to 25.53 mg/g and from 2.19 to 15.92 mg/g for non-ECM and ECM plants, respectively.

The Relationships of Carbon, Nitrogen and Phosphorus Concentration in Shrub Organs Under Different Mycorrhizal Status and Types

Carbon, nitrogen and phosphorus concentration in different shrub organs under different mycorrhizal status or types had different relationships. In the OM and FM species, the stem carbon concentration and leaf carbon concentration increased

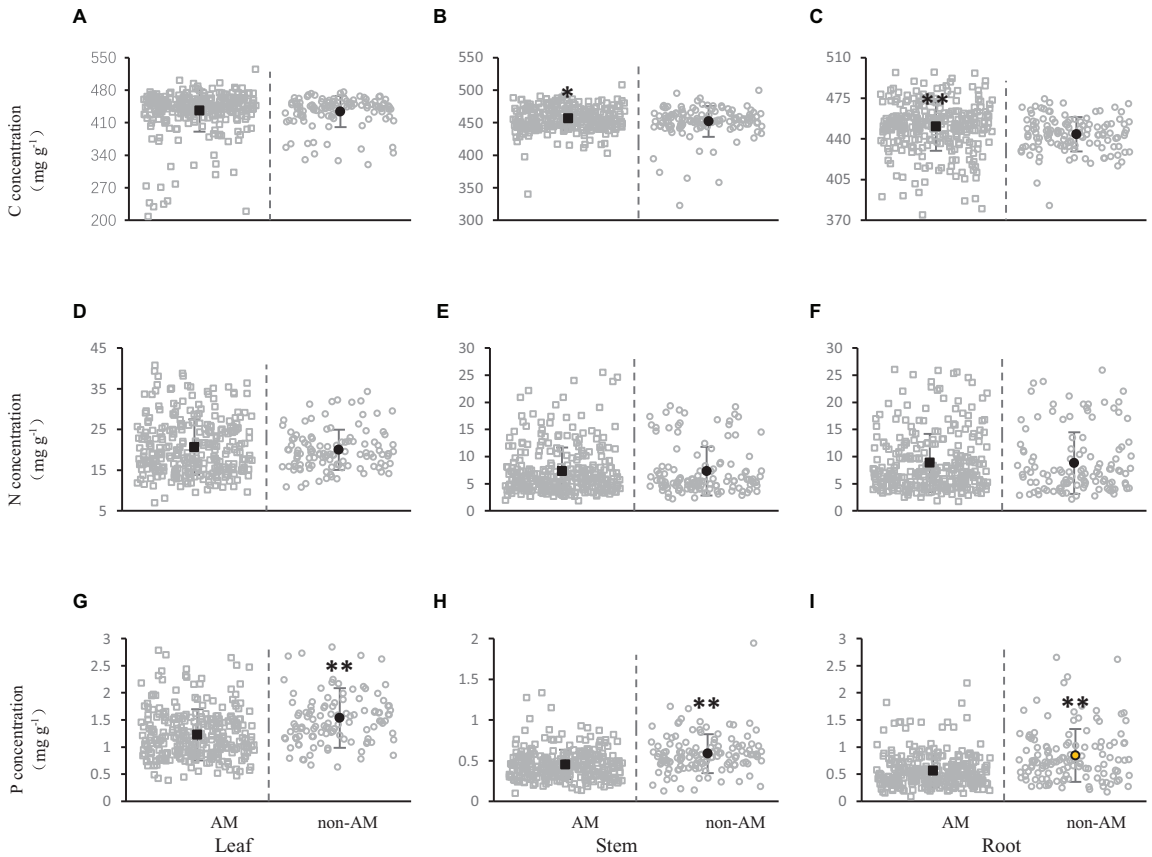

FIGURE 4 | Effect of arbuscular mycorrhizas on carbon **(A,B)**, nitrogen **(D–F)** and phosphorus **(G–I)** concentration in shrub organs [leaf **(A,D,G)**, stem **(B,E,H),** and root **(C,F,I)**]. All gray open squares represent the concentration of C, N, and P in the shrub organs of AM plants, and all gray open circles represent the concentration of elements in non-AM plants. The black dots represent the average value of C, N, and P concentration in this mycorrhizal types. The asterisks above the line bars show the results of Tukey tests at the level of $P < 0.05$. Two asterisks indicate extremely significant differences, and one asterisk indicates significant differences.

significantly with the increase of root carbon concentration (**Figures 5A,B**), leaf carbon concentration increased significantly with the increase of stem carbon concentration (**Figure 5C**). This same pattern were also seen in nitrogen and phosphorus (**Figures 5D–I**). In the NM mycorrhizal status, the stem nitrogen concentration and leaf nitrogen concentration increased significantly with the increase of root nitrogen concentration, stem phosphorus concentration increased with the increase of root phosphorus concentration (**Figures 5D,E,G**).

Among different mycorrhizal types (AM, AM+ECM, and ECM), stem carbon concentration and leaf carbon concentration increased significantly with the increase of root carbon concentration, and leaf carbon concentration increased significantly with the increase of stem carbon concentration. There was the same pattern in nitrogen and phosphorus (**Figure 6**).

DISCUSSION

The significance of this study is to compare the concentration allocation and relationship of C, N, and P in shrub organs under different mycorrhizal statuses or types, in order to provide theoretical basis and data support for seeking the most suitable mycorrhizal status or types of shrubs to accumulate basic nutrient elements.

The analysis results of this study showed that the average carbon concentration in the organs of shrubs in northern China was similar to the reported value of global terrestrial plant carbon concentration (46.4 ± 3.21%, Elser et al., 2000). It is also similar to the carbon concentration of shrubs in southern China (Zhang et al., 2019). It showed that there was little difference in carbon concentration in shrubs between northern and southern China. The average concentration of nitrogen and phosphorus in stems and roots was lower than the reported value of global terrestrial plants (18.3 and 1.42 mg g^{-1}, respectively; Reich and Oleksyn, 2004). This indicated that the concentration of nitrogen and phosphorus was relatively low in the stems and roots of Northern Chinese shrubs. Leaves have higher C, N, and P concentrations than stems and roots. Zhang's results also showed that the concentration of nitrogen and phosphorus in photosynthetic organs (leaves) was higher than in non-photosynthetic organs (stems and roots) (Zhang Q. et al., 2018). This means that the nutrient concentrations in

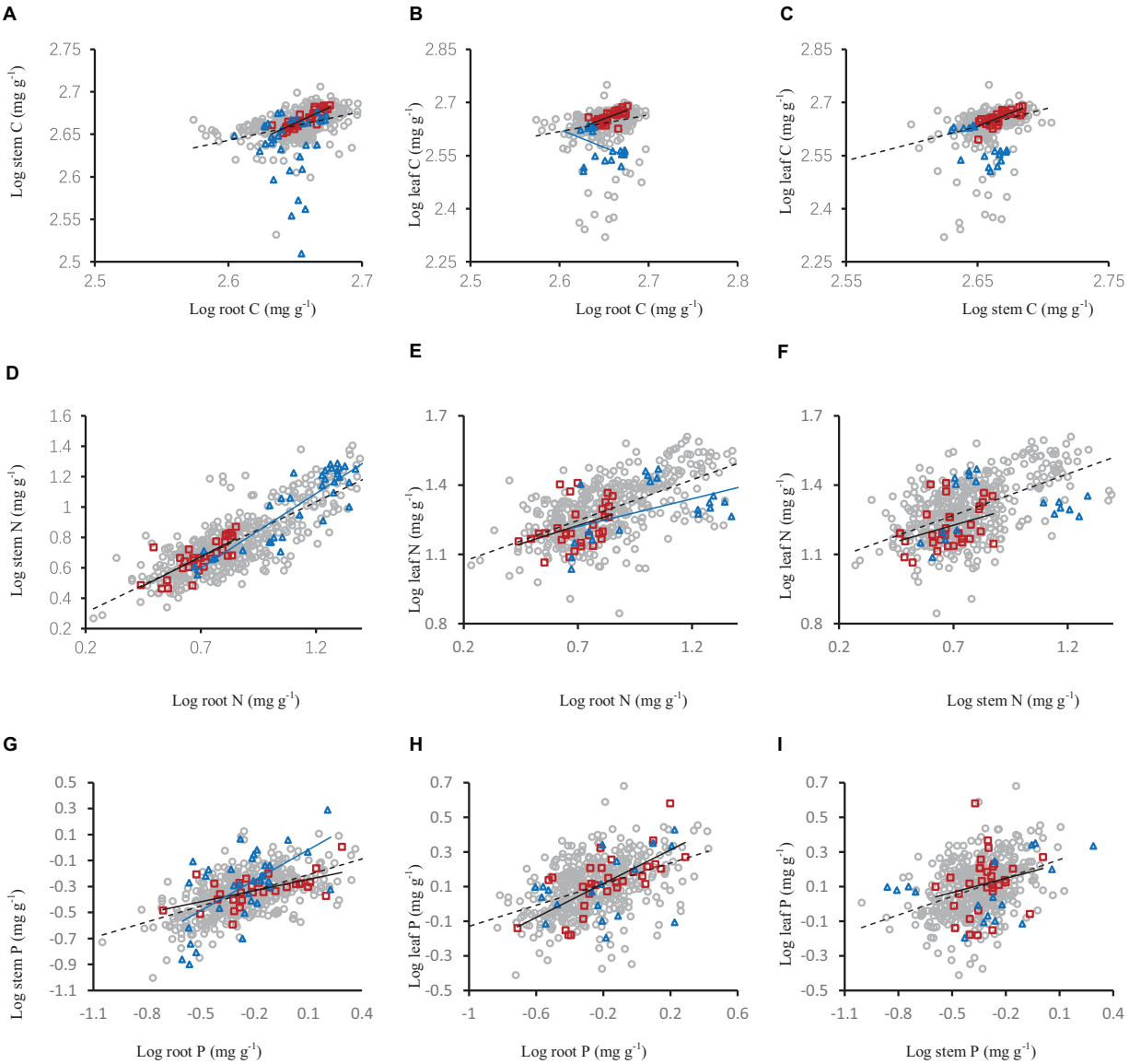

FIGURE 5 | The relationships of carbon **(A–C)**, nitrogen **(D–F)** and phosphorus concentration **(G–I)** in shrub organs (leaf, stem, and root) under different mycorrhizal status. The gray open circle and broken line means OM, red square and black solid line means FM, blue triangle and blue line means NM.

leaves of shrubs are higher than non-leaf organs. There were the same patterns in forests and grasslands (Zhao et al., 2016; Yu et al., 2017). This difference in nutrient distribution among plant organs may be related to leaf function. Leaves were the main organs for photosynthesis, respiration and water use, so they usually require higher nutrient concentrations than non-leaf organs to perform these physiological functions (He et al., 2016). For non-leaf organs such as stems and roots, their function was mainly to absorb and transport nutrient elements rather than metabolize, so they required lower nutrient concentration than leaves (Kerkhoff et al., 2006).

Mycorrhizal fungi are vital members of the plant microbiome and enhance the plant's access to nutrients (Averill et al., 2019). Fungi provide nutrition and protection to plants, and the benefits from mycorrhiza depend on the type of mycorrhiza (Bennett et al., 2017). The mycorrhizal status of plants is one of the most typical underground features, because most terrestrial plants can form mycorrhizal symbiosis (van der Heijden et al., 2015). Whether plants are OM or FM, and which mycorrhizal type they form—AM, ECM or AM+ECM, it is of great significance to the species distribution of plants on the continental scale and their response to environmental gradients (Bueno et al., 2017). In addition, the large-scale model of plant mycorrhizal status can highlight the conditions that determine the importance of mycorrhizal symbiosis in the entire ecosystem (Menzel et al., 2016). ECM and AM are two different types of mycorrhizal fungi, which, respectively, represent the adaptation of plants to low soil nutrient availability

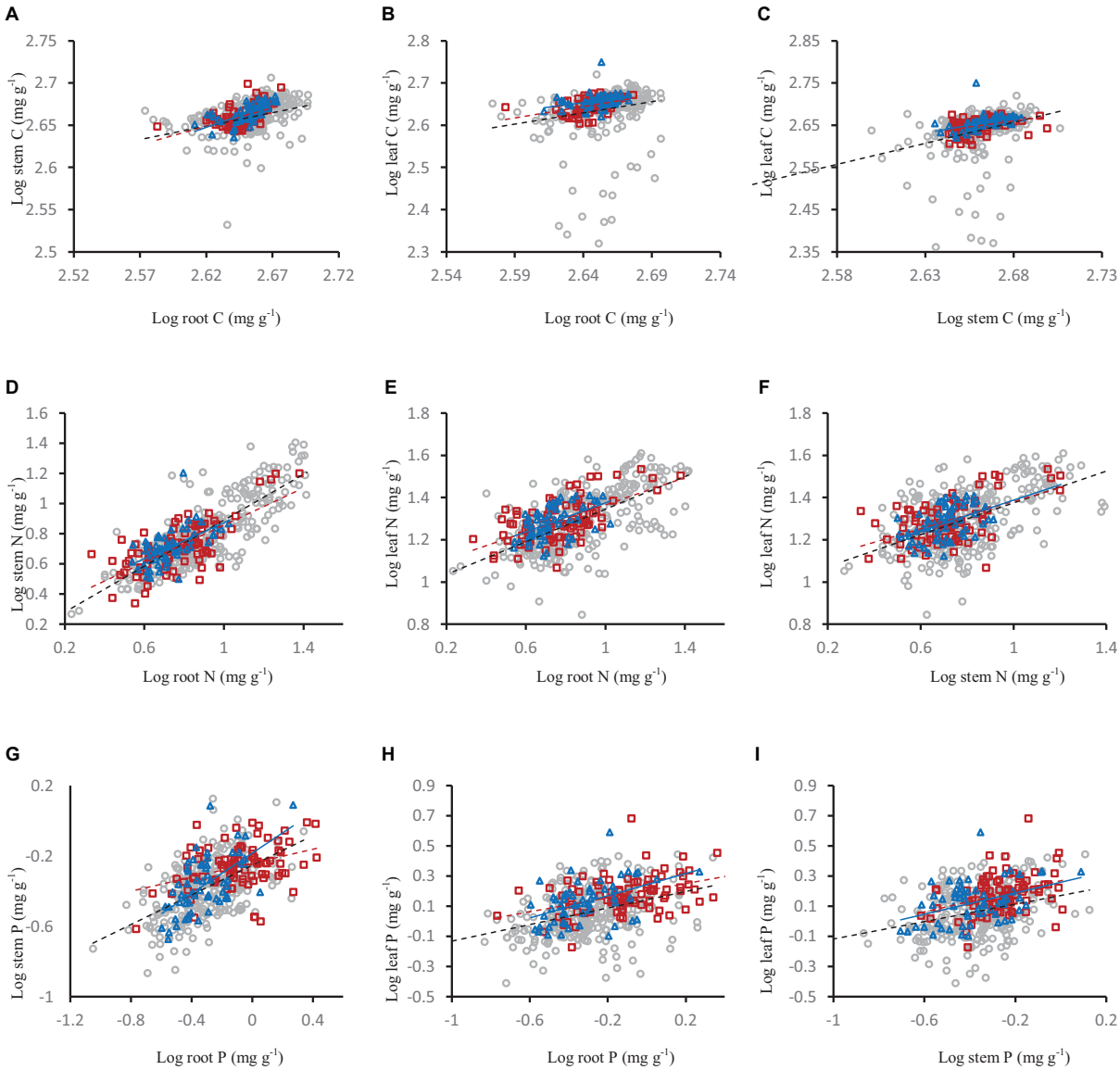

FIGURE 6 | The relationships of carbon **(A–C)**, nitrogen **(D–F)** and phosphorus concentration **(G–I)** in shrub organs (leaf, stem, and root) under different mycorrhizal types. The gray open circle and black broken line means AM, the red square and red broken line means ECM, and the blue triangle and blue line means AM+ECM.

and high soil nutrient availability (Averill et al., 2018). ECM plants were dominant in high latitude ecosystems with slow nutrient cycling, while AM plants were dominant in low latitude ecosystems with rapid nutrient cycling (Zhang H. Y. et al., 2018). In temperate zones, ECM plants are more conservative in their use of nitrogen and phosphorus than AM plants (Averill et al., 2019).

The concentration of carbon, nitrogen, and phosphorus played a crucial role in ecosystem functioning and dynamics (Kerkhoff et al., 2006; Vitousek et al., 2010). Therefore, it is necessary to study the concentration of C, N, and P. The carbon concentration in leaves, stems, and roots was highest under FM and lowest under NM. Moreover, the carbon concentration of FM plants in leaves, stems and roots was significantly the highest. The wider ecological niche and habitat of FM plants helped them cope with unstable and disturbed environments (Gerz et al., 2018). Except for roots, the carbon concentration under FM and OM was significantly higher than that under NM. It showed that the presence of mycorrhiza promote the accumulation of carbon by shrubs. Moreover, the highest carbon concentration in leaves and stems was under AM+ECM, and the highest carbon concentration in roots was under AM. The results showed that AM was better than ECM in promoting the carbon absorption of shrubs. AM plants commonly show better performance due to enhanced uptake of mineral nutrients and improved photosynthetic pigments (Smith and Smith, 2011). AM fungi were beneficial to enhance the shoot biomass and thus contribute to the accumulation of carbon (Chen et al.,

2010). The symbiotic associations between AM fungi and plants can promote nutrient absorption by host plants (Smith and Read, 2008). Mycorrhizal fungal hyphae can obtain mineral nutrients by exploring soil volume and provide these nutrients to plants in return for carbon, so the formation of AM affects the distribution of carbon in plants (Chen et al., 2007; Smith and Read, 2008). Arbuscular mycorrhizal fungi can form a symbiotic associations with approximately 71% of vascular plants in all terrestrial ecosystems (Brundrett, 2017), the data in this article also accounts for more AM mycorrhizas (66.81%). AM-dominated vegetation has higher total plant diversity than ECM-dominated vegetation or NM-dominated vegetation (Aurele et al., 2020). AM can't only help plants absorb nutrients (Bachelot et al., 2016), but can also improve plants' drought, cold and salinity resistance (Wang et al., 2018; Li et al., 2019). Although roots did not affect the absorption of carbon, they affected the transport of carbon. The nitrogen concentration in stems and roots of shrubs was highest under NM, and the nitrogen concentration under NM in stems and roots was significantly higher than that under FM and OM. It showed that mycorrhiza was not good for shrubs to absorb nitrogen, this was due to the increase in plant biomass which dilutes the N (Chen et al., 2010). In stems and roots, when there was mycorrhiza, compared with the other two typical mycorrhizal types (AM+ECM, ECM), the nitrogen concentration under AM was the highest. It showed that although mycorrhizas were not conducive to the absorption of nitrogen by shrubs, plants with AM can absorb more nitrogen than ECM. The result of ECM had lower nitrogen concentrations than AM was in contrast with the result of most previous studies (Zhang H. Y. et al., 2018; Aurele et al., 2020), but it was in line with the result of Averill (Averill et al., 2019). Averill's study showed that nitrogen in both green and senescent leaves varied with mycorrhizal types, and the nitrogen concentration in both of them showed ECM was less than AM (Averill et al., 2019). Plants related to AM mycorrhiza depend on the inorganic nitrogen resources of the soil, while ECM mycorrhiza will degrade and absorb the inorganic nitrogen in the soil (Averill et al., 2019). Previous studies have shown that the response of AM to nitrogen may be related to the availability of soil phosphorus (Johnson et al., 2003). The nitrogen concentrations in leaves, stems and roots were the lowest under AM+ECM, indicating that the co-existence of AM and ECM may produce some interaction to weaken the effect of nitrogen absorption by plants. For instance, as Aurele pointed out, the two main mycorrhizal types (AM and ECM) exhibited different, and often reciprocal, correlation (Aurele et al., 2020). The effect of ECM in promoting the absorption of phosphorus by shrubs was significantly better than that of AM. Many studies had shown that ECM exhibited greater phosphorus absorption relative to AM (Zhang H. Y. et al., 2018; Aurele et al., 2020). The reason why ECM was better than AM under certain conditions was that ECM changes the stoichiometry of the substrate by inhibiting soil nutrient mineralization, making them less available to AM (César et al., 2018). And the ECM generally exhibits positive plant soil feedback, compared with mostly negative feedback in the AM (Bennett et al., 2017), therefore, ECM may promote plant growth and thus facilitate the accumulation of phosphorus in plants. Theoretically, the symbiotic evolution of ECM may lead to the evolution of fungal phosphorus absorption characteristics, and the characteristics of phosphorus acquisition in ECM may be improved after this symbiotic evolution (Kohler et al., 2015). These all validate our first hypothesis that the statuses or types of mycorrhiza affects the allocation of carbon, nitrogen, and phosphorus in different plant organs, and this is consistent with research results of Kerkhoff et al. (2006), Yang et al. (2014), and Zhang Q. et al. (2018). This may be because different mycorrhizal types or statuses have different mechanisms to affect plants. It may also because different plant organs have different absorption mechanisms for nutrient elements, therefore, the allocation of carbon, nitrogen, and phosphorus in plant organs was different under different mycorrhizal statuses or types.

Non-AM plants had a larger range of phosphorus concentrations in leaves, stems, and roots than AM plants. We have observed that the phosphorus concentration in shrub organs (leaf, stem, and root) was extremely significantly higher in non-AM plants than in AM plants. We did further analysis to verify whether this result was related to the higher nutrient concentration in non-AM soil. The result was as expected, the soil nitrogen and phosphorus concentration of non-AM growth was extremely significantly higher than that of AM (**Supplementary Figure 1**). And the Pearson correlation analysis (**Supplementary Table 1**) showed that the phosphorus concentration in leaves, stems, and roots was significantly related to the soil phosphorus concentration. However, we did not find a correlation between stem P, root P and soil N. Research by Yang et al. (2016) also found a positive correlation between leaf P and soil P concentrations. Yang et al. (2016) speculated that this may be due to the fact that in the phosphorus-limited ecosystem [the concentration of leaf phosphorus in Northern China is significantly lower than in other parts of the world (Han et al., 2005)], plants may absorb phosphorus and precipitate it in the form of inorganic phosphorus when phosphorus in soil is abundant (Sterner and Elser, 2002), resulting a positive correlation between phosphorus in plant organs and phosphorus in soil. The mycorrhizal type was determined according to the species in this study. Mycorrhizal types affect the concentrations of carbon, nitrogen, and phosphorus (Zhang H. Y. et al., 2018; Averill et al., 2019), therefore, the concentration of carbon, nitrogen, and phosphorus will also vary with species. Changes in species composition may exacerbate the biogeographic patterns of nitrogen and phosphorus, leading to the species composition hypothesis (Peter and Jacek, 2004). Chagnon showed in his studies that different AM fungal species differ in their carbon requirements from host plants, phosphorus transfer to roots, carbon storage, and relative investment in both extracorrhizal and intracorrhizal biomass (Chagnon et al., 2013). Wang et al. (2015) showed that the concentrations of C, N, and P in stems and roots varied with species. The study by Zhang Q. et al. (2018) showed that there were significant differences in nitrogen and phosphorus concentrations between deciduous and evergreen, legume and non-legume plant species. Yang et al. (2014) also showed that there were significant differences between legumes and

non-legumes. Many studies have shown that the concentration of carbon, nitrogen, and phosphorus varies with latitude (Peter and Jacek, 2004; Fang et al., 2019), these are consistent with our findings that the concentrations of carbon, nitrogen, and phosphorus in plant organs vary with site. This result may be caused by the different soil nutrient status, shrub types and water limitation status in different sites. More importantly, the effects of site and species interactions on carbon, nitrogen, and phosphorus concentrations in plant organs should be further studied.

The carbon, nitrogen, and phosphorus in different organs are interrelated, which has been testified by numerous studies (Zhang Q. et al., 2018). Zhang showed in his research that the content of plant nutrients in different organs was closely related (Zhang Q. et al., 2018). Essentially, leaf nitrogen and phosphorus are able to regulate the rates of carbon acquisition and use (Peter and Jacek, 2004). The allocation of nutrients among organs may be an important part of plant life history strategies. For example, plants with high nutrient content in roots and stems also have higher nutrient concentrations in their leaves. The reason for this phenomenon is that the underground parts of the plant (root) absorbs nutrients and transfer them to the aboveground parts (leaf, stem). The relationship among the different nutrients in plant can predict the changes of nutrient storage. An increase in the concentration and storage of nutrients in one organ will lead to a predictable increase in the concentration and storage of nutrients in other organs (Kerkhoff et al., 2006). In FM and OM species, the correlation between nutrients (C, N, and P) in stems and roots was stronger than that in leaves. This was closely related to the fact that stems and roots were non-photosynthetic organs with similar structures and functions (Fortunel et al., 2012). Furthermore, this suggested that mycorrhiza promoted the correlation between C, N, and P in the shrub organs. What we have observed that the increase in stem nutrients was accompanied by a similar increase in root nutrients agreed with Yang (Yang et al., 2014) and Zhang (Zhang Q. et al., 2018). Regardless of the mycorrhizal statuses or types, the nutrient concentration in the lower organ of the plant usually increases with the nutrient concentration in the adjacent upper organ (for example, root vs. stem, stem vs. leaf). This result was consistent with Brouwer's hypothesis that plant organs compete for nutrients during growth, and the organs closest to the source of nutrients will be the first to obtain nutrients (Brouwer, 1983; Yang and Midmore, 2005). The nutrients will continue to be transported to the distant organs only after the organs closest to the nutrients have used up the nutrients (Yang and Midmore, 2005). Moreover, the mycorrhiza can significantly enhance the correlation between C, N, and P in different organs of shrubs.

In the future, it is still necessary to strengthen research on the effects of mycorrhizal status and types on different plant types, especially research on plant nutrient absorption, which plays an extremely important role in promoting better plant production and development. For future research, it will be quite interesting to compare the results of the study with current views on the mycorrhizal status.

CONCLUSION

The mycorrhiza promoted the accumulation of carbon in shrub organs, but inhibited the absorption of nitrogen. AM was better than ECM in promoting the absorption of carbon by shrub organs. When there were mycorrhizas in plants, compared with ECM mycorrhiza and AM+ECM mycorrhiza, AM mycorrhiza had the most obvious effect in promoting the absorption of nitrogen by shrub organs, while ECM mycorrhiza had the most obvious effect in promoting the absorption of phosphorus by shrub organs. The statuses or types of mycorrhiza were able to change the relationship of carbon, nitrogen, and phosphorus in different organs of plants. In OM and FM mycorrhizal statuses and different mycorrhizal types, nutrient concentration between shrub organs were correlated. The distribution of nutrients between different organs was not independent.

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

SY and ZS: conceptualization, validation, writing—review and editing, and visualization. SY: methodology and writing—original draft preparation. ZS: software, investigation, data curation, supervision, project administration, and funding acquisition. SY, ZS, MZ, and YL: resources. All authors contributed to the article and approved the submitted version.

FUNDING

This research was funded by NSFC (31670499 and 32171620), Scientific and technological research projects in Henan province (192102110128), Program for Science and Technology Innovation Talents in Universities of Henan Province (18HASTIT013), Key Laboratory of Mountain Surface Processes and Ecological Regulation, CAS (20160618).

SUPPLEMENTARY MATERIAL

The Supplementary Material for this article can be found online at: https://www.frontiersin.org/articles/10.3389/fpls.2021.687347/full#supplementary-material

REFERENCES

Akhmetzhanova, A. A., Soudzilovskaia, N. A., Onipchenko, V. G., Cornwell, W. K., Agafonov, V. A., Selivanov, I. A., et al. (2012). A rediscovered treasure: mycorrhizal intensity database for 3000 vascular plants species across the former Soviet Union. *Ecol. Arch.* 93, 689–690. doi: 10.1890/11-1749.1

Aurele, T., Guillermo, B., John, D., Mari, M., Leho, T., Martin, Z., et al. (2020). Asymmetric patterns of global diversity among plants and mycorrhizal fungi. *J. Veget. Sci.* 31, 355–366. doi: 10.1111/jvs.12837

Averill, C., Bhatnagar, J. M., Dietze, M. C., Pearse, W. D., and Kivlin, S. N. (2019). Global imprint of mycorrhizal fungi on whole-plant nutrient economics. *PNAS* 116:23168. doi: 10.1073/pnas.1906655116

Averill, C., Dietze, M. C., and Bhatnagar, J. M. (2018). Continental-scale nitrogen pollution is shifting forest mycorrhizal associations and soil carbon stocks. *Glob. Change Biol.* 24, 4544–4553. doi: 10.1111/gcb.14368

Bachelot, B., Uriarte, M., Mcguire, K. L., Thompson, J., and Zimmerman, J. (2016). Arbuscular mycorrhizal fungal diversity and natural enemies promote coexistence of tropical tree species. *Ecology* 98, 712–720. doi: 10.1002/ecy.1683

Bennett, J. A., Maherali, H., Reinhart, K. O., Lekberg, Y., Hart, M. M., and Klironomos, J. (2017). Plant-soil feedbacks and mycorrhizal type influence temperate forest population dynamics. *Science* 355, 181–184. doi: 10.1126/science.aai8212

Brouwer, R. (1983). Functional equilibrium: sense or nonsense? *Neth. J. Agric. Sci.* 31, 335–348. doi: 10.18174/njas.v31i4.16938

Brundrett, M. C. (2017). Global diversity and importance of mycorrhizal and non-mycorrhizal plants. *Ecol. Stud.* 230, 533–556. doi: 10.1007/978-3-319-56363-3_21

Bueno, C. G., Moora, M., Gerz, M., Davison, J., Öpik, M., Pärtel, M., et al. (2017). Plant mycorrhizal status, but not type, shifts with latitude and elevation in Europe. *Global Ecol. Biogeogr.* 26, 690–699. doi: 10.1111/geb.12582

César, T., Sara, V., Benjamin, D. S., Bruce, A. H., Richard, P. P., Peter, B. R., et al. (2018). Ecosystem responses to elevated CO2 governed by plant–soil interactions and the cost of nitrogen acquisition. *New Phytol.* 217, 507–522. doi: 10.1111/nph.14872

Chagnon, P. L., Bradley, R. L., Maherali, H., and Klironomos, J. N. (2013). A trait-based framework to understand life history of mycorrhizal fungi. *Trends Plant Sci.* 18, 1360–1385. doi: 10.1016/j.tplants.2013.05.001

Chen, B. D., Zhu, Y. G., Duan, J., Xiao, X. Y., and Smith, S. E. (2007). Effects of the arbuscular mycorrhizal fungus Glomus mosseae on growth and metal uptake by four plant species in copper mine tailings. *Environ Pollut.* 147, 374–380. doi: 10.1016/j.envpol.2006.04.027

Chen, M. M., Yin, H. B., Connor, P. O., Wang, Y. S., and Zhu, Y. G. (2010). C: N: P stoichiometry and specific growth rate of clover colonized by arbuscular mycorrhizal fungi. *Plant Soil.* 326, 21–29. doi: 10.1007/s11104-009-9982-4

Craine, J. M., Lee, W. G., Bond, W. J., Williams, R. J., and Johnson, L. C. (2005). Environmental constraints on a global relationship among leaf and root traits of grasses. *Ecology* 86, 12–19. doi: 10.1890/04-1075

Geological Publishing House (2007). *Vegetation Map of the People's Republic of China (151000,000)*. Beijing: Geological Publishing House.

Elser, J. J., Fagan, W. F., Denno, R. F., Folarin, A., Huberty, A., Interlandi, S., et al. (2000). Nutritional constraints in terrestrial and freshwater food webs. *Nature* 408, 578–580. doi: 10.1038/35046058

Fang, Z., Li, D. D., Jiao, F., Yao, J., and Du, H. T. (2019). The latitudinal patterns of leaf and soil C:N:P stoichiometry in the loess plateau of China. *Front. Plant Sci.* 10:85. doi: 10.3389/fpls.2019.00085

Fortunel, C., Fine, P. V., and Baraloto, C. (2012). Leaf, stem and root tissue strategies across 758 Neotropical tree species. *Funct. Ecol.* 26, 1153–1161. doi: 10.1111/j.1365-2435.2012.02020.x

Gerz, M., Bueno, C. G., Ozinga, W. A., Zobel, M., and Moora, M. (2018). Niche differentiation and expansion of plant species are associated with mycorrhizal symbiosis. *J. Ecol.* 106, 254–264. doi: 10.1111/1365-2745.12873

Gong, X. S., Xu, Z. Y., Lu, W., Tian, Y. Q., Liu, Y. H., Wang, Z. X., et al. (2018). Spatial patterns of leaf carbon, nitrogen, and phosphorus stoichiometry of aquatic macrophytes in the arid zone of northwestern China. *Front. Plant Sci.* 9:1398. doi: 10.3389/fpls.2018.01398

Han, W. X., Fang, J. Y., Guo, D. L., and Zhang, Y. (2005). Leaf nitrogen and phosphorus stoichiometry across 753 terrestrial plant species in China. *New Phytol.* 168, 377–385. doi: 10.1111/j.1469-8137.2005.01530.x

He, M., Song, X., Tian, F., Zhang, K., Zhang, Z., Chen, N., et al. (2016). Divergent variations in concentrations of chemical elements among shrub organs in a temperate desert. *Sci. Rep.* 6:20124. doi: 10.1038/srep20124

Jing, H., Zhou, H. X., Wang, G. L., Xue, S., Liu, G. B., and Duan, M. C. (2017). Nitrogen addition changes the stoichiometry and growth rate of different organs in *Pinus tabuliformis* Seedlings. *Front. Plant Sci.* 8:1922. doi: 10.3389/fpls.2017.01922

Johnson, N. C., Wilson, G. W. T., Bowker, M. A., Wilson, J. A., and Miller, R. M. (2010). Resource limitation is a driver of local adaptation in mycorrhizal symbioses. *PNAS* 107, 2093–2098. doi: 10.1073/pnas.0906710107

Johnson, N. C., Rowland, D. L., Corkidi, L., Egerton-Warburton, L. M., and Allen, E. B. (2003). Nitrogen enrichment alters mycorrhizal allocation at five mesic to semiarid grasslands. *Ecology* 84, 1895–1908. doi: 10.1890/0012-9658

Kerkhoff, A. J., Fagan, W. F., Elser, J. J., and Enquist, B. J. (2006). Phylogenetic and growth form variation in the scaling of nitrogen and phosphorus in the seed plants. *Am. Nat.* 168, E103–E122. doi: 10.1086/507879

Koele, N., Dickie, I. A., Jacek, O., Richardson, S. J., and Reich, P. B. (2012). No globally consistent effect of ectomycorrhizal status on foliar traits. *New Phytol.* 196, 845–852. doi: 10.1111/j.1469-8137.2012.04297.x

Kohler, A., Kuo, A., Nagy, L. G., Morin, E., Barry, K. W., Buscot, F., et al. (2015). Convergent losses of decay mechanisms and rapid turnover of symbiosis genes in mycorrhizal mutualists. *Nat. Genet.* 47, 410–415. doi: 10.1038/ng.3223

Kriszta, V., and Matthias, C. (2015). Rillig and stefan hempe. *New Phytol.* 205, 1577–1586. doi: 10.1111/nph.13236

Kubisch, P., Hertel, D., and Leuschner, C. (2016). Fine root productivity and turnover of ectomycorrhizal and arbuscular mycorrhizal tree species in a temperate broad-leaved mixed forest. *Front. Plant Sci.* 7:1233. doi: 10.3389/fpls.2016.01233

LeBauer, D. S., and Treseder, K. K. (2008). Nitrogen limitation of net primary productivity in terrestrial ecosystems is globally distributed. *Ecology* 89, 371–379. doi: 10.1890/06-2057.1

Li, A., Guo, D., Wang, Z., and Liu, H. (2010). Nitrogen and phosphorus allocation in leaves,twigs, and fine roots across 49 temperate, subtropical and tropical tree species: a hierarchical pattern. *Funct. Ecol.* 24, 224–232. doi: 10.1111/j.1365-2435.2009.01603.x

Li, D. W., Xue, L., and Zhang, W. H. (2011). Diversity and distribution of arbuscular mycorrhizal fungi in loess hilly and gully regions. *Forestry Sci.* 47, 116–122.

Li, J. Q., Meng, B., Chai, H., Yang, X. C., Song, W. Z., Li, S. X., et al. (2019). 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

Liu, G. F., Freschet, G. T., Pan, X., Cornelissen, J. H., and Li, Y. (2010). Coordinated variation in leaf and root traits across multiple spatial scales in Chinese semi-arid and arid ecosystems. *New Phytol.* 188, 543–553. doi: 10.1111/j.1469-8137.2010.03388.x

Lü, X. T., Read, S., Yu, Q., He, N. P., Wang, Z. W., and Han, X. G. (2013). Convergent responses of nitrogen and phosphorus resorption to nitrogen inputs in a semiarid grassland. *Global Change Biol.* 19, 2775–2784. doi: 10.1111/gcb.12235

Maarten, V. G., Hans, J., Jan, P., Liina, S., Liis, K., Peeters, G., et al. (2018). Olivier honnayand tobias ceulemans. *New Phytol.* 220, 1262–1272. doi: 10.1111/nph.14947

Menzel, A., Hempel, S., Manceur, A. M., Götzenberger, L., Moora, M., Rillig, M. C., et al. (2016). Distribution patterns of arbuscular mycorrhizal and non-mycorrhizal plant species in Germany. *Perspect. Plant Ecol. Evolu. Syst.* 21, 78–88. doi: 10.1016/j.ppees.2016.06.002

Peter, B. R., and Jacek, O. (2004). Global patterns of plant leaf N and P in relation to temperature and latitude. *PNAS* 101, 11001–11006. doi: 10.1073/pnas.0403588101

Reich, P. B., and Oleksyn, J. (2004). Global patterns of plant leaf N and P in relation to temperature and latitude. *Proc. Natl. Acad. Sci. U.S.A.* 101, 11001–11006.

Shi, Z. Y., Gao, S. C., and Wang, F. Y. (2008). Diversity of arbuscular mycorrhizal fungi in desert ecosystems. *Arid Zone Res.* 6, 783–789. doi: 10.13866/j.azr.2008.06.016

Shi, Z. Y., Li, K., Zhu, X. Y., and Wang, F. Y. (2020). The worldwide leaf economic spectrum traits are closely linked with mycorrhizal traits. *Fungal. Ecol.* 43:100877. doi: 10.1016/j.funeco.2019.100877

Sizonenko, T. A., Dubrovskiy, Y. A., and Novakovskiy, A. B. (2020). Changes in mycorrhizal status and type in plant communities along altitudinal and ecological gradientsa case study from the Northern Urals (Russia). *Mycorrhiza* 30, 445–454. doi: 10.1007/s00572-020-00961-z

Smith, S. E., and Read, D. J. (2008). *Mycorrhizal Symbiosis. Third Edition*. San Diego, CA: Academic Press.

Smith, S. E., and Smith, F. A. (2011). Roles of arbuscular mycorrhizas in plant nutrition and growth: new paradigms from cellular to ecosystem scales. *Ann. Rev. Plant Biol.* 62, 227–250. doi: 10.1146/annurev-arplant-042110-103846

Sterner, R. W., and Elser, J. J. (2002). *Ecological Stoichiometry: The Biology Of Elements From Molecules To The Biosphere*. Princeton, NJ: Princeton University Press.

Tang, Z. Y., Xu, W. T., Zhou, G. Y., Bai, Y. F., Li, J. X., Tang, X. L., et al. (2018). Patterns of plant carbon, nitrogen, and phosphorus concentration in relation to productivity in China's terrestrial ecosystems. *PNAS* 115, 4033–4038. doi: 10.1073/pnas.1700295114

Tarquin, N., Jan, B., Eveline, J., and Krab, M. B. (2021). Biotic interactions with mycorrhizal systems as extended nutrient acquisition strategies shaping forest soil communities and functions. *Basic Appl. Ecol.* 50, 25–42. doi: 10.1016/j.baae.2020.10.002

Treseder, K. K., Allen, E. B., Egerton-Warburton, L. M., Hart, M. M., Klironomos, J. N., Maherali, H., et al. (2018). Arbuscular mycorrhizal fungi as mediators of ecosystem responses to nitrogen deposition: a trait-based predictive framework. *J. Ecol.* 106, 480–489. doi: 10.1111/1365-2745.12919

van der Heijden, M. G. A., Martin, F. M., Selosse, M.-A., and Sanders, I. R. (2015). Mycorrhizal ecology and evolution: the past, the present, and the future. *New Phytol.* 205, 1406–1423. doi: 10.1111/nph.13288

Veresoglou, S. D., and Rillig, M. C. (2012). Suppression of fungal and nematode plant pathogens through arbuscular mycorrhizal fungi. *Biol. Lett.* 8, 214–217. doi: 10.1098/rsbl.2011.0874

Vitousek, P. M., Porder, S., Houlton, B. Z., and Chadwick, O. A. (2010). Terrestrial phosphorus limitation: mechanisms, implications, and nitrogen – phosphorus interactions. *Ecol. Appl.* 20, 5–15. doi: 10.1890/08-0127.1

Vrede, T., Dobberfuhl, D. R., Kooijman, S. A. L. M., and Elser, J. J. (2004). Functional connections among organism C:N:P stoichiometry, macromolecular composition and growth. *Ecology* 85, 1217–1229. doi: 10.1890/02-0249

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, W. Q., Sardans, J., Wang, C., Zeng, C. S., Tong, C., and Asensio, D. (2015). Ecological stoichiometry of C, N, and P of invasive *Phragmites australis* and native *Cyperus malaccensis* species in the Minjiang River tidal estuarine wetlands of China. *Plant Ecol.* 216, 809–822. doi: 10.1007/s11258-015-0469-5

Wang, Y. M., Fan, J. Q., and Shi, Z. Y. (2018). Molecular diversity of arbuscular mycorrhizal fungi in China. *Microbiol. Bull.* 45, 2399–2408. doi: 10.13344/j.microbiol.china.171093

Wassen, M. J., Venterink, H. O., Lapshina, E. D., and Tanneberger, F. (2005). Endangered plants persist under phosphorus limitation. *Nature* 437, 547–550. doi: 10.1038/nature03950

Wu, Q. S., He, J. D., Srivastava, A. K., Zou, Y. N., and Kamil, 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

Wurzburger, N., and Brookshire, E. N. (2017). Experimental evidence that mycorrhizal nitrogen strategies affect soil carbon. *Ecology* 98, 1491–1497. doi: 10.1002/ecy.1827

Yang, X., Chi, X. L., Ji, C. J., Liu, H., Ma, W., Mohhammat, A., et al. (2016). Variations of leaf N and P concentrations in shrubland biomes across northern China: phylogeny, climate, and soil. *Biogeosciences* 13, 4429–4438. doi: 10.5194/bg-13-4429-2016

Yang, X., Tang, Z., Ji, C. J., Liu, H. Y., Ma, W. H., Mohhamot, A., et al. (2014). Scaling of nitrogen and phosphorus across plant organs in shrubland biomes across Northern China. *Sci. Rep.* 4:5448.

Yang, Z., and Midmore, D. J. (2005). Modelling plant resource allocation and growth partitioning in response to environmental heterogeneity. *Ecol. Mod.* 181, 59–77. doi: 10.1016/j.ecolmodel.2004.06.023

Yu, H., Fan, J., Harris, W., and Li, Y. (2017). Relationships between below-ground biomass and foliar N: P stoichiometry along climatic and altitudinal gradients of the Chinese grassland transect. *Plant Ecol.* 218, 661–671. doi: 10.1007/s11258-017-0719-9

Zhang, H. Y., Lue, X. T., Hartmann, H., Keller, A., Han, X. G., Trumbore, S., et al. (2018). Foliar nutrient resorption differs between arbuscular mycorrhizal and ectomycorrhizal trees at local and global scales. *Global Ecol. Biogeogr.* 27, 875–885. doi: 10.1111/geb.12738

Zhang, P., Liu, X., Zhang, X. L., Zhao, W. J., Jing, W. M., and Wang, S. L. (2013). Study on the hydrological and ecological functions of shrubs in the forest-grass composite watershed of Qilian Mountains. *Arid Area Geogr.* 36, 922–929. doi: 10.13826/j.cnki.cn65-1103/x

Zhang, Q., Liu, Q., Yin, H. J., Zhao, C., Zhang, L., Zhou, G., et al. (2019). C:N:P stoichiometry of Ericaceae species in shrubland biomes across Southern China: influences of climate, soil and species identity. *J. Plant Ecol.* 12, 346–357. doi: 10.1093/jpe/rty033

Zhang, Q., Xiong, G. M., Li, J. X., Lu, Z. J., Li, Y. L., Xu, W. T., et al. (2018). Nitrogen and phosphorus concentrations and allocation strategies among shrub organs: the effects of plant growth forms and nitrogen-fixation types. *Plant Soil.* 427, 305–319. doi: 10.1007/s11104-018-3655-0

Zhao, N., Yu, G., He, N., Xia, F., Wang, Q., Wang, R., et al. (2016). Invariant allometric scaling of nitrogen and phosphorus in leaves, stems, and fine roots of woody plants along an altitudinal gradient. *Plant Res.* 129, 647–657. doi: 10.1007/s10265-016-0805-4

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.

Publisher's Note: All claims expressed in this article are solely those of the authors and do not necessarily represent those of their affiliated organizations, or those of the publisher, the editors and the reviewers. Any product that may be evaluated in this article, or claim that may be made by its manufacturer, is not guaranteed or endorsed by the publisher.

frontiers in Plant Science

ORIGINAL RESEARCH
published: 28 October 2021
doi: 10.3389/fpls.2021.742628

Effects of Mycorrhizal Colonization on Transcriptional Expression of the Responsive Factor *JERF3* and Stress-Responsive Genes in Banana Plantlets in Response to Combined Biotic and Abiotic Stresses

Younes M. Rashad[1], Waleed M. E. Fekry[2], Mohamed M. Sleem[1] and Nahla T. Elazab[3]*

[1] Plant Protection and Biomolecular Diagnosis Department, Arid Lands Cultivation Research Institute, City of Scientific Research and Technological Applications (SRTA-City), New Borg El-Arab City, Egypt, [2] Plant Production Department, Arid Lands Cultivation Research Institute, City of Scientific Research and Technological Applications (SRTA-City), Alexandria, Egypt, [3] Botany Department, Faculty of Science, Mansoura University, Mansoura, Egypt

OPEN ACCESS

Edited by:
Jan Jansa, Institute of Microbiology, Academy of Sciences of the Czech Republic (ASCR), Czechia

Reviewed by:
Silvia Proietti, University of Tuscia, Italy
Rupam Kapoor, University of Delhi, India

***Correspondence:**
Younes M. Rashad
yrashad@srtacity.sci.eg

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

Received: *16 July 2021*
Accepted: *30 September 2021*
Published: *28 October 2021*

Citation:
Rashad YM, Fekry WME, Sleem MM and Elazab NT (2021) Effects of Mycorrhizal Colonization on Transcriptional Expression of the Responsive Factor JERF3 and Stress-Responsive Genes in Banana Plantlets in Response to Combined Biotic and Abiotic Stresses. Front. Plant Sci. 12:742628. doi: 10.3389/fpls.2021.742628

Banana plants (*Musa acuminata* L.) are exposed to various biotic and abiotic stresses that affect their production worldwide. Banana plants respond to these stresses, but their responses to combined stresses are unique and differ from those to various individual stresses. This study reported the effects of the mycorrhizal colonization of banana roots and/or infection with root rot on the transcriptional expression of the responsive factor *JERF3* and stress-responsive genes (*POD, PR1*, *CHI*, and *GLU*) under different salinity levels. Different transcriptional levels were recorded in response to the individual, dual, or triple treatments. All the applied biotic and abiotic stresses triggered the transcriptional expression of the tested genes when individually applied, but they showed different influences varying from synergistic to antagonistic when applied in combinations. The salinity stress had the strongest effect when applied in combination with the biotic stress and/or mycorrhizal colonization, especially at high concentrations. Moreover, the salinity level differentially affects the banana responses under combined stresses and/or mycorrhizal colonization in addition, the mycorrhizal colonization of banana plantlets improved their growth, photosynthesis, and nutrient uptake, as well as greatly alleviated the detrimental effects of salt and infection stresses. In general, the obtained results indicated that the responses of banana plantlets under the combined stresses are more complicated and differed from those under the individual stresses depending on the crosstalks between the signaling pathways.

Keywords: arbuscular mycorrhiza, banana, *Fusarium solani*, plant resistance, salinity

INTRODUCTION

Banana (*Musa acuminata* L.) is one of the most important tropical fruit crops worldwide and a major economic commodity for some countries in international trading. In 2019, the global export of bananas was around 24.7 million tons with a total value of USD 13 billion, representing the highest export value among the international trade of fruits. In Egypt, 1.36 million tons of bananas were produced in 2019 for a production area of around 30.4 ha (FAOSTAT, 2021). Moreover, bananas have a high nutritional content of carbohydrates, proteins, potassium (K), calcium, phosphorus (P), nitrogen (N), and vitamins, in addition to their medicinal importance (Ranjha et al., 2020). However, bananas are exposed to diverse biotic and abiotic stresses which affect their production.

Banana root rot, caused by *Fusarium solani* (Mart.) Sacc., is one of the soil-borne diseases that affect crop yield. The disease symptoms include rotting and lesions in the root system that result in reducing the water and nutrient uptake and decreasing plant growth and productivity (El-Nagdi et al., 2015). Various studies were conducted to control banana root rot using chemical fungicides such as benomyl, carbendazim, mancozeb, and prochloraz, and biocontrol agents such as *Streptomyces* spp., *Bacillus* spp., *Pseudomonas* spp., *Trichoderma* spp., and *Saccharomyces* spp. (El-Deeb and El-Naggar, 2008).

Salinity is considered one of the main abiotic stresses threatening global agriculture including bananas, being a limiting factor for plant growth and productivity. The adverse effects of salinity on plant physiology and biochemistry include osmotic stress. This leads to water stress, and ion cytotoxicity stress caused by the high sodium (Na) ion uptake. The high uptake of sodium ions results in a decrease in the uptake of K and calcium ions causing the inhibition of the enzymatic activities and the cellular functions (Isayenkov and Maathuis, 2019). In addition, oxidative stress can also occur due to the production of reactive oxygen species (ROS) that attack plant tissues and DNA (Kamran et al., 2020). In contrast, plants have several defense mechanisms in response to these biotic and abiotic stresses such as the activation of specific ion channels and kinase cascades, and signaling phytohormones such as abscisic acid (ABA), jasmonic acid (JA), salicylic acid (SA), and ethylene (ET) but their efficiency and diversity differ according to the plant susceptibility to those stresses (Ben Rejeb et al., 2014). However, plant responses to combined stress are unique and may be different from their response to individual stresses. In other words, the combined stresses lead to high levels of complexity in the plant responses, which are mediated by different synergistic and/or antagonistic signaling pathways (Suzuki et al., 2014).

The ethylene response factor *JERF3*, which belongs to the *ERF* plant-specific transcription factor family, has significant roles in regulating multiple responsive genes against different biotic and abiotic stresses, as well as plant growth and development genes *via* the JA and ET signaling pathways (Pegoraro et al., 2013). Furthermore, different defensive genes are overexpressed in plants in response to various biotic and abiotic stresses such as the peroxidase gene (*POD*). The *POD* is involved in many developmental and defensive processes and has antioxidant activities by oxidizing the phenolic compounds regulating ROS and the free radicals produced by abiotic and biotic stresses (Shigeto and Tsutsumi, 2016). The antifungal gene *PR1* encodes a protein with antifungal activity and is involved in plant resistance against many fungal pathogens (Breen et al., 2017). The Chitinase gene (*CHI*) encodes the chitinase enzyme that is involved in the plant defense against different biotic and abiotic stresses via the JA-signaling pathway (Zheng et al., 2020). The Glucanase gene (*GLU*) encodes the antifungal enzyme β-1,3-glucanase and is also involved in the plant resistance against different stresses *via* the SA-signaling pathway (Gupta et al., 2013).

Arbuscular mycorrhizal fungi are obligate symbionts, which form mutualistic associations with roots of the majority of terrestrial plants. Various functional roles have been reported for arbuscular mycorrhizal fungi (AMF) colonization of the host plant including growth promotion (Rashad et al., 2020b), enhancement of nutrient uptake (El-Sharkawy et al., 2018), improvement of plant tolerance to abiotic stresses such as salinity and drought (Evelin et al., 2019), and induction of plant defense-responses against different pathogens (Aseel et al., 2019; Rashad et al., 2020a). In this regard, Yano-Melo et al. (2003) found that the colonization of banana plants with AMF, especially *Glomus clarum*, significantly reduced the salt inhibitory effect on the plant growth parameters more effectively if the salinity is $\leq$4.68 dS/m, compared with the non-mycorrhizal plants. Moreover, SuChen et al. (2012) reported a considerable reduction of up to 67% in the incidence of *Fusarium* wilt disease, caused by *F. oxysporum* f. sp. *cubense*, in banana plants colonized with *G. clarum*. However, limited studies have investigated the effects of mycorrhizal colonization on plant responses to combined biotic and abiotic stresses. The present study aimed to investigate effects of mycorrhizal colonization on transcriptional expression of the responsive factor *JERF3* and stress-responsive genes *POD*, *PR1*, *GLU*, and *CHI* in banana plantlets in response to combined and individual stresses of salinity and root rot infection. In addition, effects on the growth, biochemical parameters, and disease development of the stressed banana plantlets were studied.

MATERIALS AND METHODS

Banana Cultivar and Fungal Inocula

Tissue-cultured banana plantlets (*M. acuminata* cv. Grand Nain) were obtained from the Genetic Engineering and Biotechnology Research Institute, University of Sadat City, Egypt. An isolate of the pathogenic fungus *F. solani*, isolated from banana roots exhibiting root rot symptoms, was used in the greenhouse experiment. For the inoculum preparation, the pathogenic fungus was grown in glass flasks containing a mixture of sterilized sand and ground maize grains (1:2 v/v) and incubated at 28°C for 2 weeks.

For the AMF inoculum preparation, a mixture of AMF including *Rhizoglomus clarum* (T.H. Nicolson and N.C. Schenck) Sieverd. G.A. Silva and Oehl, *Funneliformis mosseae* (T.H. Nicolson & Gerd.) C. Walker and A. Schüßler, and *Rhizophagus aggregatus* (N.C. Schenck & G.S. Sm.) C. Walker, in the

equal ratio, was used. The AMF inoculum, which consisted of colonized root pieces and rhizospheric soil containing AMF spores and external mycelia, was propagated in pot cultures using sudangrass, at a 75% colonization index. The cultures were grown for two cycles (each of 4 months) at 25 ± 2°C, 65% humidity, and in a 12 h day /12 h night photoperiod. The sudangrass plants were then harvested and the rhizospheric soil and the colonized roots were used as the AMF inoculum.

Greenhouse Experiment

Pots (30 cm diameter) filled with sterilized soil (sand mixed with clay at a ratio of 1:2 v/v) were used in this experiment. Forty-five-day-old banana plantlets were transplanted in the prepared pots at five plantlets per pot. Two weeks after transplanting, soil infection was done by mixing the pathogen inoculum with the soil upper layer of the pots at 3% (v/v). Half of them were inoculated twice with AMF at the transplanting time (10 g per plantlet) and with a soil drench 30 days after transplantation (15 ml per plantlet) using AMF spore suspension (1×10^6 unit L^{-1}). Solutions with three levels of salinity (0.7, 2.3, and 3.5 dS m^{-1}) were applied. Ten pots were used as replicates for each treatment. All the pots were regularly irrigated as required, arranged in a factorial split–split plot design ($2 \times 3 \times 2$), i.e., two treatments of mycorrhization (+AM, and −AM), three levels of salinity (S0 = 0.7, S1 = 2.3, and S2 = 3.5 dS m^{-1}), and two treatments of infection (+ P, and −P), and kept in the greenhouse at 28/20°C day/night temperature at 70% humidity.

Quantitative Real-Time PCR (qPCR)

Two weeks after the pathogen inoculation (api), three banana plantlets were uprooted, washed under running tap water to remove the soil particles, and the total RNA was extracted from the root system using an RNeasy Mini Kit (Qiagen, Germany) according to the instructions of the manufacturer. The extracted RNA was incubated with DNase for 1 h at 37°C.

For the complementary DNA (cDNA) synthesis, a reverse transcription (RT) -PCR kit (Qiagen, Germany) was used according to the instructions of the manufacturer. The reaction mixture (20 μl) contained 2.5 μl of dNTPs (2.5 mM), 5 μl of 5X-buffer with $MgCl_2$, 4 μl of oligo (dT) primer (20 pmol. μl^{-1}), 0.2 μl of reverse transcriptase enzyme (Omniscript RT, Qiagen, Germany), and 2 μl of RNA. The PCR amplification was performed using a thermal cycler (Promega, Germany), at 42°C for 2 h and 65°C for 20 min.

The qPCR mixture (20 μl) included 1 μl of the template, 12.5 μl of SYBR Green Master Mix (Bioline, Germany), 1 μl of forward primer, 1 μl of reverse primer, and sterile RNase free water. *β*-actin was used as a reference gene (*β*-actin-F 5′-GTGGGCCGCTCTAGGCACCAA-3′, and *β*-actin-R 5′-CTCTTTGATGTCACGCACGATTTC-3′). Five pairs of primers, selected as pathway-reporter genes, were used in this study and are presented in **Table 1**. The reaction was performed using a Rotor-Gene-6000-system (Qiagen, United States) as follows: one cycle at 95°C for 10 min, 40 cycles (95°C for 20 s, 58°C for 25 s, and 72°C for 30 s). For each sample, three biological and three technical replicates were performed. The comparative CT method ($2^{-\Delta\Delta CT}$) was used to analyze the relative mRNA expression levels according to Livak and Schmittgen (2001).

TABLE 1 | Primer sequences of the five genes used in the quantitative real-time PCR (qPCR).

Gene description	Abbrev.	Accession No.	Sequence (5′-3′)
Jasmonate and ethylene-responsive factor 3	*JERF3*-F	AY383630	GCCATTTGCCTT CTCTGCTTC
	JERF3-R		GCAGCAGCATC CTTGTCTGA
Pathogenesis-related-protein 1	*PR1*-F	M69247	ACTTGGCATCCCG AGCACAA
	PR1-R		CTCGGACACCC ACAATTGCA
Chitinase class II	*PR3*-F	U30465	GCGTTGTGGTTCT GGATGACA
	PR3-R		CAGCGGCAGAA TCAGCAACA
β-1,3-glucanase	*PR2*-F	M80604	TTTCGATGCCCT TGTGGATT
	PR2-R		CGGCCAACCACT TTCCGATAC
Peroxidase	*POD*-F	X94943	CCTTGTTGGTGGG CACACAA
	POD-R		GGCCACCAGTG GAGTTGAAA

Plant Growth Evaluation

Four and 10 weeks api, three banana plantlets from each treatment were carefully uprooted, washed with tap water, and evaluated for the shoot and root lengths, shoot and root dry weights, and leaf area. The plant samples were oven-dried at 80°C for 48 h before the constant dry weights were recorded.

Estimation of Nutrients Content

The total N content in the banana leaves was estimated using the modified Kjeldahl method (Peach and Tracy, 1955). The total P content was determined by the spectrophotometric vanadium phosphomolybdate method (Wild, 1988) using a spectrophotometer (Unico 2150-UV, United States). The K and Na estimation (Cottenie et al., 1982) was carried out using a flame photometer (SLE-S-935, India), while chloride (Cl) content was estimated using the potentiometric titration method (Jackson, 2005).

Estimation of Photosynthetic Pigments

Four and 10 weeks api, the photosynthetic pigments (chlorophyll *a*, chlorophyll *b,* and carotenoids) were estimated in banana leaves using the spectrophotometric method as described by Harborne (1984).

Disease Assessment

All the banana plantlets were assessed for root rot disease incidence (DI) and severity (DS) 4 and 10 weeks api. The DS was estimated by rating the root system (primary and lateral roots) rotting and necrosis areas of each banana plantlet according to the modified 6-point grading scale of Carling et al. (1999), where

0 = no damage and 5 = full death using the following equation:

$$\text{DS } (\%) = \frac{\Sigma(ab)}{AK} \times 100$$

Where, a = number of plantlets with the same infection degree, b = infection degree, A = total number of the evaluated banana plantlets, and K = highest infection degree. DI was estimated according to the following equation:

$$\text{DI } (\%) = \frac{\text{Number of infected plantlets}}{\text{Total number of plantlets}} \times 100$$

Estimation of Mycorrhizal Colonization

For each treatment, five banana plantlets were carefully uprooted and washed with tap water, 4 and 10 weeks api to estimate the mycorrhizal colonization. The root system was cut into small pieces (1 cm) and stained with 0.05% trypan blue (Sigma, St. Louis, MO, United States) according to Phillips and Hayman (1970). The mycorrhizal colonization was estimated according to Trouvelot et al. (1986). For each treatment, forty stained segments of banana roots were mounted on glass slides in lactoglycerol and examined using a light microscope (at × 100 and × 400 magnifications) for the estimation of the frequency of root colonization (F, %), the intensity of cortical colonization (I, %), and the frequency of arbuscules (A, %).

Statistical Analyses

The data were statistically analyzed using the software CoStat (v 6.4). Comparisons between the means were made using Duncan's multiple range test at $p \leq 0.05$ (Duncan, 1955).

RESULTS

Transcript Levels of Some Stress-Responsive Genes in Response to Different Stresses

The transcriptional expressions of the responsive factor *JERF3* and four stress-responsive genes in banana roots in response to colonization with AMF and/or infection with root rot under different levels of salinity were investigated (**Figure 1**). For *JERF3*, the data obtained from the qPCR indicated that the gene expression was directly proportional to the salinity level exhibiting the inducing effect of the salinity level on the gene expression. The infection with root rot also triggered the gene expression under the salinity levels S0 and S2, but not under the salinity level S1, where the gene expression was extremely down-regulated. However, the inducing effect of the salinity stress was higher than that of the fungal infection. The colonization with AMF led to a considerable induction in the gene expression under the salinity level S0 (5.5-fold). The gene expression in the mycorrhizal banana roots under the salinity level S1 was lower than in that under S0 and S2 levels. At the same time, the gene expression was highly induced in the mycorrhizal-infected banana plantlets under the salinity level S0 (6.2-fold). The maximum gene expression was recorded in the mycorrhizal-infected banana plantlets under the salinity level S1 (6.9-fold), but the inducing effect was lower in the mycorrhizal banana plantlets infected with root rot under salinity level S2 than the other salinity levels.

For *GLU*, the obtained results indicated that the salinity level directly triggered the gene expression with the maximum triggering effect at salinity level S2 (5.5-fold). The infection with root rot induced the gene expression under different salinity levels but the gene expression was lower under salinity level S1 than under salinity levels S0 and S2. The mycorrhizal colonization led to a high induction in the gene expression under salinity levels S0 and S2, recording the highest gene expression level (6.4-fold) under salinity level S0. Downregulation of the gene expression was observed in the mycorrhizal roots under salinity level S1. The mycorrhizal colonization of the infected banana roots upregulated the gene expression under all the tested salinity levels, compared with the non-mycorrhizal treatments, but the expression level was lower under salinity level S1 than S0 and S2.

For *POD*, the gene expression was upregulated with the increase in the salinity level. The infection with root rot also upregulated the gene expression level under the salinity levels S0 and S2 but downregulated under salinity level S1. The mycorrhizal colonization of banana roots under salinity level S0 highly induced the gene expression, recording the highest gene expression (6.3-fold). The inducing effect was lower in the mycorrhizal roots under salinity level S1 than that under salinity level S2. The mycorrhizal colonization of the infected roots upregulated the gene expression, but the expression level was inversely proportional with the salinity level.

For *PR1*, the gene expression was directly proportional to the salinity level. In this regard, the highest expression was recorded under salinity level S2. The infection with root rot also induced the gene expression under salinity levels S0 and S2, but the gene expression declined under salinity level S1. The mycorrhizal colonization of the banana roots upregulated the gene expression under salinity levels S0 and S2, but not under salinity level S1. The mycorrhizal colonization of the infected roots highly upregulated the gene expression, recording the highest expression level under salinity level S0 (7.5-fold), but this inducing effect lowered with the increase in the salinity level.

For *CHI*, the gene expression was directly proportional to the salinity level. In this regard, the highest expression level was recorded under salinity level S2 (5.4-fold). The infection of the banana roots with root rot upregulated the gene expression under salinity levels S0 and S2, but not under salinity level S1. The mycorrhizal colonization of the banana roots triggered the gene expression under salinity level S0, recording the highest expression level (5.4-fold), higher than under salinity level S2, but not under salinity level S1. The mycorrhizal colonization of the infected roots induced the gene expression under different salinity levels, but the expression level was higher under salinity level S1 than S0 and both of them were higher than that under salinity level S2.

Effects on the Plant Growth

Means of the evaluated growth parameters of banana plantlets in response to the colonization with AMF and/or infection

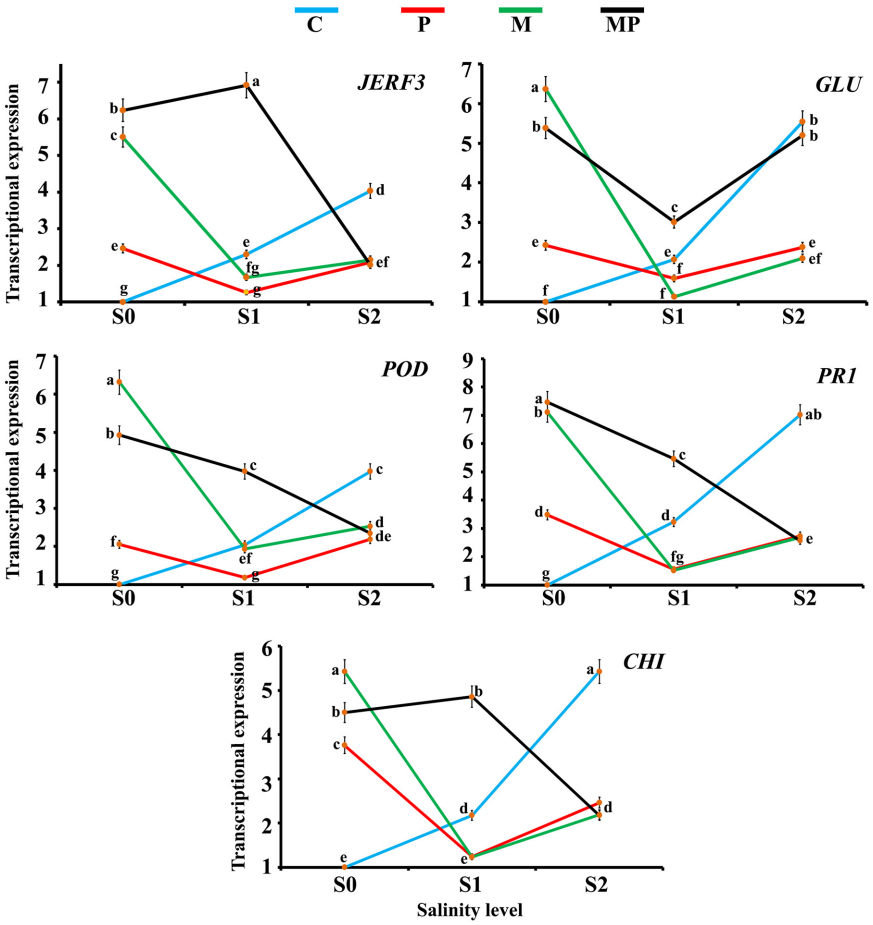

FIGURE 1 | Line charts exhibiting the transcriptional expression levels of five defense-related genes in banana roots in response to mycorrhizal colonization and/or infection with root rot under different levels of salinity. Where, C = non-mycorrhizal-uninfected plantlets, P = non-mycorrhizal-infected plantlets, M = mycorrhizal-uninfected plantlets, and MP = mycorrhizal-infected plantlets. In each subfigure, the treatments marked with the same letter are not significantly different at $p \leq 0.05$ according to Duncan's multiple range test (Duncan, 1955). The bars represent the SE. Each value represents the mean of three biological replicates, each analyzed in triplicate.

with root rot under different levels of salinity are presented in **Table 2**. The data obtained from the greenhouse experiment showed that salinity level reduced all the evaluated growth parameters including the shoot and root system lengths and dry weights. The leaf area of banana plantlets was also reduced. The rate of reduction in these parameters was directly proportional to the salinity level. Although the reduction due to salinity continued from 4 to 10 weeks api, the reduction was more effective with the increase in the plant age. The infection of banana plantlets with root rot negatively affected plant growth. The negative effects due to the infection were more evident at 10 weeks than at 4 weeks api. However, the colonization of banana roots with AMF remarkably enhanced all growth parameters achieving the highest values of these parameters compared with the non-mycorrhizal plantlets, whether at 4 or 10 weeks api. The mycorrhizal banana plantlets exhibited thicker and more vigorous roots with heavier lateral growth than the non-mycorrhizal ones. Moreover, the colonization with AMF significantly alleviated the negative effects due to the salinity stress and/or infection with root rot, when compared with the non-mycorrhizal banana plantlets subjected to any or both of these stresses. However, the mitigation effect of the AMF colonization lowered with the increase in the salinity level and was higher in the individual stresses than the combined stresses. The root system was more affected by the tested treatments than the shoot system, in particular the morphological characteristics. Varied impacts of the different individual and combined stresses on the morphology of banana root system are illustrated in **Figure 2**. The salinity level or infection with root rot negatively affected the thickness, density, and lateral growth of the banana roots. The root colonization with AMF enhanced these morphological characteristics in the unstressed banana plantlets and mitigated the negative effects in case of the stressed ones. However, the morphological effects due to the combined stresses were more severe than due to the individual stresses with the root system becoming thinner, darker in color, and longer than the banana roots under the combined stresses than under the individual stresses.

TABLE 2 | Growth parameters of banana plants in response to colonization with arbuscular mycorrhizal fungi and/or infection with *Fusarium* root rot under different levels of salinity[a].

Weeks after pathogen inoculation	Mycorrhizal status	Salinity level	Infection	Shoot length (cm)	Root length (cm)	Shoot dry wt. (g)	Root dry wt. (g)	Leaf area (cm^2)
4	−M	S0	−P	23.7 ± 3.51	40.3 ± 2.53	1.84 ± 0.36	0.43 ± 0.01	95.1 ± 1.1
			+P	19.7 ± 1.53	33.3 ± 2.23	1.54 ± 0.23	0.40 ± 0.09	91.3 ± 0.8
		S1	−P	21.7 ± 0.97	34.0 ± 3.10	1.65 ± 0.23	0.33 ± 0.07	95.1 ± 1.0
			+P	17.0 ± 1.10	26.3 ± 1.57	0.95 ± 0.06	0.31 ± 0.08	89.3 ± 0.3
		S2	−P	17.0 ± 1.30	23.0 ± 1.80	1.56 ± 0.25	0.24 ± 0.06	77.2 ± 1.4
			+P	13.7 ± 1.53	18.1 ± 2.58	0.91 ± 0.08	0.24 ± 0.05	71.2 ± 1.0
	+M	S0	−P	29.0 ± 2.30	45.7 ± 2.51	2.62 ± 0.37	0.56 ± 0.09	100.9 ± 1.3
			+P	21.7 ± 2.10	40.5 ± 2.30	1.58 ± 0.25	0.47 ± 0.08	94.6 ± 0.8
		S1	−P	23.3 ± 3.40	40.3 ± 3.12	1.67 ± 0.21	0.44 ± 0.08	99.3 ± 0.3
			+P	20.7 ± 1.63	30.1 ± 1.51	1.30 ± 0.31	0.38 ± 0.07	95.0 ± 0.3
		S2	−P	19.0 ± 2.70	25.0 ± 2.31	1.50 ± 0.22	0.35 ± 0.02	88.8 ± 1.4
			+P	15.0 ± 1.50	20.3 ± 1.56	1.22 ± 0.30	0.32 ± 0.04	82.9 ± 0.9
10	−M	S0	−P	32.0 ± 2.07	51.7 ± 3.16	4.01 ± 0.71	0.90 ± 0.05	103.2 ± 0.9
			+P	25.3 ± 2.81	40.3 ± 2.78	3.20 ± 0.63	0.82 ± 0.06	96.4 ± 0.7
		S1	−P	24.2 ± 1.68	40.7 ± 2.50	3.77 ± 0.57	0.75 ± 0.06	100.7 ± 1.0
			+P	21.1 ± 2.01	31.7 ± 1.98	2.52 ± 0.26	0.71 ± 0.02	98.4 ± 1.1
		S2	−P	21.5 ± 1.95	31.8 ± 2.04	3.03 ± 0.55	0.69 ± 0.07	94.5 ± 1.3
			+P	18.7 ± 1.14	25.5 ± 2.63	2.47 ± 0.21	0.65 ± 0.07	85.1 ± 0.9
	+M	S0	−P	38.6 ± 2.32	60.1 ± 3.44	4.58 ± 1.00	1.12 ± 0.11	114.9 ± 1.8
			+P	29.7 ± 1.98	49.3 ± 3.52	3.41 ± 0.81	0.91 ± 0.08	103.4 ± 0.9
		S1	−P	27.2 ± 1.84	47.7 ± 2.74	3.97 ± 0.80	0.87 ± 0.06	104.8 ± 0.7
			+P	23.3 ± 1.79	37.6 ± 2.35	3.07 ± 0.77	0.81 ± 0.05	102.3 ± 0.9
		S2	−P	23.7 ± 1.53	34.2 ± 2.19	3.14 ± 0.63	0.79 ± 0.07	98.3 ± 0.8
			+P	19.6 ± 1.51	27.1 ± 1.99	2.99 ± 0.58	0.70 ± 0.05	90.6 ± 0.6
LSD 0.05				3.68	4.99	0.29	0.07	3.37
Mycorrhiza				*	*	*	*	*
Salinity				***	***	***	***	**
Mycorrhiza × Salinity				*	**	*	**	**
Infection				**	**	**	**	**
Infection × Mycorrhiza				*	*	*	**	**
Infection × Salinity				**	**	**	***	*
Infection × Salinity × Mycorrhiza				**	**	**	***	**

[a]*Values are the means of three replicates ± SD, where, −AM = non-mycorrhizal, +AM = mycorrhizal, S_0 = salinity level of 0.7, S_1 = salinity level of 2.3, S_2 = salinity level of 3.5 dS m^{-1}, −P = non-infected, and +P = infected with Fusarium root rot.*
** = significant at $p < 0.05$, ** = significant at $p < 0.01$, and *** = significant at $p < 0.001$.*

Effects on the Nutrient Content

The mineral nutrient contents in the shoots of banana plantlets in response to the colonization with AMF and/or infection with root rot under different levels of salinity are presented in **Table 3**. The results from the greenhouse experiment showed that the N, P, and K contents declined in the banana leaves with the increase in the salinity level more after 10 than 4 weeks api, compared with the untreated control plantlets. The infection with root rot led to a reduction in these nutrient contents when compared with the uninfected banana plantlets. The Na and Cl contents increased in the banana leaves with the increase in the salinity level and/or due to the infection with root rot. The colonization with AMF increased the banana leaves contents of these two nutrients as well compared with the non-mycorrhizal plantlets, with a higher content after 10 than 4 weeks api. At the same time, the colonization with AMF mitigated the reduction in the N, P, and K contents due to the infection or salinity, and minimized the elevated Na and Cl contents in the banana leaves compared with the untreated control plantlets.

Effects on the Photosynthetic Pigments

The mean contents of the photosynthetic pigments in banana leaves in response to the colonization with AMF and/or infection with root rot under different levels of salinity are presented in **Table 4**. The obtained results indicated that the photosynthetic pigments in banana leaves declined due to the salinity level of the soil. Moreover, infection of banana plantlets with root rot also led to a reduction in the photosynthetic pigments. The lowest contents of Chl *a*, Chl *b*, and carotenoids were recorded for the non-mycorrhizal banana plantlets infected with

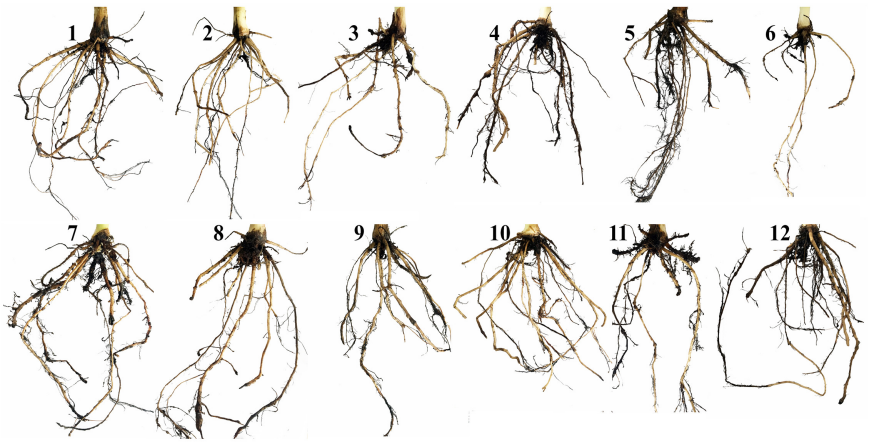

FIGURE 2 | A photograph showing the impacts of the different individual and combined stresses on the morphology of banana root system. Where, 1 = untreated control plantlets under salinity level S0, 2 = plantlets under salinity level S1, 3 = plantlets under salinity level S2, 4 = infected plantlets with root rot under salinity level S0, 5 = infected plantlets with root rot under salinity level S1, 6 = infected plantlets with root rot under salinity level S2, 7 = mycorrhizal plantlets under salinity level S0, 8 = mycorrhizal plantlets under salinity level S1, 9 = mycorrhizal plantlets under salinity level S2, 10 = mycorrhizal-infected plantlets under salinity level S0, 11 = mycorrhizal-infected plantlets under salinity level S1, and 12 = mycorrhizal-infected plantlets under salinity level S2.

root rot under salinity level S1 compared with the untreated control plantlets after 4 and 10 weeks api. In contrast, the colonization of banana roots with AMF significantly enhanced these contents in both harvests when compared with the non-mycorrhizal plantlets. The highest contents of the photosynthetic pigments were recorded for the non-mycorrhizal infected banana plantlets under the salinity level S0 after 4 and 10 weeks api. In addition, the root colonization of the infected plantlets under different salinity levels significantly lowered the negative effects resulting from these stresses compared with the non-mycorrhizal stressed plantlets.

Effects on the Disease Incidence and Severity

The disease incidence and severity of banana plantlets in response to the colonization with AMF, and/or infection with root rot under different levels of salinity are presented in **Table 5**. The results showed that salinity stress increased the DS of the infected banana plantlets compared with the unstressed-infected plantlets, whereas the DI was not affected by the salinity level, recording 100% under the three tested levels. The highest DS was recorded for the infected plantlets under salinity level S1, whether at 4 or 10 weeks api, and the lowest under salinity level S0, with an intermediate DS at salinity level S2. The colonization of the banana roots with AMF reduced both the DS and DI of the infected plantlets under the different salinity levels compared with the non-mycorrhizal plantlets at 4 and 10 weeks api. Among the mycorrhizal-infected plantlets, the banana plantlets infected with root rot under salinity level S1 recorded the highest DS and DI, compared with the other salinity levels.

Effects on the Mycorrhizal Colonization

The mycorrhizal colonization percentages of banana roots in response to the infection with root rot and/or different levels of salinity are presented in **Table 6**. No mycorrhizal colonization was detected in the banana plantlets without the AMF inoculum. In contrast, all the plantlets treated with the AMF showed varied levels of mycorrhizal colonization. The mycorrhizal colonization in the banana roots was examined using a light microscope showing typical mycorrhizal structures (**Figure 3**). The data obtained showed that the mycorrhizal colonization in banana plantlets was inversely proportional to the salinity level. The highest colonization levels were recorded for the uninfected-mycorrhizal banana plantlets under salinity level S0. The infection of banana plantlets with root rot reduced the mycorrhizal colonization in their roots under all tested salinity levels, at 4 and 10 weeks api, compared with the uninfected-mycorrhizal plantlets.

DISCUSSION

Plant responses to different biotic and abiotic stresses have been widely studied (Isayenkov and Maathuis, 2019). In this regard, various stress-responsive mechanisms have been discussed including the induction of signaling molecules, transcription factors, and/or activation of kinase cascades (Ben Rejeb et al., 2014). However, the responsive behaviors of a plant in case of the combined stresses are unique, more complicated, and differ compared with the individual stresses (Suzuki et al., 2014). In this work, effects of the mycorrhizal colonization of banana roots and/or infection with root rot on the transcriptional expression of some stress-responsive genes, selected as marker genes for different signaling pathways, were studied under different levels of salinity. The data obtained in our study revealed that the transcript level of *JERF3* increased in response to the increase in the salinity level. *JERF3* plays a vital role as a transcription activator in the JA and ET-signaling pathways regulating multiple stress-responsive genes through binding to the GCC box in

TABLE 3 | Mineral nutrient percentages in the shoots of banana plants in response to the colonization with arbuscular mycorrhizal fungi and/or infection with *Fusarium* root rot under different levels of salinity[a].

Weeks after pathogen inoculation	Mycorrhizal status	Salinity level	Infection	Nitrogen (%)	Phosphorus (%)	Potassium (%)	Sodium (%)	Chloride (%)
4	−M	S0	−P	2.55 ± 0.32	0.18 ± 0.05	4.27 ± 0.21	0.014 ± 0.001	0.73 ± 0.02
			+P	2.44 ± 0.41	0.14 ± 0.05	3.76 ± 0.12	0.021 ± 0.009	0.78 ± 0.01
		S1	−P	2.45 ± 0.21	0.14 ± 0.06	3.92 ± 0.11	0.072 ± 0.009	0.81 ± 0.02
			+P	2.34 ± 0.42	0.11 ± 0.04	3.53 ± 0.06	0.081 ± 0.004	0.92 ± 0.02
		S2	−P	2.33 ± 0.12	0.12 ± 0.03	3.35 ± 0.05	0.085 ± 0.06	0.93 ± 0.05
			+P	2.18 ± 0.31	0.10 ± 0.05	3.12 ± 0.08	0.094 ± 0.010	1.17 ± 0.09
	+M	S0	−P	3.58 ± 0.65	0.33 ± 0.04	5.30 ± 0.22	0.015 ± 0.009	0.70 ± 0.03
			+P	3.19 ± 0.28	0.24 ± 0.04	4.57 ± 0.16	0.022 ± 0.007	0.86 ± 0.02
		S1	−P	2.93 ± 0.32	0.18 ± 0.01	3.73 ± 0.08	0.138 ± 0.003	0.92 ± 0.04
			+P	2.81 ± 0.93	0.21 ± 0.02	3.41 ± 0.12	0.019 ± 0.007	0.91 ± 0.01
		S2	−P	2.76 ± 0.32	0.19 ± 0.05	3.13 ± 0.14	0.047 ± 0.005	0.94 ± 0.01
			+P	2.45 ± 0.67	0.15 ± 0.03	2.87 ± 0.09	0.089 ± 0.006	0.97 ± 0.02
10	−M	S0	−P	2.59 ± 0.02	0.19 ± 0.05	4.47 ± 0.06	0.016 ± 0.005	0.75 ± 0.05
			+P	2.52 ± 0.01	0.17 ± 0.05	4.20 ± 0.10	0.028 ± 0.006	0.86 ± 0.01
		S1	−P	2.48 ± 0.01	0.15 ± 0.04	4.13 ± 0.12	0.084 ± 0.008	0.98 ± 0.01
			+P	2.47 ± 0.02	0.16 ± 0.03	3.97 ± 0.12	0.087 ± 0.002	0.79 ± 0.03
		S2	−P	2.39 ± 0.03	0.13 ± 0.01	3.67 ± 0.05	0.088 ± 0.003	1.00 ± 0.05
			+P	2.23 ± 0.02	0.12 ± 0.02	3.41 ± 0.13	0.097 ± 0.002	1.21 ± 0.02
	+M	S0	−P	3.77 ± 0.02	0.35 ± 0.01	5.37 ± 0.18	0.015 ± 0.001	0.70 ± 0.02
			+P	3.54 ± 0.01	0.32 ± 0.03	4.87 ± 0.06	0.024 ± 0.003	0.77 ± 0.05
		S1	−P	3.19 ± 0.02	0.27 ± 0.02	4.18 ± 0.05	0.077 ± 0.003	0.82 ± 0.08
			+P	2.95 ± 0.01	0.23 ± 0.03	4.05 ± 0.10	0.074 ± 0.002	0.87 ± 0.06
		S2	−P	2.84 ± 0.02	0.21 ± 0.02	3.97 ± 0.06	0.081 ± 0.002	0.91 ± 0.08
			+P	2.71 ± 0.01	0.19 ± 0.02	3.63 ± 0.04	0.092 ± 0.006	1.02 ± 0.07
LSD 0.05				0.04	0.013	0.077	0.004	0.11
Mycorrhiza				***	***	**	***	*
Salinity				***	***	***	***	*
Mycorrhiza × Salinity				***	***	***	*	*
Infection				***	***	***	***	*
Infection × Mycorrhiza				***	***	**	***	**
Infection × Salinity				ns	ns	**	***	*
Infection × Salinity × Mycorrhiza				***	*	***	***	*

[a]*Values are the means of three replicates ± SD, where, −AM = non-mycorrhizal, +AM = mycorrhizal, S_0 = salinity level of 0.7, S_1 = salinity level of 2.3, S_2 = salinity level of 3.5 dS m^{-1}, −P = non-infected, and +P = infected with Fusarium root rot.*

** = significant at $p < 0.05$, ** = significant at $p < 0.01$, and *** = significant at $p < 0.001$.*

their promoters (Pegoraro et al., 2013). The overexpression of these genes leads to activation and improvement of plant tolerance and resistance to multiple abiotic and biotic stresses (Ku et al., 2018). For example, Zhang et al. (2010) found that the overexpression of *JERF3* in transgenic rice plants improved their tolerance to salinity and drought stresses and significantly increased their soluble sugars and proline contents by triggering some stress-responsive genes, compared with the non-transgenic plants. Our results indicated that *JERF3* was also upregulated by the infection of the banana plantlets with root rot, or mycorrhizal colonization under salinity levels S0 and S2, but not S1. This result is in agreement with those obtained by Zhang et al. (2007) who reported the overexpression of *JERF3* in wheat plants after being infected with *Blumeria graminis*, *F. graminearum*, or *Rhizoctonia cerealis*. *JERF3* was overexpressed by the individual stresses (salinity and infection) but under the combined stresses, the response was different. Under the combined stresses, the plant responses are unpredicted and may differ from those produced under individual stresses because of the interactions and crosstalks between the different signaling pathways they induce, which may be synergistic, antagonistic, or additive. However, one of the most limiting factors affecting plant responses to combined stresses is the intensity of the stress (Ben Rejeb et al., 2014). In our study, the first and superior effect when we combined the abiotic and biotic stresses is exclusively related to the abiotic stress (salinity), because the biotic stimulator (fungal infection or mycorrhizal colonization) requires more time to be established in the plant tissue resulting in delayed plant responses (Sah et al., 2016). Under the abiotic stress, the rapid accumulation of ABA, which is the primary phytohormone concerned with the perception of various abiotic stresses, modulates a set of plant responses

TABLE 4 | Photosynthetic pigments (μg g^{-1} fresh weight) of banana shoots in response to colonization with arbuscular mycorrhizal fungi and/or infection with *Fusarium* root rot under different levels of salinity[a].

Mycorrhizal status	Salinity level	Infection	Weeks after pathogen inoculation					
			4			10		
			Chlorophyll *a*	Chlorophyll *b*	Carotenoids	Chlorophyll *a*	Chlorophyll *b*	Carotenoids
−M	S0	−P	443 ± 2.53	230 ± 2.11	198 ± 1.10	487 ± 2.35	266 ± 1.99	218 ± 2.11
		+P	424 ± 1.51	219 ± 1.51	183 ± 0.95	441 ± 2.41	233 ± 1.74	196 ± 2.03
	S1	−P	436 ± 2.37	224 ± 1.77	175 ± 0.96	465 ± 2.20	241 ± 1.80	181 ± 1.49
		+P	415 ± 3.01	202 ± 1.40	139 ± 1.04	427 ± 1.79	218 ± 1.93	153 ± 1.65
	S2	−P	427 ± 2.66	215 ± 0.97	148 ± 1.21	442 ± 1.91	233 ± 2.03	161 ± 1.30
		+P	420 ± 2.15	204 ± 2.03	141 ± 0.96	431 ± 2.31	224 ± 1.99	150 ± 2.01
+M	S0	−P	486 ± 3.10	259 ± 2.14	210 ± 1.05	512 ± 3.10	284 ± 2.31	244 ± 1.44
		+P	439 ± 2.54	227 ± 1.78	193 ± 0.78	457 ± 2.27	265 ± 1.64	220 ± 1.96
	S1	−P	448 ± 2.78	240 ± 1.32	187 ± 074	481 ± 2.91	272 ± 1.97	208 ± 0.97
		+P	425 ± 1.47	218 ± 1.02	150 ± 1.17	440 ± 3.03	245 ± 2.07	178 ± 0.68
	S2	−P	438 ± 1.85	227 ± 1.05	162 ± 0.86	456 ± 2.69	269 ± 2.31	183 ± 1.01
		+P	430 ± 0.98	221 ± 1.24	158 ± 0.94	449 ± 2.85	251 ± 2.34	175 ± 1.09
LSD 0.05			7.9	6.75	2.31	8.41	9.37	4.08
Mycorrhiza			***	***	***	***	***	***
Salinity			***	***	***	***	***	***
Mycorrhiza × Salinity			***	***	***	***	***	***
Infection			**	**	**	**	**	*
Infection × Mycorrhiza			**	**	**	***	**	**
Infection × Salinity			**	***	**	***	***	**
Infection × Salinity × Mycorrhiza			***	***	**	***	***	***

[a] *Values are the means of three replicates ± SD, where, +AM = mycorrhizal, −AM = non-mycorrhizal, S_0 = salinity level of 0.7, S_1 = salinity level of 2.3, S_2 = salinity level of 3.5 dS m^{-1}, −P = non-infected, and +P = infected with Fusarium root rot.*
** = significant at $p < 0.05$, ** = significant at $p < 0.01$, and *** = significant at $p < 0.001$.*

TABLE 5 | Disease severity and incidence of banana plants in response to colonization with arbuscular mycorrhizal fungi and/or infection with *Fusarium* root rot under different levels of salinity[a].

Mycorrhizal status	Salinity level	Infection	Weeks after pathogen inoculation			
			4		10	
			Disease incidence (%)	Disease severity (%)[b]	Disease incidence (%)	Disease severity (%)
−M	S0	−P	0	0	0	0
		+P	100 [a]	40 [c]	100 [a]	66.7 [c]
	S1	−P	0	0	0	0
		+P	100 [a]	53.3 [a]	100 [a]	80 [a]
	S2	−P	0	0	0	0
		+P	100 [a]	46.7 [b]	100 [a]	73.3 [b]
+M	S0	−P	0	0	0	0
		+P	73.3 [c]	33.3 [d]	75 [c]	46.7 [e]
	S1	−P	0	0	0	0
		+P	80 [b]	46.7 [b]	100 [a]	66.7 [c]
	S2	−P	0	0	0	0
		+P	73.3 [c]	40 [c]	80 [b]	53.3 [d]

[a] *Values are the means of three replicates ± SD, where, +AM = mycorrhizal, −AM = non-mycorrhizal, S_0 = salinity level of 0.7, S_1 = salinity level of 2.3, S_2 = salinity level of 3.5 dS m^{-1}, −P = non-infected, and +P = infected with Fusarium root rot.*
[b] *Disease severity (DS) was estimated according to Carling et al. (1999).*

via the ABA-responsive element-binding protein (AREB)/ ABA-responsive element-binding factor (ABF) transcription factors activating the plant tolerance (Sah et al., 2016). The mechanisms involved include inducing stomatal closure, production of osmoprotectants, and accumulation of nitric oxide (Hewage et al., 2020). In addition, plants synthesize and accumulate ABA in

TABLE 6 | Mycorrhizal colonization of banana roots in response to infection with *Fusarium* root rot and/or different levels of salinity[a].

Mycorrhizal status	Salinity level	Infection	Weeks after pathogen inoculation					
			4			10		
			F (%)	I (%)	A (%)	F (%)	I (%)	A (%)
−M	S0	−P	0	0	0	0	0	0
		+P	0	0	0	0	0	0
	S1	−P	0	0	0	0	0	0
		+P	0	0	0	0	0	0
	S2	−P	0	0	0	0	0	0
		+P	0	0	0	0	0	0
+M	S0	−P	90.5 ± 1.0	51.6 ± 0.5	23.3 ± 0.7	96.9 ± 1.0	67.3 ± 0.6	28.1 ± 1.0
		+P	84.3 ± 06	44.3 ± 0.4	19.8 ± 0.8	89.1 ± 0.3	53.1 ± 0.8	20.2 ± 0.9
	S1	−P	87.4 ± 1.1	48.4 ± 0.8	19.1 ± 0.6	93.1 ± 0.7	61.1 ± 1.0	23.3 ± 0.5
		+P	79.2 ± 0.8	39.1 ± 1.1	16.5 ± 0.3	83.3 ± 03	49.6 ± 0.7	17.7 ± 0.7
	S2	−P	80.5 ± 1.3	40.1 ± 1.0	15.8 ± 0.9	89.1 ± 0.7	51.3 ± 1.1	19.5 ± 0.8
		+P	68.3 ± 0.9	33.7 ± 0.8	10.3 ± 0.4	71.6 ± 1.1	44.3 ± 0.9	14.3 ± 1.0
LSD 0.05			3.25	3.95	2.63	3.41	4.14	3.08
Mycorrhiza			***	***	***	***	***	***
Salinity			***	**	*	***	***	**
Mycorrhiza × Salinity			***	***	**	***	***	***
Infection			***	***	**	***	***	***
Infection × Mycorrhiza			***	**	**	***	**	**
Infection × Salinity			***	***	***	***	***	***
Infection × Salinity × Mycorrhiza			***	***	**	***	***	**

[a] *Values are the means of three replicates ± SD, where, +AM = mycorrhizal, −AM = non-mycorrhizal, S_0 = salinity level of 0.7, S_1 = salinity level of 2.3, S_2 = salinity level of 3.5 dS m^{-1}, −P = non-infected, +P = infected with Fusarium root rot, F = frequency of colonization, I = intensity of colonization, and A = frequency of arbuscules. * = significant at $p < 0.05$, ** = significant at $p < 0.01$, and *** = significant at $p < 0.001$.*

response to biotic stresses such as pathogen attacks which leads to triggering the plant immunity (Bharath et al., 2021). In this concern, the accumulation of ABA may induce stomatal closure to prevent the pathogen entry, elicit defense responses, and/or trigger callose deposition to act as a physical barrier limiting the pathogen attack and spread (Hewage et al., 2020). Biotic stress resistance is also mediated by the phytohormones SA (via the key regulator *NPR1*), JA (*via* the transcription factors *MYC*/*ERF*), and/or ET (via the ethylene-responsive factor *ERF*) (Ku et al., 2018; Backer et al., 2019). However, the role of ABA in plant resistance is more complicated for biotic stresses than for abiotic stresses. The involvement of ABA in the plant immunity against pathogen attacks may often be interlinked with the SA, JA, and ET signaling pathways, which are more dedicated to the plant defense against biotic stresses than ABA (Hewage et al., 2020). Under the combined abiotic and biotic stresses, ABA mostly antagonizes SA, JA, and ET actions elevating plant susceptibility to the pathogen, particularly at the early stages where they are not yet be induced (Nahar et al., 2012). In contrast, ET synergistically regulates the JA-signaling pathway *via* the *ERF*-branch upon the attack of necrotrophic pathogens (Li N. et al., 2019). Moreover, ABA is biosynthesized by the fungal pathogen itself as an effector molecule (Lievens et al., 2017), owing to its inducing effect on the virulence and pathogenicity capabilities of the pathogen such as appressoria formation and spore germination, in addition to their role in hindering the host resistance (Spence et al., 2015). Yazawa et al. (2012) found that minimizing the ABA levels in rice plants increased their resistance to *Magnaporthe oryzae*, the causing agent of rice blast, by impairing the pathogen penetration ability and leading to a reduction in the disease incidence. However, their inducing effect on the plant defense was also reported (Vos et al., 2019). Furthermore, ABA modulates the mycorrhizal establishment by inducing the AMF colonization at low levels but impairs it at the high ABA levels (Charpentier et al., 2014). These opposite roles make ABA a multifaceted molecule that switches from a synergistic to an antagonistic effect, and as a control point between abiotic and biotic stress (Ton et al., 2009). The probable crosstalks between different signaling pathways under the combined stresses are illustrated in **Figure 4**.

Mycorrhizal colonization, which is one of the key factors in the present study, is widely known to trigger JA-dependent plant defense responses to different pathogens (Aseel et al., 2019; Rashad et al., 2020a) and induce plant tolerance to salinity and drought (Evelin et al., 2019). The results obtained in our study showed that the overexpression of *JERF3* due to mycorrhizal colonization and/or fungal infection depended on salinity level. In other words, the limiting effect of the salinity level had superiority over the other stresses during the experiment. The overexpression of *JERF3* obtained by the treatments applied upregulated the expression of other stress-responsive genes enhancing plant tolerance against abiotic and biotic stresses indicating their important role as transcriptional

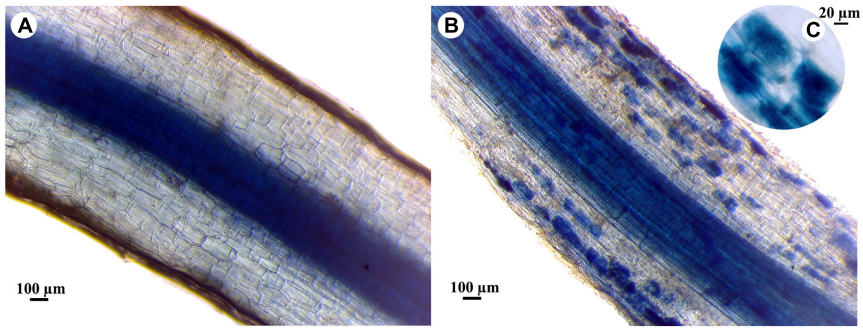

FIGURE 3 | Light micrographs showing the mycorrhizal colonization in banana roots, where, **(A)** non-mycorrhizal root, **(B)** mycorrhizal root, and **(C)** highly magnified image of colonized root showing arbuscules.

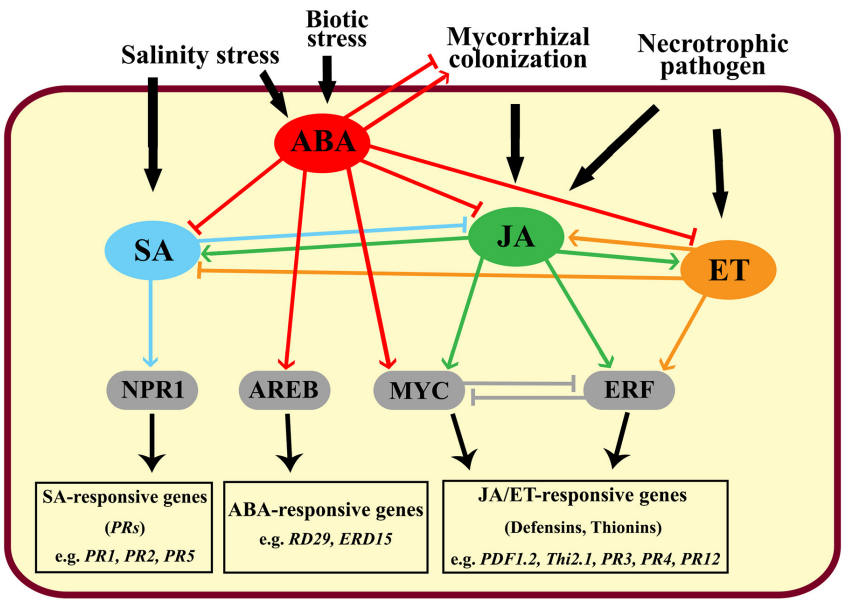

FIGURE 4 | Graphical diagram showing the crosstalks between the different signaling pathways in the plant in response to biotic and abiotic agents, where, SA: salicylic acid, ABA: abscisic acid, JA: jasmonic acid, ET: ethylene, NPR1: non-expressor of pathogenesis-related genes 1, AREB: ABA-response element binding protein, and ERF: ethylene response factor.

regulators in banana plantlets. However, the observation differed under combined stresses compared with individual stresses. The interconnections between the different signaling pathways (ABA, SA, JA, and ET) may regulate *JERF3* expression. Mycorrhizal colonization and necrotrophic infection triggered the JA and ET signaling pathway through the overexpression of *JERF3*, while the salinity stress mainly induced ABA signaling, which antagonized the JA and ET pathways, and downregulated the *JERF3* expression. The overexpression of *JERF3* obtained in this study is in accordance with the one reported by Velivelli et al. (2015) in mycorrhizal potato plantlets.

In this study, the same inducing behaviors of the applied treatments were also recorded for the *POD*, *PR1*, *CHI*, and *GLU* genes. The *POD* encoding gene is a plant-specific oxidoreductase, which is involved in many physiological processes such as lignin polymerization, plant development, resistance to pathogens attack, and tolerance to abiotic stresses. Their antioxidant activity is exerted by oxidizing the phenolic compounds regulating the ROS and free radicals produced due to abiotic and biotic stresses (Shigeto and Tsutsumi, 2016). The induction of *POD* in response to salinity stress, fungal infection, or mycorrhizal colonization has been previously reported (El-Sharkawy et al., 2018; Cai and Gao, 2020; Rashad et al., 2020a). Moreover, the modulation of AMF symbiosis to the antioxidant responses in the salinity-stressed plants was also reported by Evelin and Kapoor (2014). The overexpression of *JERF3* leads to the induction of many pathogenesis-related (PR) genes enhancing the plant resistance to many biotic and abiotic stresses (Ku et al., 2018). This result is in agreement with that obtained in this study regarding the overexpression of the *PR1*, *CHI*, and *GLU* genes. The *PR1* gene encodes a protein with antifungal activity and is involved in plant resistance against many fungal pathogens *via* the SA-signaling pathway (Breen et al., 2017). The *CHI* gene (*PR3*) encodes the chitinase enzyme, which is involved in plant defense against

different fungal infections *via* the JA-signaling pathway. The *CHI* gene catalyzes the degradation of the chitin component in the fungal cell walls by hydrolyzing the β-1,4 bonds between their subunits (Zheng et al., 2020). The *GLU* gene (*PR2*) encodes the antifungal enzyme β-1,3-glucanase, which is involved in the plant resistance to pathogen attack *via* the SA-signaling pathway, by catalyzing the hydrolysis of the β−1,3−glycosidic bond in the 1,3-glucan molecules in fungal cell walls (Gupta et al., 2013). Moreover, the induction of *CHI* and *GLU* under salinity stress has been also reported (Ohnuma et al., 2011; Su et al., 2016). In this regard, most likely, the mechanisms contributing to salt stress tolerance seem to be the inhibition of the Na^+ influx to minimize their toxicity, modulation of the cell wall metabolism to regulate root cross-linking, nutrient translocation, and osmotic changes, as well as releasing ABA from ABA-glucosyl esters (Byrt et al., 2018; Liu et al., 2019).

Our results indicated the triggering effect of AMF symbiosis on the defense responses of banana plantlets against root rot infection, even under salt-stress conditions. However, the salinity level showed a limiting effect under combined stresses, even in mycorrhizal plantlets. The biocontrol effect of mycorrhizal colonization on plant resistance against different pathogens was investigated in diverse sets of crops and pathogens (El-Sharkawy et al., 2018; Aseel et al., 2019). Once the mycorrhizal symbiosis was established in the plant root, a wide-ranging genetic reprogramming proceeded in the plant, resulting in an array of metabolic modulations and leading to an induction of both innate and adaptive plant immunity (Vangelisti et al., 2018). However, the modulation intensity depends on various factors including the arbuscular mycorrhizal fungus, the plant species, the developmental phase of symbiosis, and the surrounding environmental conditions (Jung et al., 2012). Various defense mechanisms have been discussed in this concern including cell wall lignification, the accumulation of polyphenolic compounds and defensive proteins, and the activation of antioxidant and pathogenesis-related enzymes (Abdel-Fattah et al., 2011; Rashad et al., 2020a). In addition, the growth-promoting and nutrient uptake enhancing effects to ameliorate the adverse influences of the infection have also been reported (Campo et al., 2020; Rashad et al., 2020b). On the other hand, the mycorrhizal colonization of the banana plantlets alleviated the adverse effects due to salinity stress. The different mechanisms used by AMF to trigger the salinity tolerance in plants have been discussed, including the production of osmoregulators such as proline, accumulation of antioxidant molecules, and induction of antioxidant enzymes, improvement of water uptake efficiency, and improvement of stomatal conductance (Dastogeer et al., 2020). However, the stress-mitigating effects of AMF depend on many factors such as type of the host plant, species of mycorrhizal fungus, duration and stage of colonization, environmental conditions, and stresses level, and complex interactions. In our study, mycorrhizal colonization alleviated the adverse effects of salinity and infection to some extent depending on the salinity level and its combination with the infection stress. Our results showed the enhancing effect of mycorrhizal colonization on the growth of banana plantlets under different salinity levels. The growth-promoting effect of AMF has been extensively reported in various crops (El-Sharkawy et al., 2018; Rashad et al., 2020a). The inducement of various plant growth regulators such as auxins, cytokinins, and gibberellins during root mycorrhizal colonization has been reported (Martín-Rodríguez et al., 2016). In addition, the enhancement of plant photosynthesis, cell metabolism, and water and nutrient acquisition by AMF symbiosis has been also reported (Iqbal et al., 2021), which is in agreement with our results on the enhancement of the photosynthetic pigments and nutrient contents in banana plantlets by mycorrhizal colonization.

The nutrient content of the plant affects, positively or negatively, its immunity against invading pathogens. Different physiological functions for the plant nutrients have been discussed. For example, silicon has an inducing effect on cell wall lignification and deposition of antimicrobial phenolic compounds and phytoalexins at the infection sites. These defensive mechanisms limit the fungal spread and inhibit its ability to form haustoria (Van Bockhaven et al., 2013). Calcium has a key role in pathogen recognition, cell wall stability, and inhibition of polygalacturonase enzyme. This enzyme is secreted by the pathogen as a virulence factor during the penetration stage (Dordas, 2008). In our study, the effects of different studied stresses on the contents of the three main essential nutrients in plants (N, P, and K) were investigated. In addition, the contents of Na and Cl, the main constituent ions in saline soil, were also studied. Nitrogen is one of the most important essential nutrients in plants, being the main constituent of many vital structural and functional compounds in plants such as nucleic acids, proteins, and enzymes. Moreover, it is considered the limiting nutrient for plant growth and productivity (Liu et al., 2018). Phosphorus is a major essential nutrient for plant growth. It has multiple vital functions in plants including energy transfer, photosynthetic efficiency, nutrient transfer, and genetic traits transfer to the next generations (Malhotra et al., 2018). Potassium is one of the most important essential nutrients and has many functional roles in plant metabolism and development. It acts as an enzyme activator in various physiological functions including protein synthesis, N metabolism, glucose transportation, and cell elongation. Moreover, it has vital roles in the regulation of stomatal conductance, cellular osmotic pressure, and electrolyte balance (Hu et al., 2016). In contrast, despite the fact that Na and Cl are considered micronutrients for plants, they have toxic effects on plant growth at their high concentrations. Under salinity conditions, they reduce soil water uptake, increase osmotic stress, reduce cellular ion imbalance, cause ion toxicity, and inhibit enzymatic activities and cellular functions (Isayenkov and Maathuis, 2019). In addition, oxidative stress can also occur due to the production of ROS that attacks plant tissues and DNA (Kamran et al., 2020). In general, any disturbance in the plant nutrient content, whether deficiency or oversupply, may affect plant development, productivity, stress tolerance, and immunity. Our results indicated the decline in the N, P, K contents in non-mycorrhizal banana plantlets due to infection with *F. solani*. During the infection process, the nutrition acquisition by the pathogen plays a vital role in establishing a successful infection and effective spreading. Once the necrotrophic fungus penetrates the plant cell, it produces toxins and degrading enzymes

causing the lysis of the host cell. This makes the cell nutrients available to the pathogen by direct absorption or transporters (Divon and Fluhr, 2007). In addition, the adverse effects due to the infection extend to include the destruction of the root morphology and physiology leading to a reduction in the water and nutrients uptake from soil. Our results showed that the N, P, K contents in banana leaves were adversely affected under salinity conditions due to the reduced soil nutrient availability, plant uptake efficiency, and nutrients translocation inside the plant. Whereas, the Na and Cl contents increased in the banana leaves with the increase in soil salinity. The imbalance in the nutrient contents and disturbance in the osmotic potential due to salinity stress led to growth retardation in the banana plantlets. In contrast, the mycorrhizal colonization of the banana plantlets enhanced the N, P, K contents in their leaves. This is due to the enhancing effect of AMF on nutrients uptake *via* their extraradical mycelium network, which explores larger soil areas. Moreover, AMF produces organic acids and enzymes such as phosphatases in the soil, which facilitate the nutrients released from the soil, and enhance their availability to the plant (Saia et al., 2014; Iqbal et al., 2021). Furthermore, the mycorrhizal colonization of the banana plantlets alleviated the adverse effects due to the infection and/or salinity stresses, compensated for the nutrient deficiency due to both stresses, and maintained the ionic homeostasis, which led to the enhancing effect on the banana growth. The enhancement of the plant nutrient uptake by the mycorrhizal colonization makes the plant more vigorous, and more resistant to stresses. In addition, as we mentioned before, the mycorrhizal colonization of the banana plantlets induced plant resistance against the infection with root rot and salinity stress *via* multiple defensive mechanisms. These mechanisms reduced the adverse effects due to the applied stresses.

Our results showed that the infection of the banana plantlets with necrotrophic pathogens, and/or exposure to salinity stress led to a reduction in their photosynthetic pigments content. This may be attributed to the reduction in water uptake and transport due to root damage. The water deficiency induces stomatal closure to minimize the water loss, which leads to a reduction in the carbon dioxide uptake and the photosynthesis process (Walters, 2015). Carbon starvation is another proposed mechanism during necrotrophic fungal infection. Upon infection, downregulation of the genes encoding carbon and starch metabolism occurs, which leads to a reduction in carbon assimilation and transport (Li P. et al., 2019). The mycorrhizal colonization of banana plantlets alleviates the adverse effects due to the infection and salt stresses on photosynthesis. The mechanisms used by AMF to alleviate these detrimental effects most likely include the improvement of the photosynthesis and gas exchange capacities, enhancement of the content of the photosynthetic pigment, amelioration of water and nutrient absorption and use efficiency, especially phosphorous, improvement in Na^+ exclusion capacity, and induction of antioxidant activities (Romero-Munar et al., 2019). Triggering salinity tolerance in different plants by AMF colonization has been widely studied (Evelin et al., 2019; Li et al., 2020).

One of the adverse effects of salinity stress on plants is the alteration of their root morphology. In this regard, the thinning, elongation, and discoloration of banana roots were observed under salt stress. This result is consistent with that of Arif et al. (2019) who reported up to 91% increase in the length and density of root hairs of rapeseed plants under salt stress. The banana plantlets tended to adapt to the salinity stress to decrease its negative effects. Root elongation under saline conditions is an adaptive mechanism to increase the nutrient uptake ability. On the other hand, the infection of the banana plantlets with *F. solani* led to morphological alterations in the infected roots causing necrotic lesions, pigmentation, thinning, and weakness of the root, which affect the plant growth. In contrast, mycorrhizal colonization induced some morphological alterations as adaptive mechanisms in banana roots under salinity stress and/or infection. These alterations include an increase in the root volume and lateral branching to maximize the absorptive surface area and hence uptake more water and nutrients to compensate for their deficiency due to salt stress. The thickening of the root system is another adaptive morphological alteration to decrease both Na^+ influxes into the root tissue as well as the outflow of water and nutrients outside the root tissue (Sheng et al., 2009). In addition, root thickening forms a physical barrier against infection penetration.

In conclusion, the present study indicated that the mycorrhizal colonization of the banana plantlets triggered, at varying degrees, the transcriptional expression of the stress-responsive genes *JERF3*, *POD*, *PR1*, *CHI*, and *GLU*, which mediate different signaling pathways, mainly JA. However, their inducing effect was significantly affected by the salinity level at which the symbiosis and/or infection occurred. In addition, the mycorrhizal colonization of the banana plantlets improved their growth, photosynthesis, and nutrient uptake. Moreover, the mycorrhizal colonization greatly alleviated the detrimental effects of salt and infection stresses on banana plantlets despite their negative influences on the colonization level. In general, our results indicated that the responses of banana plantlets under combined stresses are differed from those under individual stresses, and are differentially affected by the level of salinity stress.

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

YR conceived the idea and the design of the work, contributed to the implementation of the greenhouse experiment, mycorrhizal estimation, molecular investigation, and helped in writing the manuscript and photo editing. WF contributed to the design of the work, greenhouse experiments, and nutrient analyses.

MS contributed to the photosynthesis determination and molecular investigation. NE contributed to the greenhouse experiment and statistical analyses. All authors revised and approved the final manuscript.

REFERENCES

Abdel-Fattah, G. M., El-Haddad, S. A., Hafez, E. E., and Rashad, Y. M. (2011). Induction of defense responses in common bean plants by arbuscular mycorrhizal fungi. *Microbiol. Res.* 166, 268–281. doi: 10.1016/j.micres.2010.04.004

Arif, M. R., Islam, M. T., and Robin, A. (2019). Salinity stress alters root morphology and root hair traits in *Brassica napus*. *Plants* (Basel) 8:192. doi: 10.3390/plants8070192

Aseel, D. G., Rashad, Y. M., and Hammad, S. M. (2019). Arbuscular mycorrhizal fungi trigger transcriptional expression of flavonoid and chlorogenic acid biosynthetic pathways genes in tomato against tomato mosaic virus. *Sci. Rep.* 9:9692. doi: 10.1038/s41598-019-46281-x

Backer, R., Naidoo, S., and van den Berg, N. (2019). The NONEXPRESSOR OF PATHOGENESIS-RELATED GENES 1 (NPR1) and related family: Mechanistic insights in plant disease resistance. *Front. Plant Sci.* 10:102. doi: 10.3389/fpls.2019.00102

Ben Rejeb, I., Pastor, V., and Mauch-Mani, B. (2014). Plant responses to simultaneous biotic and abiotic stress: Molecular mechanisms. *Plants* 3, 458–475. doi: 10.3390/plants3040458

Bharath, P., Gahir, S., and Raghavendra, A. S. (2021). Abscisic acid-induced stomatal closure: an important component of plant defense against abiotic and biotic stress. *Front. Plant Sci.* 12:615114. doi: 10.3389/fpls.2021.615114

Breen, S., Williams, S. J., Outram, M., Kobe, B., and Solomon, P. S. (2017). Emerging Insights into the Functions of Pathogenesis-Related Protein 1. *Trends Plant Sci.* 22, 871–879. doi: 10.1016/j.tplants.2017.06.013

Byrt, C. S., Munns, R., Burton, R. A., Gilliham, M., and Wege, S. (2018). Root cell wall solutions for crop plants in saline soils. *Plant Sci.* 269, 47–55. doi: 10.1016/j.plantsci.2017.12.012

Cai, Z.-Q., and Gao, Q. (2020). Comparative physiological and biochemical mechanisms of salt tolerance in five contrasting highland quinoa cultivars. *BMC Plant Biol.* 20:70. doi: 10.1186/s12870-020-2279-8

Campo, S., Martín-Cardoso, H., Olivé, M., Pla, E., Catala-Forner, M., Martínez-Eixarch, M., et al. (2020). Effect of root colonization by arbuscular mycorrhizal fungi on growth, productivity and blast resistance in rice. *Rice* 13:42. doi: 10.1186/s12284-020-00402-7

Carling, D. E., Pope, E. J., Brainard, K. A., and Carter, D. A. (1999). Characterization of mycorrhizal isolates of Rhizoctonia solani from an orchid, including AG-12, a new anastomosis group. *Phytopathology* 89, 942–946. doi: 10.1094/PHYTO.1999.89.10.942

Charpentier, M., Sun, J., Wen, J., Mysore, K. S., and Oldroyd, G. E. D. (2014). Abscisic acid promotion of arbuscular mycorrhizal colonization requires a component of the PROTEIN PHOSPHATASE 2A complex. *Plant Physiol.* 166, 2077–2090. doi: 10.1104/pp.114.246371

Cottenie, A., Verlo, M., Kjekens, L., and Camerlynch, R. (1982). Chemical analysis of plant and soil. *Lab. Anal. Agrochem.* 42, 280–284.

Dastogeer, K. M. G., Zahan, M. I., Tahjib-Ul-Arif, M., Akter, M. A., 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

Divon, H. H., and Fluhr, R. (2007). Nutrition acquisition strategies during fungal infection of plants. *FEMS Microbiol. Lett.* 266, 65–74. doi: 10.1111/j.1574-6968.2006.00504.x

Dordas, C. (2008). Role of nutrients in controlling plant diseases in sustainable agriculture. A review. *Agron. Sustain. Dev.* 28, 33–46. doi: 10.1051/agro:2007051

Duncan, D. B. (1955). Multiple range and multiple F Tests. *Biometrics* 11, 1–42. doi: 10.2307/3001478

El-Deeb, H. M., and El-Naggar, M. A. (2008). Biological control of root rot disease on banana (Musa acuminate Colla) cv. Williams. *Pakistan J. Agric. Agric. Eng. Vet. Sci.* 24, 46–52.

El-Nagdi, W. M. A., Abd El-Khair, H., and El-Ghonaimy, A. M. (2015). Field application of biological control on root-knot nematode and Fusarium root rot fungus in Banana Cv. Grand Naine. *Middle East J. Agric. Res.* 4, 545–554.

El-Sharkawy, H. H. A., Rashad, Y. M., and Ibrahim, S. A. (2018). Biocontrol of stem rust disease of wheat using arbuscular mycorrhizal fungi and Trichoderma spp. *Physiol. Mol. Plant Pathol.* 103, 84–91. doi: 10.1016/j.pmpp.2018.05.002

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., 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

FAOSTAT (2021). *Statistics Division*. Available Online at: http://www.fao.org/faostat/en/#data/QC

Gupta, P., Ravi, I., and Sharma, V. (2013). Induction of β-1,3-glucanase and chitinase activity in the defense response of Eruca sativa plants against the fungal pathogen Alternaria brassicicola. *J. Plant Interact.* 8, 155–161. doi: 10.1080/17429145.2012.679705

Harborne, J. B. (1984). *Phytochemical methods: A guide to modern techniques of plant analysis*. London: Chapman and Hall.

Hewage, K., Yang, J. F., Wang, D., Hao, G. F., Yang, G. F., and Zhu, J. K. (2020). Chemical manipulation of abscisic acid signaling: a new approach to abiotic and biotic stress management in agriculture. *Adv. Sci. (Weinh)* 7:2001265. doi: 10.1002/advs.202001265

Hu, W., Jiang, N., Yang, J., Meng, Y., Wang, Y., Chen, B., et al. (2016). Potassium (K) supply affects K accumulation and photosynthetic physiology in two cotton (*Gossypium hirsutum* L.) cultivars with different K sensitivities. *Field Crop. Res.* 196, 51–63. doi: 10.1016/j.fcr.2016.06.005

Iqbal, M. T., Ahmed, I. A. M., Isik, M., Sultana, F., and Ortaş, I. (2021). Role of mycorrhizae inoculations on nutrient uptake in rice grown under aerobic and anaerobic water management. *J. Plant Nutr.* 44, 550–568. doi: 10.1080/01904167.2020.1845375

Isayenkov, S. V., and Maathuis, F. J. M. (2019). Plant salinity stress: Many unanswered questions remain. *Front. Plant Sci.* 10:80. doi: 10.3389/fpls.2019.00080

Jackson, M. (2005). *Soil chemical analysis*. Madison, WI: Parallel Press, University Of Wisconsin-Madison Libraries, 498.

Jung, S. C., Martinez-Medina, A., Lopez-Raez, J. A., and Pozo, M. J. (2012). Mycorrhiza-induced resistance and priming of plant defenses. *J. Chem. Ecol.* 38, 651–664. doi: 10.1007/s10886-012-0134-6

Kamran, M., Parveen, A., Ahmar, S., Malik, Z., Hussain, S., Chattha, M. S., et al. (2020). An overview of hazardous impacts of soil salinity in crops, tolerance mechanisms, and amelioration through selenium supplementation. *Int. J. Mol. Sci.* 21:148. doi: 10.3390/ijms21010148

Ku, Y. S., Sintaha, M., Cheung, M. Y., and Lam, H. M. (2018). Plant hormone signaling crosstalks between biotic and abiotic stress responses. *Int. J. Mol. Sci.* 19:3206. doi: 10.3390/ijms19103206

Li, N., Han, X., Feng, D., Yuan, D., and Huang, L. J. (2019). Signaling crosstalk between salicylic acid and ethylene/Jasmonate in plant defense: Do we understand what they are whispering? *Int. J. Mol. Sci.* 20:671. doi: 10.3390/ijms20030671

Li, P., Liu, W., Zhang, Y., Xing, J., Li, J., Feng, J., et al. (2019). Fungal canker pathogens trigger carbon starvation by inhibiting carbon metabolism in poplar stems. *Sci. Rep.* 9:10111.

Li, Z., Wu, N., Meng, S., Wu, F., and Liu, T. (2020). Arbuscular mycorrhizal fungi (AMF) enhance the tolerance of Euonymus maackii Rupr. At a moderate level of salinity. *PLoS One* 15:e0231497. doi: 10.1371/journal.pone.0231497

Lievens, L., Pollier, J., Goossens, A., Beyaert, R., and Staal, J. (2017). Abscisic acid as pathogen effector and immune regulator. *Front. Plant Sci.* 8:587. doi: 10.3389/fpls.2017.00587

Liu, X. G., Yu, Y., Liu, Q., Deng, S., Jin, X. B., Yin, Y. J., et al. (2019). A Na2CO3-responsive chitinase gene from Chinese wildrye improve pathogen resistance and saline-alkali stress tolerance in transgenic tobacco and maize. *bioRxiv* 11:504. doi: 10.1101/707281

Liu, Z., Gao, J., Gao, F., Liu, P., Zhao, B., and Zhang, J. W. (2018). Photosynthetic characteristics and chloroplast ultrastructure of summer Maize response to different nitrogen supplies. *Front. Plant Sci.* 9:576. doi: 10.3389/fpls.2018.00576

Livak, K. J., and Schmittgen, T. D. (2001). Analysis of relative gene expression data using real-time quantitative PCR and the 2-ΔΔCT method. *Methods* 25, 402–408. doi: 10.1006/meth.2001.1262

Malhotra, H., Vandana, Sharma, S., and Pandey, R. (2018). "Phosphorus nutrition: plant growth in response to deficiency and excess," in *Plant nutrients and abiotic stress tolerance*, eds M. Hasanuzzaman, M. Fujita, H. Oku, K. Nahar, and B. Hawrylak-Nowak (Springer: Singapore), 171–190.

Martín-Rodríguez, J. A., Huertas, R., Ho-Plágaro, T., Ocampo, J. A., Turečková, V., Tarkowská, D., et al. (2016). Gibberellin–abscisic acid balances during arbuscular mycorrhiza formation in tomato. *Front. Plant Sci.* 7:1273. doi: 10.3389/fpls.2016.01273

Nahar, K., Kyndt, T., Nzogela, Y. B., and Gheysen, G. (2012). Abscisic acid interacts antagonistically with classical defense pathways in rice-migratory nematode interaction. *New Phytol.* 196, 901–913. doi: 10.1111/j.1469-8137.2012.04310.x

Ohnuma, T., Numata, T., Osawa, T., Mizuhara, M., Lampela, O., Juffer, A. H., et al. (2011). A class V chitinase from Arabidopsis thaliana: gene responses, enzymatic properties, and crystallographic analysis. *Planta* 234, 123–137. doi: 10.1007/s00425-011-1390-3

Peach, K., and Tracy, M. V. (1955). *Modern Methods of Plant Analysis*. Heidelberg, Berlin: Springer, 258–261.

Pegoraro, C., da Rosa Farias, D., Mertz, L. M., dos Santos, R. S., da Maia, L. C., Rombaldi, C. V., et al. (2013). Ethylene response factors gene regulation and expression profiles under different stresses in rice. *Theor. Exp. Plant Physiol.* 25, 261–274. doi: 10.1590/s2197-00252013000400004

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. doi: 10.1016/s0007-1536(70)80110-3

Ranjha, M. M. A. N., Irfan, S., Nadeem, M., and Mahmood, S. (2020). A comprehensive review on nutritional value, medicinal uses, and processing of banana. *Food Rev. Int.* 2020, 1–27. doi: 10.1080/87559129.2020.1725890

Rashad, Y. M., Abbas, M. A., Soliman, H. M., Abdel-Fattah, G. G., and Abdel-Fattah, G. M. (2020b). Synergy between endophytic Bacillus amyloliquefaciens GGA and arbuscular mycorrhizal fungi induces plant defense responses against white rot of garlic and improves host plant growth. *Phytopathol. Mediterr.* 59, 169–186. doi: 10.14601/Phyto-11019

Rashad, Y., Aseel, D., Hammad, S., and Elkelish, A. (2020a). Rhizophagus irregularis and Rhizoctonia solani differentially elicit systemic transcriptional expression of polyphenol biosynthetic pathways genes in sunflower. *Biomolecules* 10, 1–20. doi: 10.3390/biom10030379

Romero-Munar, A., Baraza, E., Gulías, J., and Cabot, C. (2019). Arbuscular mycorrhizal fungi confer salt tolerance in giant reed (*Arundo donax* l.) plants grown under low phosphorus by reducing leaf NA+ concentration and improving phosphorus use efficiency. *Front. Plant Sci.* 10:843. doi: 10.3389/fpls.2019.00843

Sah, S. K., Reddy, K. R., and Li, J. (2016). Abscisic acid and abiotic stress tolerance in crop plants. *Front. Plant Sci.* 7:571. doi: 10.3389/fpls.2016.00571

Saia, S., Benítez, E., García-Garrido, J. M., Settanni, L., Amato, G., and Giambalvo, D. (2014). The effect of arbuscular mycorrhizal fungi on total plant nitrogen uptake and nitrogen recovery from soil organic material. *J. Agric. Sci.* 152, 370–378.

Sheng, M., Tang, M., Chen, H., Yang, B., Zhang, F., and Huang, Y. (2009). Influence of arbuscular mycorrhizae on the root system of maize plants under salt stress. *Can. J. Microbiol.* 55, 879–886. doi: 10.1139/w09-031

Shigeto, J., and Tsutsumi, Y. (2016). Diverse functions and reactions of class III peroxidases. *New Phytol.* 209, 1395–1402. doi: 10.1111/nph.13738

Spence, C. A., Lakshmanan, V., Donofrio, N., and Bais, H. P. (2015). Crucial roles of abscisic acid biogenesis in virulence of rice blast fungus magnaporthe oryzae. *Front. Plant Sci.* 6:1082. doi: 10.3389/fpls.2015.01082

Su, Y., Wang, Z., Liu, F., Li, Z., Peng, Q., Guo, J., et al. (2016). Isolation and characterization of ScGluD2, a new sugarcane beta-1,3-Glucanase D family gene induced by Sporisorium scitamineum, ABA, H2O2, NaCl, and CdCl2 stresses. *Front. Plant Sci.* 7:1348. doi: 10.3389/fpls.2016.01348

SuChen, L., ChaoYi, W., and ChingChung, S. (2012). Using arbuscular mycorrhizal fungus and other microorganisms for control of Fusarium wilt of banana. *J. Taiwan Agric. Res.* 61, 241–249.

Suzuki, N., Rivero, R. M., Shulaev, V., Blumwald, E., and Mittler, R. (2014). Abiotic and biotic stress combinations. *New Phytol.* 203, 32–43. doi: 10.1111/nph.12797

Ton, J., Flors, V., and Mauch-Mani, B. (2009). The multifaceted role of ABA in disease resistance. *Trends Plant Sci.* 14, 310–317. doi: 10.1016/j.tplants.2009.03.006

Trouvelot, A., Kough, J., and Gianinazzi, V. (1986). Mesure du taux de mycorhization VA d'un systeme radiculaire. Recherche de methodes d'estimation ayant une signification fonctionnelle. *Physiol. Genet. Aspects Mycorrhizae* 1986, 217–221.

Van Bockhaven, J., De Vleesschauwer, D., and Höfte, M. (2013). Towards establishing broad-spectrum disease resistance in plants: Silicon leads the way. *J. Exp. Bot.* 64, 1281–1293. doi: 10.1093/jxb/ers329

Vangelisti, A., Natali, L., Bernardi, R., Sbrana, C., Turrini, A., Hassani-Pak, K., et al. (2018). Transcriptome changes induced by arbuscular mycorrhizal fungi in sunflower (*Helianthus annuus* L.) roots. *Sci. Rep.* 8:4. doi: 10.1038/s41598-017-18445-0

Velivelli, S. L., Lojan, P., Cranenbrouck, S., de Boulois, H. D., Suarez, J. P., Declerck, S., et al. (2015). The induction of Ethylene response factor 3 (ERF3) in potato as a result of co-inoculation with *Pseudomonas* sp. R41805 and Rhizophagus irregularis MUCL 41833 - a possible role in plant defense. *Plant Signal. Behav.* 10:e988076. doi: 10.4161/15592324.2014.988076

Vos, I. A., Verhage, A., Watt, L. G., Vlaardingerbroek, I., Schuurink, R. C., Pieterse, C. M. J., et al. (2019). Abscisic acid is essential for rewiring of jasmonic acid-dependent defenses during herbivory. *bioRxiv* 2019:747345. doi: 10.1101/747345

Walters, D. R. (2015). "Photosynthesis in attacked plants and crops," in *Physiological responses of plants to attack*, ed. D. R. Walters (Hoboken, NJ: Wiley-Blackwell), 41–87.

Wild, A. (1988). *Russell's Soil Conditions and Plant Growth*. Harlow: Longman Scientific and Technical.

Yano-Melo, A. M., Saggin, O. J., and Maia, L. C. (2003). Tolerance of mycorrhized banana (*Musa* sp. cv. Pacovan) plantlets to saline stress. *Agric. Ecosyst. Environ.* 95, 343–348. doi: 10.1016/S0167-8809(02)00044-0

Yazawa, K., Jiang, C. J., Kojima, M., Sakakibara, H., and Takatsuji, H. (2012). Reduction of abscisic acid levels or inhibition of abscisic acid signaling in rice during the early phase of Magnaporthe oryzae infection decreases its susceptibility to the fungus. *Physiol. Mol. Plant Pathol.* 78, 1–7. doi: 10.1016/j.pmpp.2011.12.003

Zhang, H., Liu, W., Wan, L., Li, F., Dai, L., Li, D., et al. (2010). Functional analyses of ethylene response factor JERF3 with the aim of improving tolerance to drought and osmotic stress in transgenic rice. *Transgenic Res.* 19, 809–818. doi: 10.1007/s11248-009-9357-x

Zhang, Z., Yao, W., Dong, N., Liang, H., Liu, H., and Huang, R. (2007). A novel ERF transcription activator in wheat and its induction kinetics after pathogen and hormone treatments. *J. Exp. Bot.* 58, 2993–3003. doi: 10.1093/jxb/erm151

Zheng, T., Zhang, K., Sadeghnezhad, E., Jiu, S., Zhu, X., Dong, T., et al. (2020). Chitinase family genes in grape differentially expressed in a manner specific to fruit species in response to Botrytis cinerea. *Mol. Biol. Rep.* 47, 7349–7363. doi: 10.1007/s11033-020-05791-y

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.

Publisher's Note: All claims expressed in this article are solely those of the authors and do not necessarily represent those of their affiliated organizations, or those of the publisher, the editors and the reviewers. Any product that may be evaluated in this article, or claim that may be made by its manufacturer, is not guaranteed or endorsed by the publisher.

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